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Reconstitution of Carbon Monoxide-Binding Particles after Removal of Heme by Serum Albumin

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SUMMARY

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The nature of cytochrome P-420 present in "CO-binding particles" was studied. This problem was approached by investigating the transferability of heme from CO-binding particles, containing only P-420, obtained through the treatment of microsomal fractions with steapsin, to human serum albumin. In addition, the reconstitution of cytochrome P-420 from "heme-depleted" protein derived from CO-binding particles was investigated. Tritium-labeled cytochrome P-450 was prepared by treatment of rats with [3,5-^{3}H]δ-aminolevulinic acid. The heme of cytochrome P-450 was not transferred to albumin. However, up to 83% of the heme present in CO-binding particles was transferred to albumin after 40 min of incubation. The resulting heme-albumin complex was identified as ferrihemalbumin (methemalbumin) by the criteria of millimolar extinction coefficient, CO difference spectrum, and fractionation profile. CO-binding particles, from which heme had been removed by albumin, could be reconstituted by treatment with methemalbumin. The resulting CO-binding particles showed millimolar extinction coefficients and CO difference spectra indistinguishable from control CO-binding particles. These results suggest that the cytochrome P-420 present in CO-binding particles could represent several complexes of heme and heme-binding sites of a protein, preferentially that of denatured apoprotein of cytochrome P-450, rather than one structural entity. Finally, in regard to the nature of heme-apoprotein interactions in CO-binding particles, it appears that, in comparison to cytochrome P-450, the heme-apoprotein interaction in these particles is modified so that the heme moiety is exposed to the surroundings. Thus, in CO-binding particles, proteins with high heme-binding affinity can readily remove the heme from its apoprotein.

INTRODUCTION

The unusual spectra properties of cytochrome P-450 have generally been attributed to the nature of interaction of this hemoprotein with the membranes of the endoplasmic reticulum. However, when these interactions are disturbed by the treatment of microsomal membranes with a variety of agents (1–5), cytochrome P-450 is converted to a modified form, termed cytochrome P-420, which possesses the usual spectral characteristics of *b*-type cytochromes. The term "CO-binding particles,"

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originally used by Omura and Sato (2), commonly refers to the microsomal preparation devoid of cytochrome b_5 and possessing a high specific activity of cytochrome P-420. These particles are obtained when the microsomal fraction is treated with steapsin, usually 0.2% at 37° for 1 hr. Labeled CO-binding particles have been used extensively in the study of various aspects of microsomal hemoproteins, such as their turnover rate and distribution pattern (6–9).

In the present study the nature of the cytochrome P-420 present in CO-binding particles has been investigated. Previous workers have demonstrated the dissociation and exchange of heme among hemoproteins (10, 11). This knowledge was utilized in the present studies, in which it has been demonstrated that the heme of cytochrome P-420, obtaining by treatment of microsomal fractions with steapsin, is readily transferable to a heme-binding protein, albumin. Furthermore, reconstitution studies were carried out which showed that, after removal of heme from the CO-binding particles by albumin, the "heme-depleted" protein thus obtained could be restored to a CO-binding hemoprotein with properties indistinguishable from those attributed to cytochrome P-420.

MATERIALS AND METHODS

Male Sprague-Dawley rats (180–250 g) were employed as experimental animals. All chemicals were purchased from Sigma Chemical Company unless otherwise indicated.

The heme portion of cytochrome P-450 was labeled according to the method of Levin *et al.* (8). [3,5-^{3}H]δ-Aminolevulinic acid HCl[1] with a specific activity of 510 mCi/mmole (New England Nuclear) was injected intravenously at a dose of 40 μCi/100 g of body weight. Two hours later the animals were decapitated and the livers were removed. CO-binding particles, i.e., cytochrome P-420-rich microsomal pellets devoid of cytochrome b_5, were prepared as follows. Livers were perfused with cold 0.9% NaCl and homogenized in 5 volumes of 0.15 M KCl and 0.05 mM Tris, pH 7.4. Microsomal pellets were isolated from 9000 × *g* supernatant fractions by centrifugation at 105,000 × *g* at 4° for 1 hr, washed once with KCl-Tris buffer, and stored overnight at −10°. The microsomal pellets were resuspended in phosphate buffer (0.1 M, pH 7.4) to a protein concentration of 4–4.5 mg/ml and were treated aerobically with 0.2% steapsin at 37° for 1 hr. In order to prevent the possible photooxidation of heme, the incubations were carried out in the absence of light. The incubation mixture was then centrifuged at 200,000 × *g* at 4° for 2 hr. The reduced minus CO difference spectrum and cytochrome b_5 content were determined before and after steapsin treatment. This treatment released about 95% of cytochrome b_5, quantitatively converted cytochrome P-450 to P-420, and solubilized 10–15% of the cytochrome P-420. The microsomal pellets were washed once with phosphate buffer and resuspended to the original volume in phosphate buffer. Human serum albumin was added to aliquots of the microsomal suspension to final concentrations of 2, 5, 10, 20, and 40 mg/ml. In most experiments the mixtures were incubated at 25° for various times. The mixtures were then centrifuged at 105,000 × *g* at 4° for 90 min. The transfer of heme from CO-binding particles to albumin was monitored by measuring the redistribution of radioactivity between the cytochrome P-420 pellet and the supernatant fraction, using the scintillation mixture of Bray (12), and by measuring the reduced minus CO difference spectra of both the pellet and supernatant fraction. A second aliquot of CO-binding particles in phosphate buffer was treated as described above, except that no albumin was added.

