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Studies on Heme Transfer from Microsomal Hemoproteins to Heme-Binding Plasma Proteins

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SUMMARY

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The transfer of heme from cytochrome P-450 to human serum albumin and rabbit apohemopexin was investigated. These studies were conducted using both membrane-bound and solubilized cytochrome P-450 preparations, as well as the modified form of the hemoprotein, cytochrome P-420. Data indicate that the plasma heme-binding proteins used in this study were unable to remove heme from either membrane-bound or solubilized preparations of cytochrome P-450; in contrast, the heme of cytochrome P-420 present in the above preparations was readily transferred to both serum albumin and apohemopexin. These findings suggest that a barrier exists for heme transfer from cytochrome P-450 to the heme-binding proteins. This barrier is attributed to the shielding effect exerted on the prosthetic group by the tertiary structure of the apoprotein. This shielding effect in turn imposes selectivity on the substances which may come in contact with the heme moiety of the cytochrome. Furthermore, hemopexin was found, in all of the preparations studied, to have a greater affinity for for the prosthetic group of cytochrome P-420 than serum albumin. Accordingly, hemopexin is indicated as the possible intra- and intercellular heme transfer protein for the microsomal hemoprotein, cytochrome P-420.

INTRODUCTION

Previous studies demonstrating that the heme of cytochrome P-420, but not that of cytochrome P-450, was transferable to serum albumin (1) led us to propose (2) a scheme (Scheme 1) for the catabolism of cytochrome P-450. In this scheme, following the conversion of cytochrome P-450 to cytochrome P-420, a heme-binding protein would serve to carry the heme of cytochrome P-420 to the heme-degrading enzymes. Subsequently this heme-protein complex serves as a substrate for the heme oxygenase system (3), which is responsible for the degradation of the heme moiety, ferriprotoporphyrin IX (4), of cytochrome P-450. Serum albumin was the only heme-binding protein utilized in the previous transfer studies. It has been shown (5–7) that the heme-binding capacity

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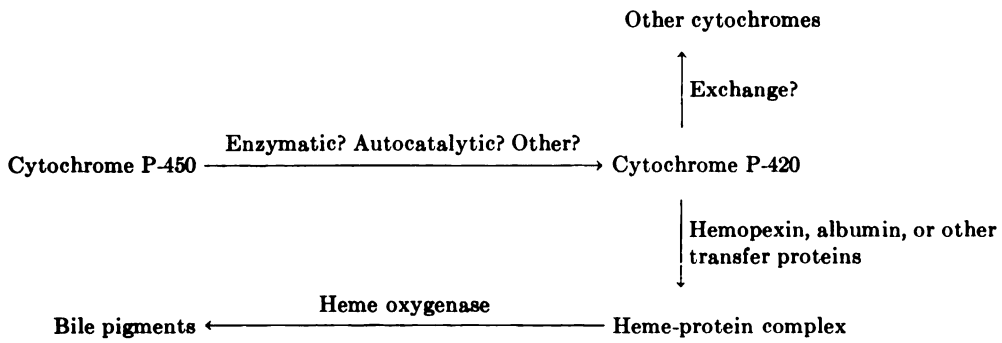

SCHEME 1

of another serum protein, hemopexin, is greater than that of albumin. Furthermore, it has been demonstrated that the heme of cytochrome P-450$_{cam}$ is readily transferable to hemopexin (8).

In the present study the transfer of heme from both membrane-bound and solubilized cytochrome P-450, as well as the heme of CO-binding particles (cytochrome P-420) to apohemopexin and serum albumin, was investigated. The data presented further support the hypothesis that in the mammalian system the molecular conformation of cytochrome P-450 is such that the heme group is very tightly bound or is not readily exposed to the environment (1). However, alterations in the hemoprotein which lead to the formation of the modified form of the cytochrome, cytochrome P-420 (9), result in increased accessibility or in lower affinity for heme. Thus proteins with high heme-binding affinity that come in contact with this portion of the molecule would be able to remove the heme from the apoprotein of the cytochrome.

MATERIALS AND METHODS

Male Sprague-Dawley rats were used. Unless otherwise indicated, all chemicals used were purchased from Sigma Chemical Company. Rabbit hemopexin and apohemopexin were prepared according to a previously described procedure (10).

