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Kinetics of the Interaction of Hemin Liposomes with Heme Binding Proteins†

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ABSTRACT: As a model for the transport of hemin across biological membranes, sonicated phosphatidylcholine liposomes with incorporated hemin were characterized. The interaction of the hemin liposomes with the heme binding proteins albumin, apomyoglobin, and hemopexin was examined as a function of liposome charge and cholesterol content. In all cases, there was an almost complete transfer of hemin from liposome to protein; a rapid phase and a slow phase were observed for the transfer. For negatively charged liposomes (with 11% dicetyl phosphate), the rapid and slow phases showed observed rates of transfer of ca. 2 and 0.01 s^{-1}, respectively, for all three proteins. The presence of cholesterol in the liposomes decreased the observed rates by a factor of 2, and positively charged liposomes (with 11% stearylamine) showed about one-fifth the observed rates of negatively charged liposomes. The observed rates were independent of protein concentration, indicating that the rate-determining step is hemin efflux from the lipid bilayer. The hemin interaction with the phospholipid bilayer is suggested to be primarily hydrophobic with some electrostatic character. The two phases are suggested to arise from two different populations of hemin within the liposomes and are interpreted as arising from two different orientations of hemin within the bilayer.

The final step of the biosynthesis of heme[1] is catalyzed by the enzyme ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1) located on the matrix side of the inner mitochondrial membrane (Granick & Beale, 1978). Following this, heme must be transported across the inner and outer mitochondrial membranes to be combined with apoproteins to assemble the heme proteins and enzymes located elsewhere in the cell. Similarly, since the heme precursor protoporphyrinogen is

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[1] Abbreviations: PC, phosphatidylcholine; CtP, dicetyl phosphate; StA, stearylamine; PS, phosphatidylserine; chol, cholesterol; Hm, hemin; P, protein; P_i, inorganic phosphate; Me_2SO, dimethyl sulfoxide; CF, carboxyfluorescein; L, liposomes. "Heme" is used to denote iron protoporphyrin IX, irrespective of oxidation state, whereas "hemin" refers specifically to the 3+ oxidation state.

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synthesized in the mitochondrial intermembrane space, either protoporphyrinogen or protoporphyrin must be transported across the inner mitochondrial membrane to provide substrate for ferrochelatase. The mechanisms by which heme and porphyrins are transported across membranes are not yet understood. There is evidence for the involvement of cytosolic heme binding proteins in the intracellular transport of heme in liver parenchymal cells (Yoda & Israels, 1972; Ketterer et al., 1976; Grandchamp et al., 1981; Davies et al., 1982). Metalloporphyrin efflux from mitochondria is greatly facilitated by the presence of heme binding proteins, particularly hemopexin, ligandin, and apohemoglobin (Husby et al., 1980, 1981). While the participation of cytosolic proteins in intracellular heme transport is possible, there is no evidence for the existence of an intrinsic mitochondrial membrane protein responsible for heme passage. In fact, for the assembly of cytochrome *c*, the apoprotein (synthesized on ribosomes) appears to be translocated across the outer mitochondrial membrane before it can be combined with its heme moiety on the inner surface of the outer mitochondrial membrane (Hennig et al., 1983).

For the liver plasma membrane, a receptor protein has been characterized which interacts with circulating heme–hemopexin and transfers heme into the liver cell (Smith & Morgan, 1981). Hemopexin serves as a scavenger for heme arising during intravascular hemolysis from dissociated hemoglobin subunits and binds heme at a single binding site with high affinity [K_D for hemin–hemopexin $\sim 10^{-13}$ M (Hrkal et al., 1974; Muller-Eberhard & Morgan, 1975)]. In addition to this receptor-mediated heme uptake, there is evidence for at least two other processes by which heme can enter the hepatocytes (Smith & Morgan, 1981).

In order to gain understanding of the mechanisms of the transport of heme across biological membranes, we have undertaken a study of the interaction of hemin, liposomes, and heme binding proteins. Hemin liposomes (i.e., phospholipid vesicles in which hemin is entrapped) have been prepared by several workers. Tipping et al. (1979) reported that phosphatidylcholine bilayers have a high affinity for hemin; they and others (Ginsburg & Demel, 1983) have shown that the interaction of hemin with the phospholipid bilayer has a large hydrophobic component. We are now reporting on the characterization of several types of hemin liposomes and the kinetics of efflux of hemin from these liposomes in the presence of the proteins albumin, apomyoglobin, and hemopexin.