In another experiment both the heme and protein of the microsomal hemoproteins were labeled using the method of Kuriyama *et al.* (13). Uniformly labeled [^{14}C]L-arginine with a specific activity of 20 mCi/mmole was administered at a dose of 5 μCi/100 g of body weight via the tail vein; 2 hr later [^{3}H]ALA was injected (40 μCi/100 g of body weight; specific activity, 510

[1] The abbreviations used are: [^{3}H]ALA, [3,5-^{3}H]δ-aminolevulinic acid; HSA, human serum albumin.

mCi/mmole) via the tail vein. Two hours after the last injection the livers were removed and CO-binding particles were prepared as described above.

The possibility of transfer of labeled heme from cytochrome P-450 to albumin was also studied. Rats were treated with [^{3}H]ALA as before. Microsomes were prepared as described above and washed once with KCl-Tris buffer. The absence of hemoglobin contamination from the microsomal preparation was verified by measuring spectrally the formation of carboxyhemoglobin upon exposure to carbon monoxide. After resuspension in phosphate buffer, cytochrome b_5 content as well as the reduced minus CO difference spectrum was measured. To one aliquot of the microsomal suspension, albumin was added to a final concentration of 2%; a second aliquot served as a control. The mixtures were incubated at 25° for up to 40 min and centrifuged at 105,000 × g at 4° for 90 min; heme transfer was determined by measuring the redistribution of tritium counts between the supernatant fraction and the microsomal pellet, the cytochrome b_5 content, and the reduced minus CO difference spectrum of the microsomal fraction.

Reconstitution of the CO-binding particles after heme removal by HSA treatment was also investigated. CO-binding particles were suspended in phosphate buffer; one aliquot was incubated with 2% HSA for 30 min at 25°, while the control aliquot did not contain HSA. Following centrifugation at 105,000 × g at 4° for 90 min, the pellets were resuspended in phosphate buffer to one-half the original volume. Samples were taken for determination of heme content and for spectral studies. The remainder of each aliquot was divided into two parts. To each part was added an equal volume of hematin or methemalbumin solution (see below). The final concentration of heme in all cases was 0.29 mM. The mixtures were maintained at 25° for 10 min, after which they were centrifuged at 105,000 × g for 1 hr. The pellets were washed and resuspended in phosphate buffer. The heme content and reduced minus CO difference spectra were measured.

The cytochrome b_5 content was determined using sodium dithionate or NADH as the reducing agent and an extinction coefficient of 185 $mM^{-1}cm^{-1}$ (1) between 412 and 425 nm. The contents of cytochrome P-450 and P-420 were determined from the reduced minus CO difference spectra (2), using extinction coefficients of 91 $mM^{-1}cm^{-1}$ between 450 and 490 nm for cytochrome P-450 and 110 $mM^{-1}cm^{-1}$ between 422 and 490 nm for cytochrome P-420. The reduced minus CO difference spectra between 419 and 490 nm of the HSA-containing supernatant fraction were determined using sodium dithionite as the reducing agent. Spectra were recorded on a Aminco DW-2 dual-beam spectrophotometer at room temperature, using 1-cm cuvettes.