Preparation of labeled cytochrome P-450. The heme moiety of cytochrome P-450 was labeled by treating rats (150–200 g) with an intravenous injection of [3,5-^{3}H]δ-aminolevulinic acid (specific activity, 510 mCi/mmole; New England Nuclear) at a dose of 40 μCi/100 g of body weight (1). After 2 hr the animals were decapitated, and the livers were removed, perfused with 0.9% sodium chloride solution, and homogenized. Microsomal fractions were prepared using 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, as previously described (1).

Transfer of heme from membrane-bound cytochrome P-450. The microsomal suspension was divided into three aliquots. The protein content of each aliquot was adjusted to 4.5 mg/ml. To one aliquot rabbit apohemopexin was added to a final concentration of 0.1%. To a second aliquot human serum albumin, essentially free of fatty acids (prepared by Sigma Chemical Company), was added to a final concentration of 0.1%. The third aliquot, in Tris-HCl buffer, served as the control. These mixtures were incubated for 30 min at 25°. The incubation mixtures were then centrifuged at 105,000 × *g* for 90 min. The microsomal pellets thus obtained were resuspended in Tris-HCl buffer (0.05 M, pH 7.4) to the original volume. The redistribution of heme between the supernatant and microsomal fractions was investigated by column chromatography, spectral, and radioactive studies.

Three milliliters of the supernatant fraction were applied to a DEAE-Sephadex A-50 column (1.6 × 26 cm) equilibrated with Tris-HCl buffer (0.05 M, pH 7.4) and eluted with a gradient of 500 ml of 0–0.5 M KCl in Tris-HCl buffer. The flow rate was about 20 ml/hr, and 10-ml fractions were collected.

Spectral studies were carried out on a DW-2 Aminco dual-beam spectrophotometer. The cytochrome P-450 content was

determined using an extinction coefficient of 91 mM^{-1} cm^{-1} between 450 and 490 nm (11). The cytochrome b_5 content was measured utilizing NADH as the reducing agent and an extinction coefficient of 185 mM^{-1} cm^{-1} between 412 and 424 nm (12). The heme-hemopexin complex concentration was obtained using an extinction coefficient of 110 mM^{-1} cm^{-1} for the difference in absorption between 413 and 500 nm (13). The concentration of protoheme was determined by the pyridine-hemochromogen method of Paul *et al.* (14); an extinction coefficient of 32.4 mM^{-1} cm^{-1} was used for reduced minus oxidized difference spectra between 557 and 575 nm.

Radioactivity of samples was measured utilizing Aquasol Universal liquid scintillation "cocktail" (New England Nuclear) and a Packard scintillation counter. The background radioactivity was about 20 cpm. The redistribution of radioactivity between the supernatant and microsomal fractions was determined according to the following formula:

$$\frac{\text{Supernatant } {}^3\text{H cpm/ml}}{\text{Supernatant } {}^3\text{H cpm/ml} + \text{pellet } {}^3\text{H cpm/ml}} \times 100$$

Protein concentration was measured by the method of Lowry *et al.* (15).

Transfer of heme from solubilized cytochrome P-450 particles. Immature rats (50–60 g) received daily intraperitoneal injections of phenobarbital (100 mg/kg) for 3 days. The animals were killed 17 hr after the last injection. Microsomal fractions were prepared as described above. Cytochrome P-450 was solubilized according to the method of Lu *et al.* (16). The content of cytochrome P-450 was measured as described above; the highest specific activity was found in the 0–45% ammonium sulfate fraction. The cytochrome b_5 content of this fraction was measured by the addition of exogenous NADH–cytochrome b_5 reductase.[2] Aliquots of the solubilized cytochrome P-450 (0–45%) fraction, containing 4.5 mg of protein per milliliter in Tris-HCl buffer mixture (0.1 M, pH 7.7; glycerol, 20%; dithiothreitol, 0.1 mM), were incubated at 25° for 30 min with 1 mg/ml of apohemopexin, 1 mg/ml of serum albumin, or the Tris buffer mixture alone.

The reduced minus CO difference spectrum of each mixture was measured before and after incubation. The incubation mixtures were chromatographed on DEAE-Sephadex A-50 columns (1.6 × 26 cm); 3 ml of sample were applied to the column. The columns were equilibrated with Tris buffer mixture and eluted with a linear gradient of 0–0.5 M KCl in the Tris buffer mixture described above. Spectral studies were conducted on the eluents, and the protoheme content of the fractions was determined as described above.

