Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 193-198

COVALENT BINDING TO APOPROTEIN IS A MAJOR FATE OF HEME IN A VARIETY OF REACTIONS IN WHICH CYTOCHROME P-450 IS DESTROYED[1]

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Received May 15, 1986

SUMMARY — The heme in rat liver microsomal cytochrome P-450 was labeled with ^{14}C or ^{3}H and the microsomes were fractionated after in vitro incubations with a variety of agents known to destroy cytochrome P-450 heme. A major fraction of the heme label was irreversibly bound to apoprotein in all cases, including incubations with fluroxene, 1-octene, vinyl bromide, trichloroethylene, vinyl chloride, parathion, cumene hydroperoxide, NaN_3, or iron-ADP complex. Label was also extensively bound to apoprotein when purified and reconstituted cytochrome P-450 was incubated with NADPH and vinyl chloride. This process appears to be widespread and involved to a significant extent in the cytochrome P-450 heme destruction observed with many compounds.

P-450 hemoproteins are involved in the oxidation of a wide variety of substrates. Many of the mechanism-based inactivators of these enzymes destroy the heme prosthetic group (1,2). A wide variety of chemicals act as such "suicide" substrates. The only products of such P-450 heme inactivation that have been identified are the N-alkylporphyrins (1-3). However, the fraction of the degraded heme which is accounted for by these compounds has not been measured. In our own work on the identification of products of the peroxidative degradation of P-450 heme in the absence of substrates, only a fraction could be recovered as the products we identified; most of the radiolabel could be accounted for as being covalently bound to apoprotein (4,5). Pohl and his associates have recently reported that such irreversible attachment is observed during

[1] This work was supported in part by USPHS grants ES 01590, ES 02205, and ES 00267. F.P.G. is a Burroughs-Wellcome Scholar in Toxicology (1983-1988) and was also supported by USPHS Career Development Award ES 00041 (1978-1983) while portions of the work were done.

Abbreviations used: P-450, liver microsomal cytochrome P-450; BHT, butylated hydroxytoluene; di-12 GPC, L-α-1,2-dilauroyl-sn-glycero-3-phosphocholine; and DOC, sodium deoxycholate.

0006-291X/86 $1.50

the reduction of CCl_4 and the oxidation of allylisopropylacetamide (6,7). The generality of such heme attachment to apoprotein was examined here.

MATERIALS AND METHODS

Microsomes were prepared from livers of adult, male Sprague-Dawley rats that had been treated with phenobarbital and had been administered [3,5-^{3}H]-, [4-^{14}C]-, or [5-^{14}C]-labeled 5-aminolevulinic acid to label P-450 (4,5,8). P-450 (P-450$_{PB-B}$ form) and NADPH-P-450 reductase were purified as described elsewhere for these studies (9,10).

The procedure for the fractionation of heme products is based upon the method of Schwartz et al. (11) and presented in detail elsewhere (4). The overall recovery of radioactivity was greater than 90%. Briefly, fraction 1 contains propentdyopents and maleimides (4,5), fraction 2 contains bilirubin, fraction 3 contains biliverdin, fraction 5 contains intact heme, and fraction 6 contains radioactivity covalently bound to apoprotein. In all cases examined the migration of the radioactivity in fraction 5 was concident with heme in thin layer chromatography (silica gel G, benzene-CH_3OH-CH_3CO_2H, 90:10:2, v/v). The material in fraction 6 was successively washed (three times each) with 0.2% HCl in acetone, 10% (w/v) Cl_3CCO_2H, and 95% (w/v) aqueous ethanol.

RESULTS

Incubation of hydroperoxides with P-450 results in the formation of several water-soluble propentdyopents and maleimides (fraction 1) and most of the radioactivity in P-450 heme becomes irreversibly bound to apoprotein (4,5); these results were confirmed in an experiment with cumene hydroperoxide (Table 1). The oxidation of a number of terminal olefins, including fluroxene and 1-octene, has been shown to be accompanied by the loss of P-450 heme and the formation of N-alkyl porphyrin derivatives (1,3,12). Fractionation of labeled heme after incubation of microsomes with NADPH and any of four different olefins resulted in the recovery of the bulk of the degraded radioactivity in the water-soluble fraction 1 and the irreversibly-bound fraction 6. Parathion oxidation leads to the release of sulfur (13), and these incubations resulted in the destruction of heme and covalent attachment of the heme label to proteins.