Experimental Procedures

Materials. Purified egg L-α-phosphatidylcholine (in chloroform–methanol solution with 0.1% butylated hydroxytoluene), stearylamine, dicetyl phosphate, cholesterol, hemin chloride, and human serum albumin were purchased from Sigma and used without further purification. Carboxyfluorescein was purchased from Eastman and recrystallized from ethanol–water with decolorizing charcoal; Ultragel AcA 34 was purchased from LKB, Bio-Gel A 1.5M was from Bio-Rad, and Sephadex G-50 and Sepharose 4B were from Sigma. Hemopexin was prepared either according to Hrkal & Muller-Eberhard (1971) or according to Vretblad & Hjorth (1977) with equivalent results. Apomyoglobin was prepared from sperm whale myoglobin (Sigma) by the procedure of Adams (1977) and was shown to migrate as a single well-defined band on a sodium dodecyl sulfate gel.

Preparation of Liposomes. Liposomes were prepared by rotary evaporation of a chloroform solution of the appropriate lipid mixture; the dried lipid film was suspended in pH 7.4 0.1 M KP_i/Cl buffer (0.02 M potassium phosphate/0.08 M potassium chloride) to a final concentration of 20 mg/mL lipid. The milky suspension was vortexed for 2–3 min, sonicated under nitrogen with a microtip-equipped Heat Systems–Ultrasonics Model W-375 sonicator at 60–75 W for 25 min at 20–25 °C, and centrifuged at 50000*g* for 30 min to precipitate large multilamellar structures and titanium particles. The liposomes in the slightly turbid supernatant could be stored up to 3 weeks at 4 °C. Phospholipid concentrations were determined by a modification of the Fiske-Subbarow technique (Naito, 1975) and generally showed a phospholipid content corresponding to 70–80% of the original weight of phospholipid. Liposomes containing carboxyfluorescein (CF) were prepared as described above except that the suspending buffer contained 0.02 M neutralized CF; after sonication and centrifugation, the CF liposomes were passed through a Sephadex G-50 column and collected in the void volume.

To prepare hemin liposomes, hemin was dissolved in 0.1 M NaOH and the pH adjusted to ca. 7.5 with 0.1 M pH 7 KP_i/Cl buffer. This hemin solution, prepared fresh daily, was combined with liposomes (ca. 20× by weight, uncorrected) and sonicated under nitrogen for 7–10 min by using the cup horn attachment at 60–75 W (or alternatively the microtip, in which case centrifugation is necessary to remove titanium). The liposomes were passed through an Ultragel AcA 34 gel filtration column, and the hemin liposomes were collected in the void volume and used within 24 h. Hemin concentrations were measured by the pyridine hemochrome method (Fuhrhop & Smith, 1975). Passage through the gel filtration column was shown not to alter the hemin/lipid ratio, by measurement of hemin and phospholipid concentrations.

Spectral and Kinetic Measurements. All spectra were recorded on a Varian 2290 UV–visible spectrophotometer. Fluorometric measurements were performed on an Aminco-Bowman spectrofluorometer at excitation wavelengths of 490 and 280 nm and emission wavelengths of 520 and 340 nm for CF and albumin, respectively. Rapid kinetic measurements were performed on a Nortech stopped-flow spectrophotometer interfaced with a Cromenco microcomputer by monitoring the increase in absorbance at 405 nm (ϵ = 95 mM^{-1}) for hemin–albumin, 409 nm (ϵ = 90 mM^{-1}) for metmyoglobin, and 415 nm (ϵ = 109 mM^{-1}) for hemin–hemopexin. Observed rates were obtained from the least-squares-calculated slopes of the plots of ln $(A_\infty - A)$ vs. time; the plots were generally linear over 3–4 half-lives. The observed rates, k_1^{obsd} and $k_{1'}^{obsd}$, for albumin were calculated by the method of Provencher (1976a,b).

Results

Preliminary Control Experiments. CF liposomes were used to evaluate the stability of liposomes under the conditions used. CF is a self-quenching fluorophore useful for monitoring liposome stability, since leakage of CF from liposomes leads to an increase in fluorescence (Gregoriadis & Davis, 1979). Dilution of CF liposomes under stopped-flow conditions did not lead to CF release, and the CF liposomes were also stable to incubations with albumin, since these treatments did not lead to an increase in fluorescence within 30 min.

Characterization of Hemin Liposomes. Hemin liposomes of the compositions indicated in Table I were prepared. Most work described below was performed with preparations A and B, negatively charged liposomes, to approximate the charge distribution of mitochondrial membrane lipids. For all preparations, the incorporation of hemin into liposomes gave rise to a shift in the hemin Soret peak from ca. 380 nm (for "free" hemin) to 395 nm (ϵ = 56 mM^{-1}). This peak indicates the presence of monomeric or dimeric hemin and is also observed

Table I: Composition of Hemin Liposomes

prepn	lipid	phospholipid/Hm (M/M) ratio	total lipid/Hm (M/M) ratio
A	PC/CtP, 8/1	14	18
B	PC/CtP/chol, 8/1/3	11	22
C	PC/StA, 8/1	14	18
D	PC/StA/chol, 8/1/3	11	22
E	PC	16	16
F	PS	16	16

in detergent micelles (Simplicio, 1972). Hemin partitions efficiently into liposomes of all compositions (A–F) and partitions into liposomes of compositions A and B even without sonication. Furthermore, neither raising the ionic strength to 1 M with KCl or resonicating previously isolated hemin liposomes led to the formation of free hemin on gel filtration.