Transfer of heme from CO-binding particles (cytochrome P-420). The heme portion of cytochrome P-450 was labeled by treating the rats (150–200 g) with [^{3}H]ALA[3] as described above. Microsomal fractions and subsequently CO-binding particles, the cytochrome P-420-rich microsomal particles devoid of cytochrome b_5 (12), were prepared as previously indicated (1). The microsomal pellets were anaerobically incubated in the dark with 0.2% steapsin at 37° for 1 hr. Thereafter the incubation mixture was centrifuged at 200,000 × *g* for 2 hr. The CO-binding particles thus obtained were resuspended in Tris-HCl buffer (0.05 M, pH 7.4) and centrifuged at 200,000 × *g* for 1 hr in order to remove the steapsin. The CO-binding particles were resuspended in Tris buffer (4.5 mg of protein per milliliter), and aliquots were incubated at 25° for 30 min with rabbit apohemopexin or human serum albumin (1 mg/ml). The incubation mixtures were centrifuged at 200,000 × *g* for 1 hr, and the redistribution of heme between the supernatant fraction and the CO-binding particles was determined by column chromatography of the supernatant fractions as well as spectral and radioactive studies according to the methods outlined earlier. Three milliliters of the supernatant fraction were chromatographed on a DEAE-Sephadex A-50 column (1.6 × 26 cm), which was equilibrated with Tris buffer and eluted with

[2] The NADH–cytochrome b_5 reductase was kindly supplied by Dr. D. J. Lorusso.

[3] The abbreviation used is: ALA, δ-aminolevulinic acid.

a linear gradient of 0–0.5 M KCl in Tris-HCl buffer. The cytochrome P-420 content was measured using sodium dithionate as the reducing agent and an extinction coefficient of 110 mM^{-1} cm^{-1} for the difference in absorption between 420 and 490 nm (11).

RESULTS

Transfer of heme from membrane bound cytochrome P-450 to serum albumin and apohemopexin. Table 1 shows the transfer of tritium-labeled microsomal heme to albumin and apohemopexin. After centrifugation of the microsomal fraction (4.5 mg of protein per milliliter; 0.67 nmole of cytochrome P-450 per milligram of protein) which had been incubated with 0.1% albumin or 0.1% apohemopexin at 25° for 30 min, the microsomal contents of cytochrome P-450 and b_5 remained unchanged. However, decreases of 11% and 15% were observed in the radioactivity of the microsomal pellet in the presence of serum albumin and apohemopexin, respectively. This radioactivity was recovered in the supernatant fraction containing the added proteins.

TABLE 1

Transfer of microsomal heme to serum albumin and serum hemopexin

[^{3}H]ALA (40 μCi/100 g of body weight; 510 mCi/mmole) was administered intravenously to male rats, and the animals were killed 2 hr later. Microsomal fractions (4.5 mg of protein per milliliter; 25,000 cpm/ml) were prepared and equilibrated at 25° for 30 min with human serum albumin or rabbit apohemopexin (0.1%). Thereafter the incubation mixtures were centrifuged, and the redistribution of radioactivity between the supernatant and microsomal pellet was determined as described in MATERIALS AND METHODS. The spectral studies were conducted as described in the text.

Fraction	Cytochrome P-450	Cytochrome b_5	Radioactivity
	nmoles/mg protein	*nmoles/mg protein*	*% total cpm*
Microsomes + buffer			
Supernatant	0	0	5
Pellet	0.67	0.85	95
Microsomes + albumin			
Supernatant	0	0	11
Pellet	0.64	0.85	89
Microsomes + hemopexin			
Supernatant	0	0	15
Pellet	0.68	0.88	85

In order to determine the composition of the radioactivity lost from the microsomal pellet, the hemopexin-containing supernatant fraction was chromatographed on a DEAE-Sephadex A-50 column using a linear gradient of 0–0.5 M KCl in Tris-HCl buffer. As shown in Fig. 1, the pattern of elution of radioactivity followed the elution profile of hemopexin, which indicates that the radioactivity removed from microsomes was associated with this protein.