In these experiments (Table 1) in which the heme was labeled with 5-amino-[4-^{14}C]-levulinic acid, a significant fraction of the label appeared in the protein fraction (6) with the microsomes which were not incubated with substrates. However, the fraction of radioactivity recovered in fraction 6 was increased after incubation with any of the compounds listed. In order to reduce the background radioactivity in some of the fractions, the experimental conditions were modified, 5-amino-[3,5-^{3}H]-levulinic

Table 1

Distribution of ^{14}C-labeled Microsomal Heme in Fractions Following Incubation with Various Compounds[a]

Compound added	Percent total ^{14}C recovered in each fraction					
	1	2	3	4	5	6
Cumene hydroperoxide (10 mM)	17	4	0.5	1	6	71
Fluroxene (1 mM)	17	2	0.3	0.5	44	36
1-Octene (10 mM)	30	1	0.3	0.4	26	42
Vinyl bromide (10 mM)	19	1	0.3	0.5	41	37
Trichloroethylene (10 mM)	16	0.7	0.2	0.5	54	28
Parathion (0.1 mM)	18	1	0.3	0.5	28	51
None[b]	7	0.2	0.1	0.4	75	18

[a] Microsomal hemeproteins were labeled by i.v. administration of 5-amino-[4-^{14}C]-levulinic acid. The microsomes contained 2.31 nmol P-450, 2.79 nmol heme, and 3.40 nCi ^{14}C per mg of protein. The compounds were incubated (at the indicated concentrations) with the liver microsomes (2 mg protein/ml), 50 mM potassium phosphate buffer, 1 mM EDTA, 20 μM BHT, and an NADPH generating system containing 10 mM glucose-6-phosphate, 0.5 mM $NADP^+$, and 2 IU yeast glucose-6-phosphate dehydrogenase/ml for 30 min at 37°C in the dark (total volume 2.0 ml). Samples were fractionated as described under Materials and Methods.

[b] The NADPH-generating system was deleted.

acid was used as the source of the heme label, and the P-450 was purified and reconstituted for the incubations (Table 2). As previously noted (4,5), incubation of microsomal or reconstituted P-450 with NADPH in the absence of catalase (inhibited by NaN_3) leads to the production of water-soluble propentdyopents and maleimides and the attachment of heme to apoprotein (label in fractions 1 and 6). During the oxidation of vinyl chloride by P-450 (14), heme also appeared to become bound to apoprotein. These experiments were done in the presence of catalase (to inhibit H_2O_2 accumulation) (4,14) and EDTA and BHT (to block lipid peroxidation) (15). Label from heme also became covalently attached to microsomal protein in an iron-dependent lipid peroxidation system, although such binding was seen to a lesser degree in the reconstituted system.

Table 2

Distribution of ^{3}H-labeled Heme in Fractions Following Incubation of Microsomes or Purified Cytochrome P-450 with Various Compounds[a]

System	Percent total ^{3}H recovered in each fraction					
	1	2	3	4	5	6
Microsomes, NaN_3, NADPH[b]	26	4	< 0.1	< 0.1	44	26
Reconstituted P-450, NADPH[b]	44	2	0.3	0.5	33	30
Microsomes, vinyl chloride, NADPH[c]	7	2	< 0.1	< 0.1	75	16
Reconstituted P-450, vinyl chloride, NADPH[c]	14	1	0.1	< 0.1	74	11
Microsomes, Fe-ADP, NADPH[d]	13	7	< 0.1	1	61	17
Reconstituted P-450, Fe-ADP, NADPH[d]	6	0.5	< 0.1	< 0.1	92	2
Microsomes[e]	2	< 0.1	< 0.1	< 0.1	92	5
Reconstituted system[e]	3	0.7	< 0.1	< 0.1	98	< 0.1

[a] All incubations were carried out for 30 min at 37°C in the dark in 50 mM potassium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (pH 7.7) buffer containing 15 mM $MgCl_2$ in total volumes of 3.0 ml for microsomal incubations and 6.0 ml for incubations utilizing reconstituted systems. The incubated samples were fractionated as described under Materials and Methods.