Methemalbumin was prepared by a modification of the method described by Rosenfeld and Surgenor (14). A solution of 7.5 mg of crystalline bovine hemin chloride in 1.6 ml of 0.1 N NaOH was prepared, and the pH was adjusted to 7.4 with 1 N HCl. The volume was brought to 20 ml with phosphate buffer (0.1 M, pH 7.4) containing 4% HSA. The final concentration of heme was 0.58 mM; this resulted in the formation of a heme-albumin complex with a 1:1 molar ratio. This molar ratio was verified spectrally and by pyridine-hemochromogen determination. Hematin solution was prepared in the same manner except that serum albumin was not added. The methemalbumin solution was maintained at 25° for 0.5 hr to assure equilibrium. Hematin solution was added to the CO-binding particles immediately after dilution with phosphate buffer because of the instability of the ferriprotoporphyrin at pH 7.4 in the absence of protein.

The concentration of heme was determined by the pyridine-hemochromogen method as described by Paul *et al.* (15), using the reduced minus oxidized difference spectrum between 557 and 575 nm and an extinction coefficient of 32.4 $mM^{-1}cm^{-1}$.

For isolation of heme from the protein, the acid-acetone technique of Fox and Thomson (16) was used. The absolute spectrum of the isolated hemin in acetone or chloroform was recorded.

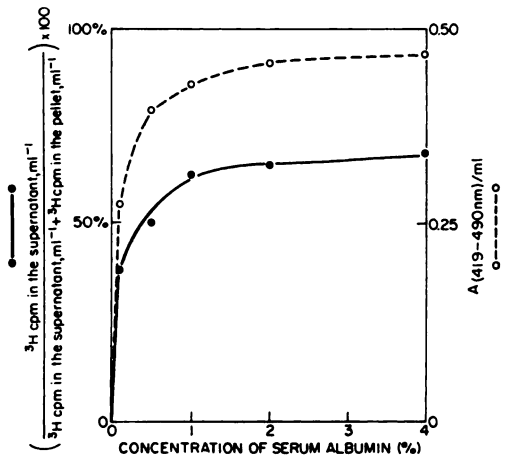

FIG. 1. *Effect of serum albumin concentration on heme removal from CO-binding particles*

Microsomal hemoproteins were labeled by treating rats intravenously with [^{3}H]ALA (40 μCi/100 g of body weight; 510 mCi/mmole). CO-binding particles were prepared by incubating microsomal fractions (4.5 mg of protein per milliliter; 0.77 nmole of cytochrome P-450 per milligram of protein) with 0.2% steapsin for 1 hr at 37°. The CO-binding particles thus obtained were incubated with various concentrations of albumin at 25° for 10 min. At the end of the incubation time the microsomes were resedimented and washed once. Dithionite-reduced minus CO difference spectra of the supernatant fractions were measured as described by Omura and Sato (2). The percentage of tritium activity transferred from CO-binding particles to albumin was determined as described in MATERIALS AND METHODS.

The CO-binding particles, the albumin-containing supernatant, and methemalbumin were subjected to gel filtration on Sephadex G-100 columns (2.5 × 36 cm). Three-milliliter samples were applied to the columns, which were equilibrated and eluted with phosphate buffer (0.5 M, pH 7.4). The flow rate was adjusted to 15–20 ml/hr, and 5-ml fractions were collected. The void volume was determined by use of blue dextran.

The transfer of radioactive heme and protein to albumin is expressed as

$$\left[\frac{^3\text{H or } ^{14}\text{C cpm in supernatant } (\text{ml}^{-1})}{^3\text{H or } ^{14}\text{C cpm in supernatant } (\text{ml}^{-1}) + {}^3\text{H or } {}^{14}\text{C cpm in precipitate } (\text{ml}^{-1})}\right] \times 100$$

In experiments employing both ^{3}H and ^{14}C the radioactivity was determined by the channels ratio method of Okita *et al.* (17). Protein was determined by the method of Lowry *et al.* (18).

RESULTS

The effect of HSA concentration on the transfer of heme from CO-binding particles to albumin is shown in Fig. 1. CO-binding particles were prepared by treatment of the microsomal fraction (4.5 mg of protein per milliliter; 0.77 nmole of cytochrome P-450 per milligram of protein) with steapsin and were incubated with HSA at concentrations of 0.1, 0.5, 1, 2, and 4%. The radioactivity in the supernatant fraction increased with increasing albumin concentration. In addition, the reduced minus CO difference spectrum of the supernatant fraction showed an increase in absorption at 419 nm with increasing HSA concentrations. This increase in absorption in the supernatant fraction parallels the increase in radioactivity transferred to the supernatant fraction. Heme transfer to albumin was maximal at albumin concentrations of 2% and higher. Therefore 2% albumin was used in subsequent experiments.