Transfer of heme from solubilized cytochrome P-450 to albumin and apohemopexin. A solubilized cytochrome P-450 preparation with a specific activity of 1.8 nmoles/mg of protein was prepared from livers of phenobarbital-treated, immature rats. The heme content was 2.3 nmoles/mg of protein, measured as pyridine-hemochromogen. The cytochrome b_5 content of the preparation, measured by the addition of NADH–cytochrome b_5 reductase, was less than 0.02 nmole/mg of protein. Aliquots of the cytochrome P-450 preparation were incubated at 25° for 30 min with 1 mg/ml of serum albumin or apohemopexin; thereafter the reduced minus CO difference spectra of the incubation mixtures were measured. The cytochrome P-450 content of incubation mixtures was identical with that of the control incubated with Tris-HCl buffer mixture (data not shown). Subsequently the incubation mixtures were chromatographed. The elution profiles of the albumin- and hemopexin-containing incubation mixtures are shown in Figs. 2 and 3, respectively. As shown, upon incubation of solubilized cytochrome P-450 fractions with the heme-binding proteins, some heme was transferred from the solubilized cytochrome P-450 fractions to the exogenous heme-binding proteins. The extent of heme transferred in the presence of apohemopexin (0.6 nmole/mg of protein) was markedly greater than in the presence of albumin (0.2 nmole/mg of protein) (peaks I of Figs. 2 and 3). As in the previous experiment, the cytochrome P-450

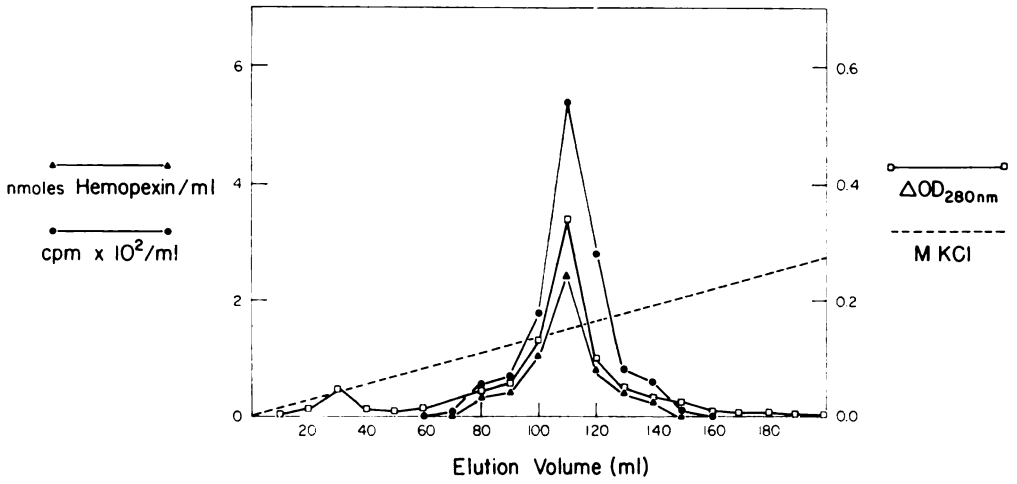

FIG. 1. *Chromatographic profile of hemopexin after equilibration with microsomal fraction*

Tritium-labeled microsomal hemoproteins were prepared by intravenous injection of [^{3}H]ALA into rats (40 μCi/100 g of body weight; 510 mCi/mmole). After 2 hr the animals were killed, and microsomal fractions (4.5 mg of protein per milliliter; 25,000 cpm/ml) were prepared and incubated with 0.1% hemopexin at 25° for 30 min. Following centrifugation, the hemopexin-containing supernatant fraction was chromatographed on a DEAE-Sephadex A-50 column equilibrated with Tris-HCl buffer (0.05 M, pH 7.4) and eluted with a linear gradient of 0–0.5 M KCl in Tris-HCl buffer. The hemopexin concentration was measured as described in MATERIALS AND METHODS.

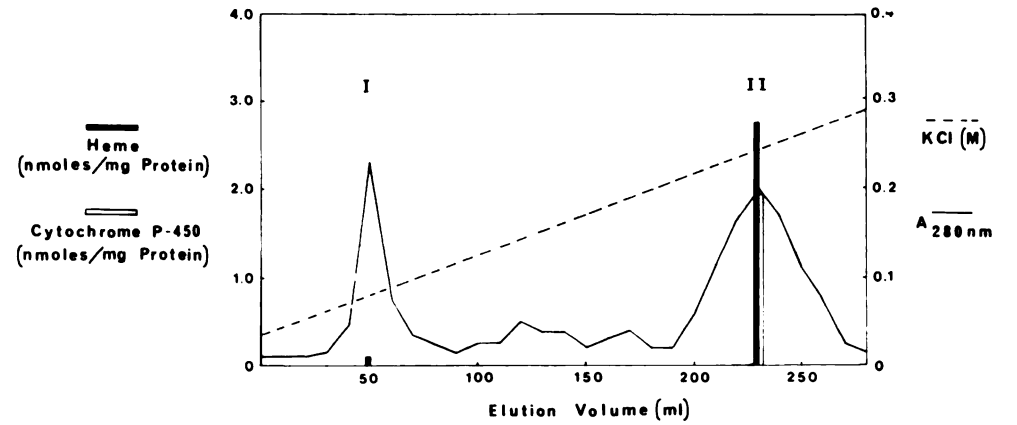

FIG. 2. *Chromatographic profile of solubilized cytochrome P-450 after incubation with serum albumin*