[b] The microsomal system contained 2 mg microsomal protein/ml (2.25 nmol P-450 and 3.17 nCi ^{3}H/mg protein), 1 mM EDTA, 20 μM BHT, 1 mM NaN_3, and an NADPH-generating system, as described under Table 1. The reconstituted system contained 2.35 μM $P\text{-}450_{PB\text{-}B}$ (2.03 nCi ^{3}H/ml, 0.36 mg protein/ml), 0.51 μM NADPH-P-450 reductase, 40 μM di-12 GPC, 0.25 mM DOC, 1 mM EDTA, 20 μM BHT, 1 mM NaN_3, and 1 mM NADPH.

[c] The vinyl chloride systems were as in footnote b, except that NaN_3 was omitted and the head space (10 ml) contained 50% vinyl chloride in air; in addition, bovine catalase (17 μg/ml) was added to the reconstituted system.

[d] The microsomal lipid peroxidation system contained microsomes and the NADPH system as before, as well as 2 mM ADP and 0.2 mM $FeCl_3$. The reconstituted lipid peroxidation system contained 2.35 μM $P\text{-}450_{PB\text{-}B}$, 0.51 μM NADPH-P-450 reductase, 40 μM di-12 GPC, 0.25 mM DOC, 1 mM NADPH, 17 μg catalase/ml, 2 mM ADP, 0.2 mM $FeCl_3$, and 0.3 mg egg lecithin/ml (added as sonicated vesicles).

[e] The control microsomal system contained microsomes (2 mg protein/ml), 1 mM EDTA, and 20 μM BHT; the control reconstituted system contained 2.3 μM P-450, 40 μM di-12 GPC, 0.25 mM DOC, 1 mM EDTA, 20 μM BHT, and 17 μg bovine catalase/ml.

In other experiments P-450 heme was labeled in the methene bridges by administration of 5-amino-[5-^{14}C]-levulinic acid to rats. Incubations of the microsomes were done with NADPH and iron-ADP, NaN_3, vinyl chloride, vinylidene chloride, allyl isopropylacetamide, norethindrone, or cumene hydroperoxide and the head space was analyzed for ^{14}CO production (16). In no case was a significant fraction of the methene bridge ^{14}C released as ^{14}CO, although spleen heme oxygenase released CO from labeled methemalbumin quantitatively under these conditions. (Unlabeled CO was produced from vinylidene chloride and trichloroethylene during oxidation (17,18) and complexed with reduced P-450 to yield a 450 nm complex.)

Attempts were made to observe spectral changes during the microsomal incubations with all of the compounds mentioned in Tables 1 and 2. With the exception of the 450 nm peaks formed in the case of the compounds that were converted to CO (vide supra), none of the spectra were particularly distinguishing. The α, β, and Soret bands were decreased (in difference spectra) but no new distinguishable peaks were formed. The material in fraction 6 had a brown color, which upon solubilization yielded a broad visible absorption band. The bound material is not due to modification of nucleophiles by methyl vinyl maleimide (5). Efforts have been made to digest the material with proteases and isolate the chromophore.

DISCUSSION

The heme of P-450 can be degraded in several ways. Released from the apoprotein, heme can be oxidized by coupled oxidation or by the enzyme heme oxygenase--this path results in the release of a methene carbon as CO and the formation of biliverdin, which can be reduced to bilirubin. Peroxidative degradation occurs in the presence of hydroperoxides, including H_2O_2 or strong oxygen donors such as iodosylbenzene, and yields propentdyopents, maleimides, and HCO_2H (5). During the oxidation of terminal olefins (3) and several other types of compounds such as 1,4-dihydropyridines (19) and aminobenztriazoles (20), the heme reacts with electrophilic enzyme-substrate intermediates to produce N-alkyl porphyrins.

We have previously reported that labeled heme becomes covalently attached to apo-P-450 during peroxidative heme degradation (4,5). More recently such covalent

attachment has been demonstrated during the metabolism of CCl_4 (6) and allylisopropylacetamide (7). The work in this paper demonstrates that such binding accompanies the mechanism-based heme loss which occurs during the oxidation of a variety of P-450 substrates. The basis of this binding is yet unclear, nor is the similarity between the heme-protein adducts known in the various systems. Whether the substrate (e.g., olefin etc.) participates in the linkage in any or all of these reactions is not known, as is the role of this phenomenon in the in vivo loss of apo-P-450 which follows mechanism-based inactivation (21). Nevertheless this process seems to be a major aspect of the mechanism-based inactivation of P-450s.

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