The time dependency of heme transfer to HSA is shown in Fig. 2. CO-binding particles were incubated with albumin for 0, 10, 20, or 40 min, after which the samples were centrifuged at 105,000 × *g* for 90 min. The results are represented as tritium counts transferred to the supernatant fraction from CO-binding particles. As shown, 87% of the total tritium counts of cytochrome P-420 were transferred to albumin (2%) within 40 min of incubation (25°); in contrast, in the absence of HSA, only 9% of the radioactivity present in control CO-binding particles was removed within the same length of time. The time dependency of heme transfer from cytochrome P-420 to HSA was also monitored spectrally. The reduced minus CO difference spectrum of the CO-binding particles after incubation at 25° with albumin and that of the corresponding supernatant fractions were measured. The increase in absorption at 419 nm in the supernatant

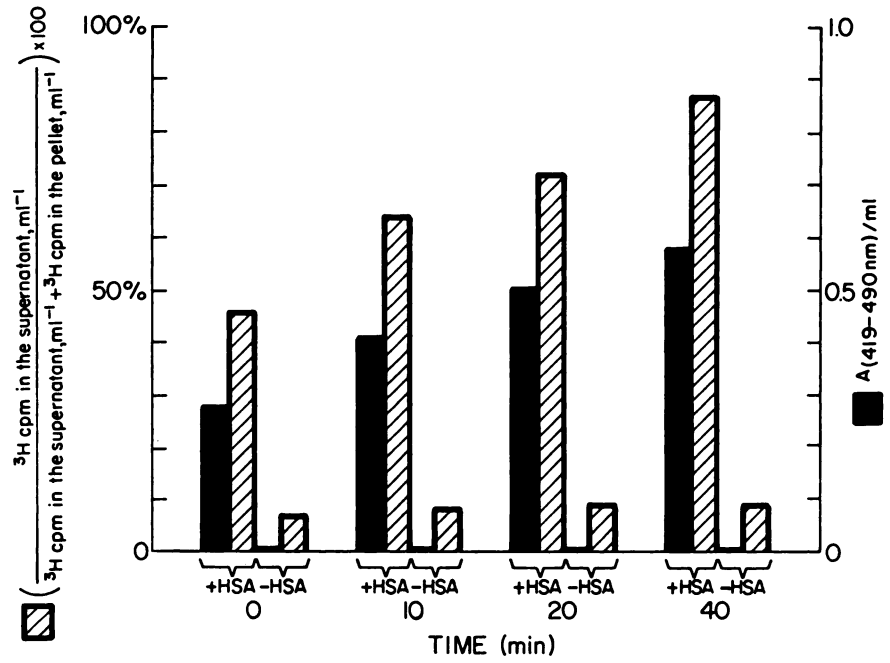

FIG. 2. *Time course of heme transfer from CO-binding particles to serum albumin*
Tritium-labeled CO-binding particles, prepared as described in Fig. 1, were incubated with 2% albumin at 25° for the times shown above. At the end of the respective time periods, the particles were resedimented and washed, and both the reduced minus CO difference spectrum and radioactivity were measured as described in MATERIALS AND METHODS.

was accompanied by a concomitant decrease in absorption at 422 nm in the CO-binding particles.

The transfer of heme from CO-binding particles to albumin was found to be temperature-dependent. When the above study was conducted at 0°, the same pattern of transfer was observed; however, the magnitude of heme transfer was reduced so that in 40 min only 38% of the tritium counts were transferred to albumin while the controls decreased to 5%.

The possibility of heme transfer from cytochrome P-450 was also studied. Microsomal fractions were incubated with 2% albumin for 0, 10, 20, and 40 min and subsequently centrifuged. The microsomal contents of cytochrome b_5 and cytochrome P-450 were determined before and after incubation. Figure 3 shows that the cytochrome P-450 content of the microsomal fraction remains virtually constant during the 40-min incubation period, in the presence or absence of albumin. However, a slight decrease in the levels of radioactivity in the presence of albumin was observed. In addition, the microsomal content of cytochrome b_5 did not change during the incubation period.