Rat liver cytochrome P-450 was solubilized according to the method of Lu *et al.* (16). The 0–45% ammonium sulfate fraction (1.8 nmoles of cytochrome P-450 per milligram of protein) was incubated with 0.1% serum albumin at 25° for 30 min. The incubation mixture was chromatographed on a DEAE-Sephadex A-50 column equilibrated with Tris-HCl buffer (0.05 M, pH 7.7) mixture, the composition of which is described under MATERIALS AND METHODS, and eluted with a linear gradient of 0–0.5 M KCl in Tris-HCl buffer mixture. Heme and cytochrome P-450 were determined as described in MATERIALS AND METHODS.

content of the fraction remained the same after both treatments (peaks II of Figs. 2 and 3; 2.1 nmoles/mg of protein). The reduced minus CO difference spectrum of peaks II of both Figs. 2 and 3 is shown in Fig. 4. This figure shows that although the cytochrome P-450 content of both fractions remained the same, there was a decrease in

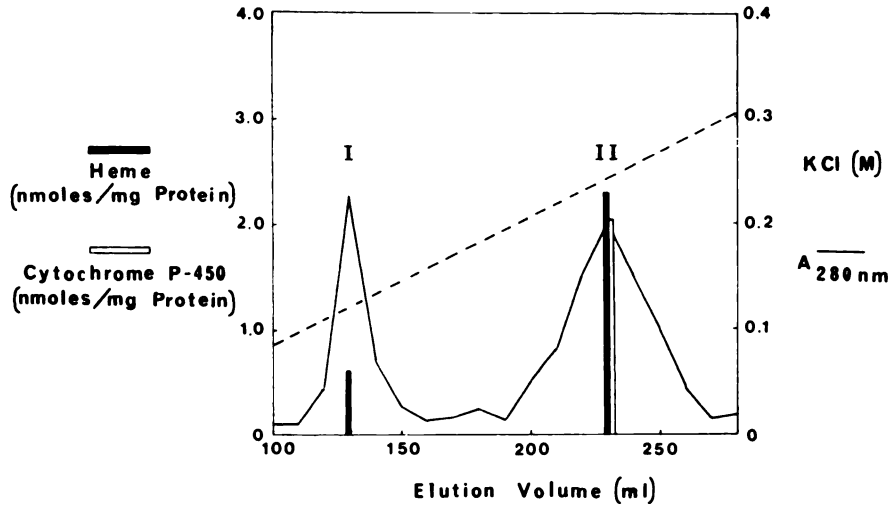

FIG. 3. *Chromatographic profile of solubilized cytochrome P-450 after incubation with apohemopexin*
Solubilized rat liver cytochrome P-450 was incubated with 0.1% serum apohemopexin. Conditions were the same as described for Fig. 2.

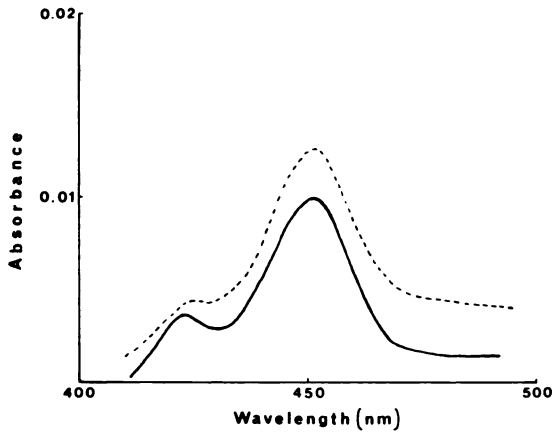

FIG. 4. *CO difference spectrum of solubilized cytochrome P-450 after incubation with plasma heme-binding proteins*
Solubilized cytochrome P-450 preparations with a specific activity of 1.8 nmoles/mg of protein were obtained from rat liver microsomal fractions (16). Aliquots of the 0–45% ammonium sulfate fraction (4.5 mg of protein per milliliter) were incubated with 0.1% human serum albumin or rabbit apohemopexin at 25°C for 30 min. The incubation mixtures were chromatographed on a DEAE-Sephadex A-50 column. The reduced minus CO difference spectrum of peak II was measured as described in MATERIALS AND METHODS. ——, after incubation with serum albumin; - - -, after incubation with apohemopexin.

