REMOVAL OF HEME FROM CYTOCHROME P-450$_{CAM}$ BY HEMOPEXIN AND APOMYOGLOBIN ASSOCIATED WITH LOSS OF P-450 HYDROXYLASE ACTIVITY[1]

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Cytochrome P-450$_{CAM}$ in purified form loses Sorret spectrum and enzymatic activity when incubated at 25° with heme deficient hemopexin or horse apomyoglobin and the ferriheme spectra of the latter appear. The addition of ferriprotoporphyrin IX restores the P-450$_{CAM}$ methylene hydroxylase activity, and indicates a capacity to bind more than the ca. 0.6 moles heme in the enzyme as currently prepared.

A soluble P-450 type cytochrome, P-450$_{CAM}$, isolated from Pseudomonas putida grown on camphor and shown to function in methylene hydroxylation by Katagiri, Ganguli and Gunsalus, 1968 (1,2), possesses properties in common with the mammalian P-450 cytochromes (3). The three subunits of the P. putida hydroxylase are available, pure, in quantity and are of relatively low molecular weight, thus permitting a consideration of the relationship of heme to activity. The characteristic visible spectra of metmyoglobin (4), hemopexin (5) and cytochrome P-450$_{CAM}$ (1) and the accessibility of heme deficient forms of the first two have provided a mechanism for determining affinity, quantity and identity of the ferriheme moiety of P-450$_{CAM}$.

MATERIALS AND METHODS

Cytochrome P-450$_{CAM}$ was purified by the method of Katagiri et al. (1); human hemopexin by a modification of the procedure of Muller-Eberhard, et al.

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(6) and apomyoglobin according to Teale (7) from horse myoglobin purchased from Seravac Laboratories, England.

The heme contents of the three proteins were measured by the titration method of Heide et al. (5) using the maxima of the Sorret bands, i.e. in nano meters, P-450$_{CAM}$ 416, hemopexin 411 and myoglobin 408. For reference the alkaline pyridine hemochrome was measured according to Gallagher et al. (8). We measured heme binding by reconstituted P-450$_{CAM}$ with ^{59}Fe- or ^{14}C-heme, respective specific activities 0.8 x 10^7 and 1.9 x 10^7. dpm μmole^{-1}. The heme was added in excess and the mixture incubated in 20 mM phosphate buffer pH 7.4, (hereafter termed buffer) at 37° for 10 minutes; then excess, unbound heme removed by DEAE-Sephadex treatment as described subsequently.

The measurement of heme transfer from P-450$_{CAM}$ to either hemopexin or apomyoglobin was accomplished by incubating the P-450 and heme-acceptor protein at a molar ratio of 1 or of 2 in buffer at 25° for 30 to 45 minutes. The proteins were then separated at 4° on a DEAE-Sephadex A50 column (1.5 x 28 cm.) using buffer to elute P-450 and buffer containing 0.25 M KCl for the second protein. The respective protein fractions were concentrated by pressure ultrafiltration using a Diaflo membrane, and the completeness of separation verified by polyacrylamide gel electrophoresis according to Davis (9).

The biological activity of P-450$_{CAM}$ prior to and following the experiments was measured by the methylene hydroxylase assay system reported by Katagiri et al. (1).

RESULTS

Interaction of cytochrome P-450$_{CAM}$ with human serum hemopexin (HHx) or with horse apomyoglobin (ApoMb) lead to regeneration of the characteristic Sorret absorption bands of the latter two with decrease in the 416 and 360 nano meter absorption bands of the cytochrome, as shown in Figure 1. With ApoMb, at approximately equimolar concentration to the P-450, the absorption maximum shifts from 416 to 408 and is increased (ε mM myoglobin 153 at 408 (4), P-450 $<$ 100 at 416); the shoulder at 360 disappears almost completely.