Various experiments were conducted in order to determine the nature of the heme transferred to albumin. The results of dual-label studies are shown in Fig. 4. In these experiments the apoprotein portion of microsomal cytochrome P-450 was labeled with ^{14}C, and the heme moiety with ^{3}H. Dual-labeled CO-binding particles were prepared by treatment of the microsomal fraction with steapsin. After 10 min of incubation of the CO-binding particles with 2% albumin, protein transfer was complete, while the heme transfer to albumin continued, indicating transfer of free heme. This observation was confirmed by use of gel filtration chromatography. Control CO-binding particles and the albumin-containing supernatant fractions after equilibration with CO-binding particles were chro-

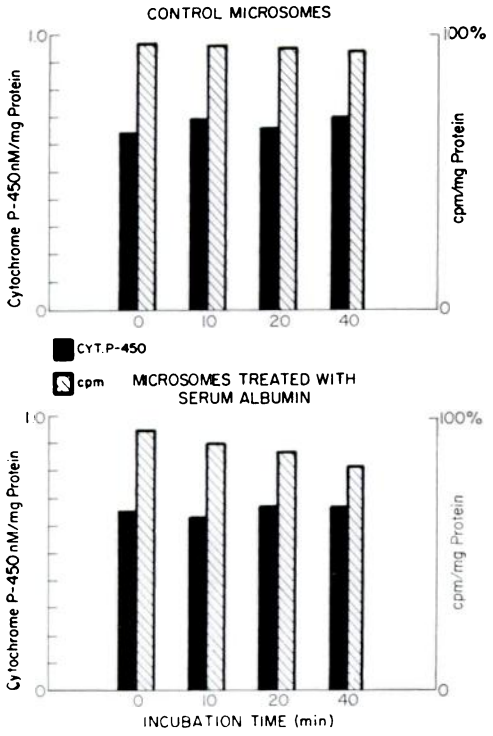

FIG. 3. *Time course of heme transfer from microsomal P-450 to serum albumin*

Rats were treated intravenously with [^{3}H]ALA (40 μCi/100 g of body weight; 510 mCi/mmole), and 2 hr later the animals were killed. Washed microsomal fractions were prepared and incubated with 2% albumin at 25° for the periods indicated. At the end of the incubation period the microsomes were washed once, the reduced minus CO difference spectra were recorded according to the method of Omura and Sato (2), and the radioactivity remaining in the microsomes was determined as described in MATERIALS AND METHODS.

matographed on a Sephadex G-100 column. The recovery of ^{3}H counts from the column was about 60% for both the CO-binding particles and albumin-containing supernatant fractions. As shown in Fig. 5, the elution profile of tritium counts in the case of the albumin-containing supernatant fraction clearly indicates the presence of a new species of radioactive heme-containing material (peaks I and II vs. peaks III and IV). In addition, this different species contained most of the radioactive heme removed from the CO-binding particles. A small amount of radioactivity was eluted at the void volume, corresponding to that of control CO-binding particles. The elution profile of methemalbumin, recorded as absorption at 280 nm (peak V), resembled that of the albumin supernatant fractions, indicating that this species is probably a heme-albumin complex. The extinction coefficients of both methemalbumin (peak V) and peak II were found to be 61 $mM^{-1}cm^{-1}$. The Soret band of the reduced minus CO difference spectra of both methemalbumin and peak II was at 419 nm. The maxima of the Soret, β, and α bands in the reduced difference spectra of methemalbumin and peak II were at 430, 535, and 567 nm, respectively; the ratio

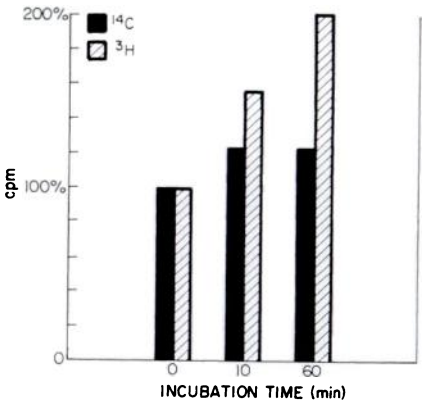

FIG. 4. *Comparative transfer of heme and protein from CO-binding particles*

Rats were treated intravenously with uniformly labeled [^{14}C]L-arginine (5 μCi/100 g of body weight; 20 mCi/mmole); 2 hr later [^{3}H]ALA (40 μCi/100 g of body weight; 510 mCi/mmole) was also injected intravenously. Two hours after the last injection the animals were killed and CO-binding particles prepared as described in MATERIALS AND METHODS. The CO-binding particles were incubated with 2% albumin at 0° for the times shown. After centrifugation at 105,000 $\times$ g for 90 min, the transfer of labeled heme and protein to albumin was determined according to the formula given in MATERIALS AND METHODS. Sixteen and fourteen per cent of the ^{3}H and ^{14}C counts, respectively, were transferred to the supernatant fraction at zero time. These values are normalized to 100%; the increase in radioactivity in the supernatant fractions with time is compared with that obtained at zero time.