the peak at 422 nm when incubated with apohemopexin. This finding indicates that the source of heme removed from the solubilized cytochrome P-450 particles was the cytochrome P-420 produced during solubilization procedure.

Transfer of heme from CO-binding particles to serum albumin and apohemopexin. Cytochrome P-450 was quantitatively converted to cytochrome P-420 by incubating the microsomal fraction with 0.2% steapsin at 37° for 1 hr under nitrogen. Table 2 shows that upon equilibration of the CO-binding particles with the heme-binding proteins at 25° for 30 min there was a substantial decrease in the heme content of the CO-binding particles as measured by the pyridine-hemochromogen spectrum (54% and 64% upon incubation with serum albumin and apohemopexin, respectively). Concomitant decreases in the radioactivity of the CO-binding particles, amounting to 41% and 45% after treatment with serum albumin and apohemopexin, respectively, were observed. It should be noted that the loss of heme from CO-binding particles, when measured by the pyridine-hemochromogen method and by radioactivity determinations, does not exhibit an exact stoichiometric relationship. This is attributed to methodologi-

TABLE 2

Loss of heme from CO-binding particles to serum albumin and serum hemopexin

Adult male rats received an intravenous injection of [^{3}H]ALA (40 μCi/100 g of body weight; 510 mCi/mmole), and 2 hr later the animals were killed. CO-binding particles (4.5 mg of protein per milliliter) were prepared by incubating microsomal fractions with steapsin (0.2%) at 37° for 1 hr under nitrogen and centrifugation at 200,000 × *g* for 2 hr. This treatment released cytochrome b_5 and quantitatively converted cytochrome P-450 to cytochrome P-420. The CO-binding particles were equilibrated with human serum albumin or rabbit apohemopexin (0.1%) at 25° for 30 min. Thereafter the incubation mixtures were centrifuged at 200,000 × *g* for 1 hr and spectral as well as radioactivity studies were conducted as described in MATERIALS AND METHODS.

Treatment	Protoheme	Radioactivity
	nmoles/mg protein	*% total*
CO-binding particles + buffer	1.99	87
CO-binding particles + albumin	0.93	59
CO-binding particles + hemopexin	0.70	55

cal difficulties, which, at present, cannot be resolved. However, the results obtained by both methods are supportive.

The chromatographic pattern of hemopexin after equilibration with CO-binding particles (Fig. 5) shows that the elution pattern of hemopexin paralleled that of the tritium counts. Figure 6 shows that intact heme molecules were removed from the microsomal membranes as the eluted hemopexin exhibited a visible absorption spectrum characteristic of a heme-hemopexin complex (10): the oxidized form showed a Soret band at 413 nm with poorly defined α- and β-bands. The difference spectrum of the reduced form exhibited an absorption maximum at 427.5 nm with an α-band at 557 nm and the β-band at 530 nm. The reduced plus CO vs. oxidized difference spectrum showed a Soret band and α- and β-bands at 417, 572, and 540 nm, respectively. The reduced plus CO vs. reduced difference spectrum exhibited a Soret band at 421 nm, with no detectable α- or β-band. Evidence for the ligation of CO with the reduced heme-hemopexin complex has been reported (17).