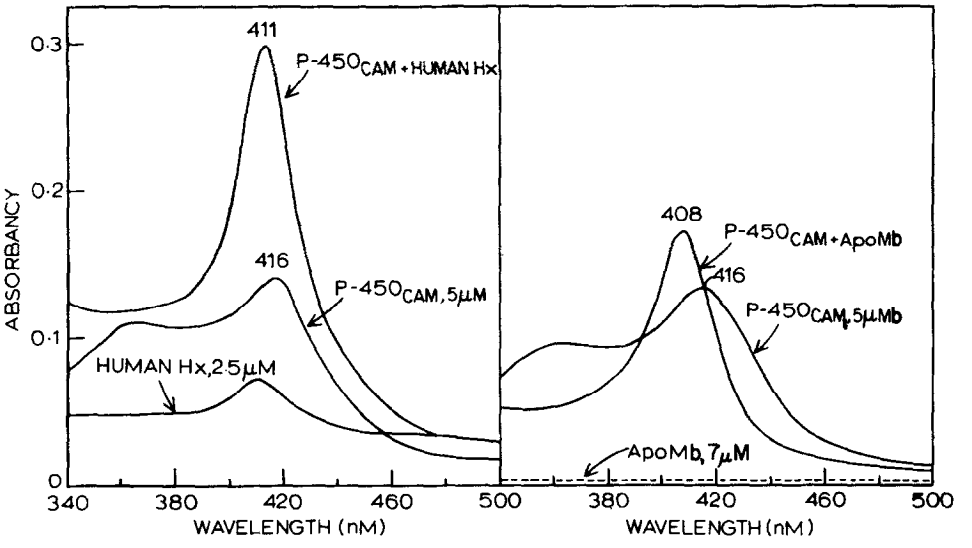

Figure 1. Heme Transfer from Cytochrome P-450$_{CAM}$ to Human Hemopexin (HHx) and to Apomyoglobin (ApoMb).
20 mM phosphate buffer, pH 7.4, 25°, 30 min.

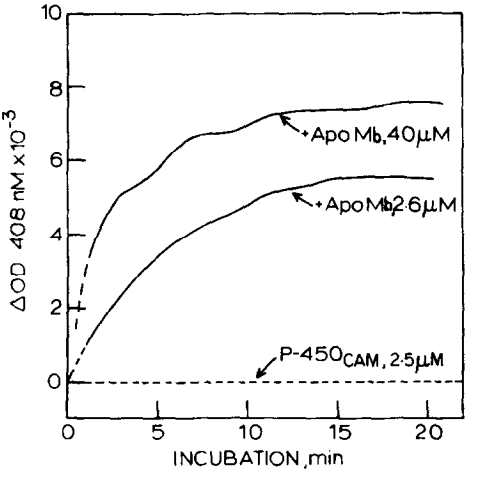

Figure 2. Time Dependence of Heme Transfer from P-450$_{CAM}$ to Apomyoglobin (ApoMb).
20 mM phosphate buffer, pH 7.4, 25°.

Therefore, Mb has the higher affinity for heme. With HHx at half mole per mole P-450 the shift in wavelength of the maximum is less (416 to 411) and

the increase in intensity is greater. The larger increase in absorption is presumably due to the higher heme affinity of HHx than Mb and the greater overlap of absorption maxima of the HHx-P-450 pair (εmM HHx $\approx$ 127).

The data in Figure 2 indicate the time dependence of the transfer of the heme from P-450 to ApoMb. At 25°, the reaction is relatively slow, half exchange occurs in 2 to 5 minutes and equilibrium is approached in 20 min. For Mb-P-450 both the time dependence and the equilibrium are indicated in Figure 2.

A decrease in the hydroxylase activity of P-450 accompanies the transfer of heme to hemopexin, or to apomyoglobin, and is partially restored by ferriprotoporphyrin IX. The relevant data are given in Tables 1 and 2. Table 1 shows the heme content of the P-450 and the hemopexin used and the residual heme on each, after 30 minutes incubation at equimolar concentration and separation on a DEAE-Sephadex column. The P-450 contained 0.58 moles heme per 47,000 grams protein, the approximate molecular weight indicated by acrylamide gel electrophoresis. After incubation the heme was decreased to 0.3 mole with greater than 50 percent loss in biological activity. A concomitant increase occurred in heme-hemopexin from 0.15 to 0.37 moles per mole (72,000 grams protein). Increase in enzyme activity of the P-450 separated from the hemopexin after incubation occurred on titration with ferriprotoporphyrin IX. The heme uptake by P-450 was also monitored by titration according to Heide et al.

Table 1

Transfer of Ferriheme from Cytochrome $P\text{-}450_{CAM}$ to Hemopexin

Protein	Conc.* (mg/ml)	Heme per Mole 0 min	Heme per Mole 30 min + DEAE	Δ Heme per mole	Δ Heme μM
$P\text{-}450_{CAM}$	1.8	0.57	0.30	-0.27	-10.3
HHx	3.2	0.15	0.37	+0.22	+9.4

*P-450 M. wt. ca. 47 K = 38 μM; Hemopexin 72 K = 44 μM.