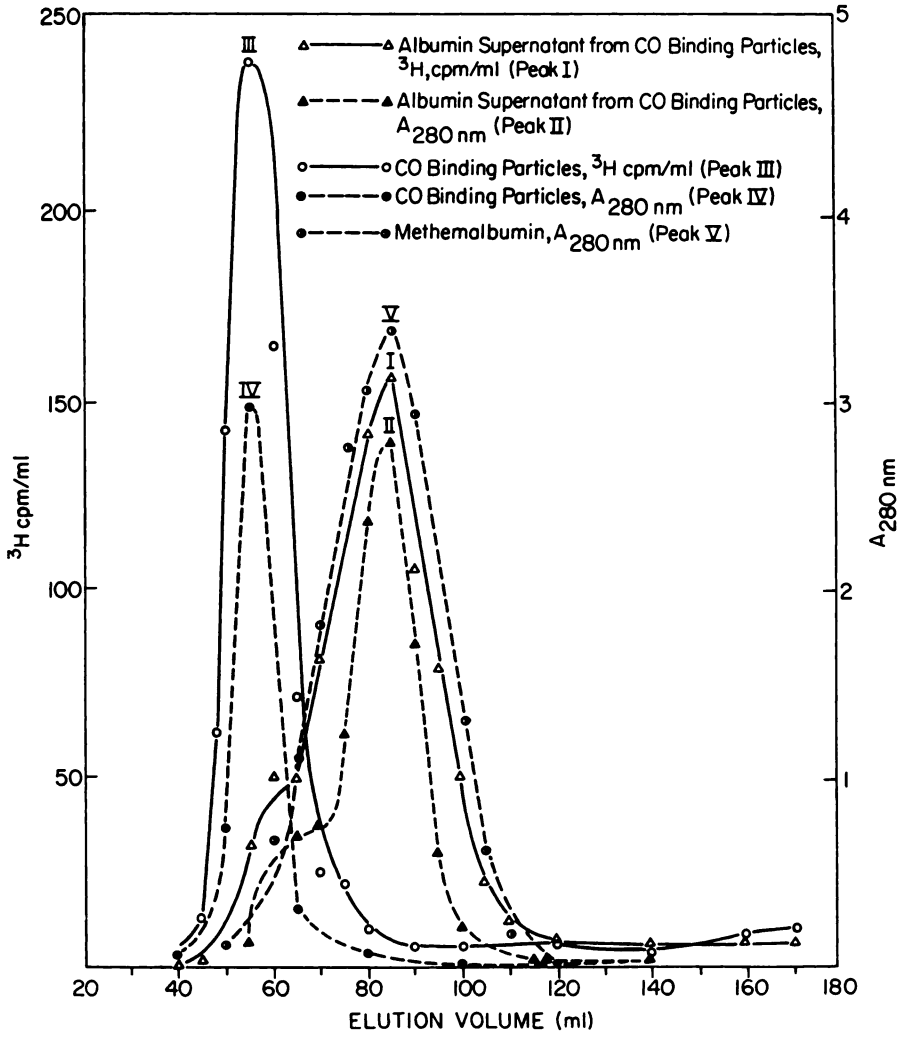

FIG. 5. *Elution profile of CO-binding particles, albumin after equilibration with CO-binding particles, and methemalbumin*

Tritium-labeled CO-binding particles were prepared as described in MATERIALS AND METHODS, and an aliquot was incubated with 2% albumin for 30 min at 25°. Following centrifugation the albumin-containing supernatant fraction was applied to a Sephadex G-100 column (V_0 = 50–55 ml) as described in MATERIALS AND METHODS. CO-binding particles equilibrated with phosphate buffer served as a control. Methemalbumin, prepared as described in MATERIALS AND METHODS, was similarly chromatographed. The elution pattern was monitored by measuring absorption at 280 nm and tritium activity.

of α and β bands between the respective peaks and 500 nm was 2:1 (Fig. 6).