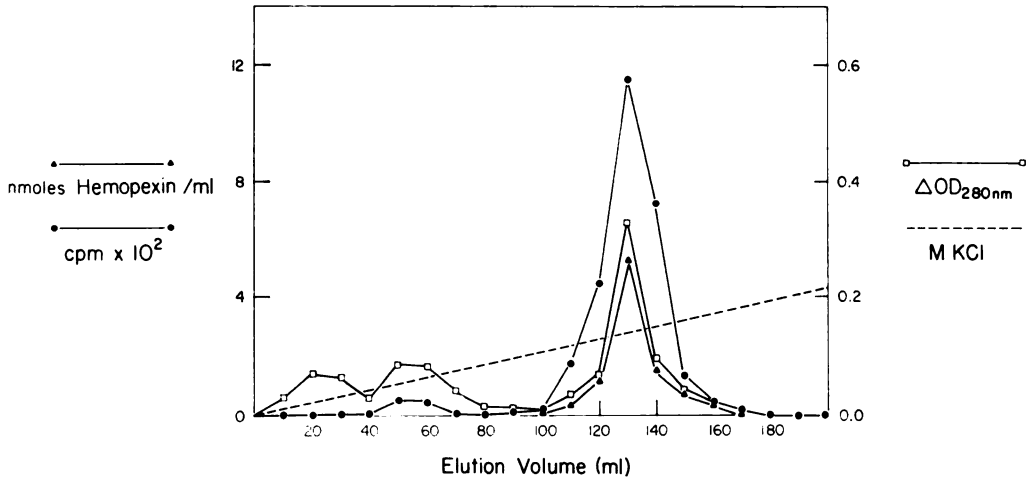

FIG. 5. *Chromatographic profile of hemopexin after equilibration with CO-binding particles*

Tritium-labeled CO-binding particles were prepared by treating the microsomal fraction with steapsin (0.2%) at 37° for 1 hr under anaerobic conditions. Apohemopexin (0.1%) was equilibrated with the particles at 25° for 30 min and chromatographed on a DEAE-Sephadex A-50 column. The column was eluted with a linear concentration gradient of 0–0.5 M KCl in Tris Buffer (0.05, pH 7.4).

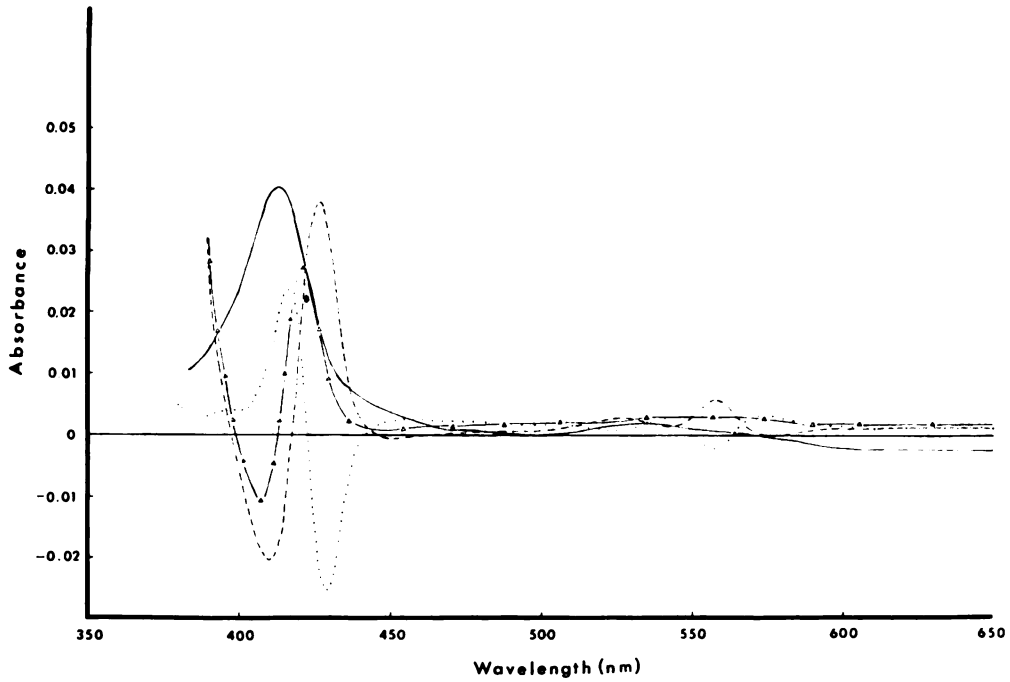

FIG. 6. *Absorption spectra of hemopexin eluted from DEAE-Sephadex A-50*

CO-binding particles were prepared from tritium-labeled rat liver microsomal cytochrome P-450 as described in MATERIALS AND METHODS. CO-binding particles (4.5 mg of protein per milliliter; 3.9 nmoles of cytochrome P-420 per milligram of protein) were incubated with rabbit apohemopexin (0.1%) at 25° for 30 min. After centrifugation at 200,000 × *g* for 1 hr, the supernatant fraction was chromatographed on a DEAE-Sephadex A-50 column and spectral studies were conducted on the eluent. ——, oxidized (absolute); - - -, reduced (difference); △——△, reduced plus CO (difference); · · · ·, reduced minus CO (difference).