Table 2

Effect of Hemopexin on Methylene Hydroxylase Subunit Activities

Subunit	Conc. (mg.)	Incubn. (min.)	Hydroxylase Activity - μmoles/min[1] Untreated	+Heme, 5μM	+Hx[2]	+Hx + Heme[3]
$P\text{-}450_{CAM}$	0.1	0	1.5			
		30	0.8	1.3	0.4	0.8
Reductase	2.0	30	1.2	1.5	0.2	
Redoxin	0.2	10	0.4	1.2	0.8	

1 Each component incubated in buffer at 37° for time indicated, the other assay components added, and hydroxylation rate followed by DPNH oxidation; suitable blanks were subtracted.

2 Human hemopexin, 25 μM; equimolar rabbit hemopexin gave values of 0.3 and 0.4.

3 HHx 25 μM; heme 40-280 μM; high heme concentrations uncoupled the system.

(5); when ^{59}Fe or ^{14}C-heme was used, the binding was confirmed by appearance of radioactivity in the protein fraction. The spectral changes in P-450 with loss in biological activity on standing, and the appearance of P-420 are still imperfectly understood (10) and may account for our failure to recover fully the enzyme activity.

Table 2 shows the effect on hydroxylase activity on incubating hemopexin, heme, or both, with each of the three subunits of the P-450 hydroxylase system. The P-450 activity decreases slowly on incubation at 37° in buffer; hemopexin in 2-200 mole excess diminishes P-450 activity in a time dependent reaction. Heme preserves, and in some samples, enhances P-450 activity; whereas hemopexin in the presence of excess heme has little effect. Excess heme affects the assay system, thus interfering with quantitation. Rabbit and human hemopexin are equally effective, in diminishing P-450 activity (see footnote to Table 2); in both cases the activity is restored by heme.

The protective effect of heme on redoxin and reductase and the inactiva-

tion of reductase during incubation with hemopexin require further study. The reductase flavoprotein has been purified and is stabilized by 10 percent glycerol, or as concentrated enzyme solution, but is currently less well understood than either of the two iron proteins.

DISCUSSION

Confirmation of the identity of ironprotoporphyrin IX as the prosthetic group of cytochrome P-450$_{CAM}$ requires the reconstitution of the apoenzyme by heme. Although the P-450 is part of a soluble enzyme system (1) it shows some of the instability under oxidizing conditions observed with other cytochromes. The present study adapts to P-450, the mild conditions of heme removal which are used for the exchange of heme between hemoglobin and albumin, (11) as well as among human and rabbit albumins and hemopexins (12). The function of P-450 in methylene hydroxylation was drastically reduced on heme removal, although the latter was incomplete, and could be reconstituted on addition of iron-protoporphyrin IX. Thus, heme is essential for the biological activity of this enzyme. Furthermore, the heme content of the purified, active P-450 was found to be only ca. 0.6 mole per mole of enzyme, which was homogeneous by polyacrylamide gel electrophoresis. Other cytochromes of the P-450 type derived from bacterial (Appleby, 13) or mammalian (Lu and Coon, 14; Imai and Sato, 3) enzyme systems and the mammalian cytochrome b_5 (Strittmatter and Ozols, 15) carry iron protoporphyrin IX as the prosthetic group. A stoichiometric relation of heme to protein was found for cytochrome b_5 but has not been reported for the other cytochromes. Whether the 0.6 molar ratio of heme to protein, consistently found in current preparations of P-450 is an artifact arising during the isolation procedure remains to be investigated. Nevertheless, a reduction of the heme content of P-450 below the 0.6 molar ratio resulted in loss of its biological activity, thus indicating that at least this ratio is essential for the integrity of the protein.

The ready exchangeability of heme between a protein of the cytochrome P-450 type and a serum protein such as hemopexin was unexpected. The

question of whether an exchange of heme may also occur *in vivo* deserves exploration. It is interesting to speculate whether certain types of cytochromes may serve as tissue receptor sites for circulating plasma heme (Muller-Eberhard, et al. 16) and thus contribute to the dynamics of the heme pool.

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