Several experiments were conducted in order to determine the nature of the linkage between the heme, removed from CO-binding particles, and albumin. Attempts were made to remove the heme from albumin by dialysis. The albumin supernatant fraction was dialyzed at 4° overnight against phosphate buffer (0.1 M, pH 7.4). Less than 2% of the tritium counts was lost from the heme-albumin complex. The albumin supernatant fraction and the CO-binding particles were also subjected to extraction with either chloroform or acidified ether; in both instances only about

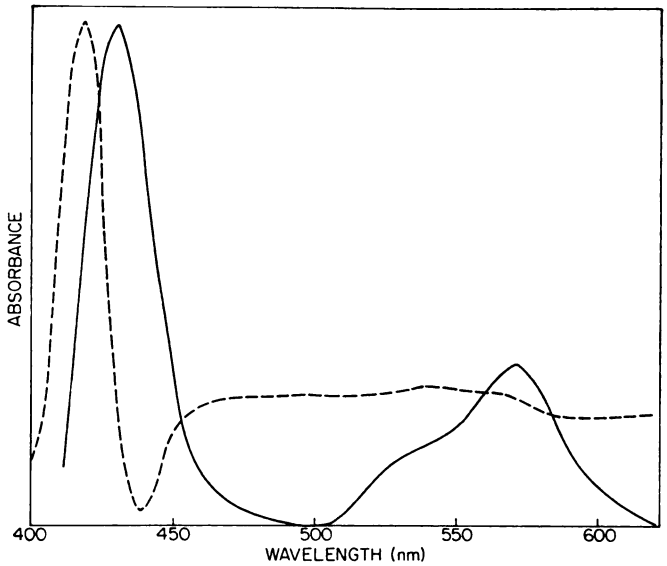

FIG. 6. *Reduced minus CO difference spectrum* (——) *and reduced difference spectrum* (—) *of methemalbumin and supernatant fraction of CO-binding particles after serum albumin treatment*

Methemalbumin was prepared as described in MATERIALS AND METHODS with a 1:1 ratio of heme to protein. CO-binding particles were incubated with serum albumin (2%, 25°, 30 min). Difference spectra of methemalbumin and the supernatant fraction were determined as described in MATERIALS AND METHODS.

1% of the radioactive heme was extracted. However, heme could be extracted successfully from albumin by the acid-acetone technique, and the spectra of heme in acetone and in chloroform showed Soret bands at 365 nm and 375 nm, respectively. Similar results were obtained with authentic hemin in the solvents mentioned above.

The results of reconstitution studies on albumin-extracted CO-binding particles are shown in Table 1. The CO-binding particles were incubated with 2% serum albumin for 30 min, during which time about 73% of the heme was removed from the particles. This was accompanied by a marked change in the millimolar extinction coefficient of the reduced minus CO difference spectrum between the Soret bands and 490 nm. However, the absorption maximum of the reduced minus CO difference spectrum remained the same (Table 1, compare experiments 1 and 2). Treatment of CO-binding particles with phosphate buffer produced no change in either the extinction coefficient or the absorption maximum (Table 1, compare experiments 1 and 3). After removal of heme from the CO-binding particles the resulting "heme-depleted" protein was equilibrated with methemalbumin (1:1 heme to protein molar ratio with a final heme concentration of 0.29 mM) or hematin solution (0.29 mM). After equilibration of the heme-depleted CO-binding particles with methemalbumin the specific activity increased 9-fold over that of the original CO-binding particles; concomitantly the extinction coefficient of the reduced minus CO difference spectrum reverted to its original value, with the absorption maximum of the CO difference spectrum remaining at 421.5 nm (Table 1, compare experiments 1 and 4). In contrast, when the heme-depleted protein was equilibrated with hematin, the specific activity increased about 6-fold, the extinction coefficient of the hemoprotein decreased to 60 $mM^{-1}cm^{-1}$, and the Soret band of the reduced minus CO difference spectrum showed a broad peak at about 420 nm. The increase in specific activity of CO-binding particles when equilibrated with methemalbumin is comparable to that of the heme-depleted CO-binding hemoprotein, with no change in either the

Soret band or the extinction coefficient of the reduced minus CO difference spectrum (Table 1, compare experiments 1 and 6). Hematin treatment of CO-binding particles resulted in similar changes: the specific activity increased to about 160 nmoles/mg of protein, and the Soret band again showed a broad peak at about 423 nm and an extinction coefficient of 49 $mM^{-1}cm^{-1}$.