DISCUSSION

We (2) have recently proposed the presence of a "heme transfer protein" that serves to carry heme from cytochrome P-420 to the major site of heme oxygenase activity. We further suggested that the conversion of cytochrome P-450 to cytochrome P-420 occurs *in vivo*. This conjecture was based on the observation that the heme moiety of cytochrome P-450, in contrast to that of cytochrome P-420, could not be transferred to human serum albumin *in vitro*. Hemopexin, a heme-binding plasma protein with a much higher affinity for heme than albumin (6, 7), has been shown to remove heme from cytochrome $P\text{-}450_{cam}$ (8), which is a soluble cytochrome isolated from *Pseudomonas putida* (18). Therefore we investigated the transfer of heme to hemopexin from mammalian microsomal cytochrome P-450, and a solubilized form thereof, as well as steapsin-treated microsomes, i.e., CO-binding particles.

The present study shows that hemopexin, like albumin, was unable to remove the heme moiety from cytochrome P-450. Treatment with albumin or apohemopexin (Table 1) did not change the microsomal content of cytochrome P-450, membrane-bound or solubilized. Furthermore, the heme which was transferred to the exogenous heme-binding proteins was not lost from cytochrome b_5, since the content of this cytochrome in the intact microsomal preparation was not altered in the presence of the added heme-binding proteins. Similarly, the very minute quantity of this cytochrome present in the solubilized microsomal fraction was not al-

tered upon incubation with serum albumin or apohemopexin. Therefore cytochrome P-420, which was present in the intact or solubilized microsomal fractions, could be implicated as the source of the heme transferred from these preparations. That cytochrome P-420 was the actual source of the heme transferred to the exogenous proteins is further supported by the experiments showing that upon conversion of cytochrome P-450 to cytochrome P-420, the heme of the latter species is readily transferable to the added heme-binding proteins.

This finding indicates that a barrier exists for heme transfer. Such a barrier may be attributed to (a) the tertiary structure of the cytochrome P-450 molecule, the apoprotein of which encompasses the heme moiety; such a molecular configuration would impose selectivity on the substances which could come in contact with the prosthetic group of the molecule; (b) the location of cytochrome P-450 in the endoplasmic reticulum such that the molecule is "buried" within the membrane; and (c) the combination of both (a) and (b), namely, molecular conformation and mode of association with the membrane. The data presented here, as well as in our previous studies (1, 2), support the first hypothesis. If location within the membrane constituted the barrier to heme transfer, the heme of solubilized cytochrome P-450 should be transferable; however, this was not observed. Regardless of the nature of the barrier, it is apparent that the binding energy of heme and apocytochrome P-450 must be greater than that of heme and apocytochrome P-420. Furthermore, the binding energy of hemopexin for heme is estimated to be about 10^{-8} (6), and that of cytochrome P-450, about 10^{-6} (19). Thus, if affinities were important determinants of the barrier to heme transfer, one would expect apohemopexin to remove the heme from cytochrome P-450; again, this was not observed. From these findings it may be speculated that cytochrome P-450 is localized on the outer surface of the microsomal vesicles, with the prosthetic group of the molecule being shielded from the surroundings by the tertiary structure of the apoprotein. This could explain why the heme of intact cytochrome P-450 is not transferable to the heme-binding proteins while that of the denatured form, cytochrome P-420, is readily transferable. Strittmatter *et al.* (20) have shown that cytochrome b_5 is localized on the outer surface of the microsomal vesicles. Furthermore, a synergistic effect of NADH, which by reducing cytochrome b_5 may provide the second reducing equivalent required for cytochrome P-450-dependent drug oxidation, has been shown (21–23). This would imply that, in order for the synergistic effect to take place, a structural proximity must exist between these cytochromes. It is interesting that even solubilized cytochrome P-450 did not give up its heme moiety, which further supports the importance of the structural configuration of the molecule.

As expected, the transfer of heme from CO-binding particles to apohemopexin was markedly greater than to albumin. Although hemopexin and albumin are so-called secretory proteins, it is conceivable that hemopexin and albumin may serve as intermediary proteins in the intracellular transport of heme from cytochrome P-420 to heme oxygenase (3). At this point, although there is no firm evidence for the natural occurrence of cytochrome P-420 *in vivo*, the possibility that this form may serve as an intermediate in the degradation of heme is most intriguing.

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