DISCUSSION

Following the elucidation of the role of cytochrome P-450 in hepatic microsomal drug metabolism (19), many laboratories have studied various aspects of the nature of this hemoprotein. Some of the problems which have been rather extensively investigated relate to the kinetics of heme incorporation and turnover of this hemoprotein. In these studies microsomal preparations are routinely treated with steapsin in order to release cytochrome b_5. The product of this treatment is referred to as "CO-binding particles," which possess a high content of CO-binding hemoproteins, mainly in a form exhibiting a Soret band at about 422 nm, corresponding to the absorption maximum of cytochrome P-420. Cooper *et al.* (20) have suggested that possibly cytochrome P-420 is composed of more than one hemoprotein structure. The findings reported here show that the physical characteristics exhibited by the CO-binding pigments, although they may implicitly denote a structural unit, are manifested by more than one unique combination of heme and protein.

The ready transferability of heme from CO-binding particles to albumin, which was demonstrated in the results presented above (Figs. 1 and 2), provided a simple means by which the heme-depleted protein of CO-binding particles could be prepared with negligible loss of other components of CO-binding particles (Figs. 4–6). As shown in this study, it is possible to reconstitute CO-binding particles by treatment of the heme-depleted CO-binding particles with methemalbumin. These reconstituted CO-binding particles were indistinguishable from untreated CO-binding particles with respect to their absorptivity and reduced minus CO difference spectra (Table 1).

TABLE 1

Reconstitution of CO-binding particles after removal of heme with serum albumin

CO-binding particles were prepared by treatment of liver microsomes with 0.2% steapsin. Heme was removed from the CO-binding particles by treatment with serum albumin (2%, 25°, 30 min). The resulting "heme-depleted" protein was equilibrated with methemalbumin (1:1 heme to protein molar ratio) or hematin; the final heme concentration in both cases was 0.29 mM. The extinction coefficient and the reduced minus CO difference spectrum were measured as described under MATERIALS AND METHODS.

Treatment	Extinction coefficient	Absorption maximum	Specific activity
	mM^{-1} cm^{-}	*nm*	*nmoles heme/mg protein*
1. CO-binding particles	109	421.5	2.6
2. CO-binding particles after HSA treatment	65	421.0	0.7
3. CO-binding particles after phosphate buffer wash	108	421.5	3.7
4. Expt 2 after methemalbumin treatment	109	421.5	23.5
5. Expt 2 after hematin treatment	60	420.0[a]	159.3
6. Expt 3 after methemalbumin treatment	109	421.5	26.4
7. Expt 3 after hematin treatment	49	423.0[a]	158.8

[a] Broad.

The finding that the specific activity of the heme-depleted CO-binding particles increased several fold when reconstituted indicates that multiple heme-binding sites may be present on the apoproteins; these sites may be partially unoccupied because of the normal tertiary structure of the protein. However, upon treatment of the microsomal membranes with steapsin, these sites are made available for heme binding. The specific activities of both the control and heme-depleted CO-binding particles after treatment with methemalbumin were of the same magnitude, indicating that a

limited number of specific heme-binding sites are available. That the increase in specific activity of the CO-binding particles is due to nonspecific heme binding seems unlikely, since this may be expected to result in a lowered absorptivity such as that seen when CO-binding particles are treated with hematin (Table 1).

It can be concluded that the cytochrome P-420 in the CO-binding particles represents a complex between heme and heme-binding sites of the denatured apoprotein of cytochrome P-450. However, the reconstitution studies indicate that the heme-apoprotein complex no longer represents a unique hemoprotein in which a rigid molar ratio of heme to protein is necessitated. In other words, the denatured apoprotein of cytochrome P-450 possesses additional heme-binding sites which may become occupied, as suggested by the increase in specific activity of the CO-binding particles upon reconstitution.

Furthermore, the present study may offer a suggestion as to the nature of the heme-apoprotein interaction in CO-binding particles. It appears that in cytochrome P-450 the association of heme with its apoprotein is such that it renders this moiety inaccessible to albumin (Fig. 3). However, the heme present in CO-binding particles is readily to transferred to albumin, indicating that treatment of the microsomal membranes with steapsin modifies the interactions between the heme moiety of cytochrome P-420 and its surroundings in such a way that the propionic acid groups of the heme are more accessible for binding to albumin while the porphyrin structure remains bound to the apoprotein. These contentions are supported by the observations of Keilin (21) that the link between ferriprotoporphyrin IX and albumin is through the carboxylic acid side chains and that mesohematin, which also possesses a propionic acid side chain, reacts with albumin; in contrast, etiohematin, which lacks carboxylic acid groups, fails to react with albumin. The possibility of heme iron–albumin binding seems unlikely, since the absorption spectrum of the ferriprotoporphyrin-albumin complex (methemalbumin) is greatly different from that of the hemochromogen complexes, in which a coordination bond is formed between the heme iron and the ligands.

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