An attempt was made to measure the stoichiometry of hemin–phospholipid binding. This was done by preparation of negatively charged hemin liposomes with hemin/lipid ratios from 1/2 up to 1/14 M/M and measuring the amount of hemin incorporation into liposomes by gel filtration. Accurate measurements were not possible because of the high tendency of hemin to aggregate; therefore, Scatchard plots of the results showed a great degree of scatter. Nevertheless, the results appear to indicate that between four and five phospholipid molecules were required to bind one hemin, an observation similar to the results of Tipping et al. (1979). For preparations with lipid/hemin ratios <6 M/M, gel filtration on Bio-Gel A 1.5M allowed resolution of two populations of hemin–lipid species. This finding indicates that under the conditions employed, hemin liposomes may be in equilibrium with hemin–lipid micelles of lower molecular weight. For preparations with lipid/hemin ratios >10 M/M, no low molecular weight species were detectable; therefore, preparations with ratios indicated in Table I were used for all further experiments.

Hemin is well-known to catalyze lipid peroxidation (Kaschnitz & Hatefi, 1975); we observed lipid peroxidation at very high ratios of lipid/hemin (>50 M/M)(Cannon & Waitkus, 1983). However, in the presence of butylated hydroxytoluene, and at the lipid/hemin ratios presented in Table I, no lipid peroxidation was observed.

Interaction with Heme Binding Proteins. When hemin liposomes of the compositions listed in Table I were incubated with albumin, hemopexin, or apomyoglobin, the hemin was rapidly and almost completely transferred from the liposome to the protein. On incubation of hemin liposomes (preparation A) with 2 equiv of albumin for 5 min, passage through an Ultragel AcA 34 column allowed separation of hemin–albumin from liposomes which still contained 5–15% of the original amount of hemin incorporated. This observation indicates that the liposome bilayer structure remains intact in the presence of albumin during this time scale. There are reports that albumin binds phospholipids and causes leakage of liposome contents with $t_{1/2} \sim 1$ h (Zborowski et al., 1977).

To examine the reversibility of the transfer process, hemin–albumin was prepared (Hm/Alb = 0.2–5 M/M) and incubated with liposomes (A or B) at 25 °C, with ratios of phospholipid/hemin = 15–30 M/M. At ratios of Hm/Alb >1, a shift in the Soret absorbance maxima to ca. 395 nm could be observed, reflecting the transfer of hemin to liposome. This spectral change was not accompanied, however, by an increase in the fluorescence intensity of albumin. For experiments involving Hm/Alb ratios <1, little change in absorbance or fluorescence could be observed.

Kinetics of Transfer of Hemin from Liposomes to Protein. Upon mixing of hemin liposomes with protein, an increase in absorbance was observed at the absorption maximum of the hemin–protein complex. For all three proteins, a rapid phase and a slow phase were observed; the results are summarized in Table II. For negatively charged hemin liposomes (A and B), the kinetics for albumin were generally more complex than those for apomyoglobin or hemopexin; the rapid portion itself showed two phases, with k_{obsd} values of 2.6 and 0.4 s^{-1} for each. The presence of cholesterol in the liposomes lowered the rate of the rapid phase by about a factor of 2. The ratio of the magnitudes of the absorbance change during the rapid and slow phases was also affected by cholesterol: the slow effect decreased in size when cholesterol was present.

The observed rates were shown to be independent of protein concentration. For hemopexin and apomyoglobin, the same rates were observed over a range of protein/hemin ratios of 1–20 and for albumin over a range of 1–40. The relative magnitudes of the two effects were somewhat affected by the protein/hemin ratio. With an excess of hemin, e.g., protein/hemin = 0.4, the slow effect (k_2) disappeared for hemopexin and albumin.

In the presence of >0.01 M caffeine, hemin has been shown to be monomeric (Gallagher & Elliot, 1967). If hemin liposomes (A) are prepared in the presence of 0.025 M caffeine, the Soret peak shifts from 395 to 402 nm, indicating that a hemin–caffeine complex has formed. However, since the hemin species formed under these conditions still elutes in the void volume of Ultragel AcA 34, it can be concluded that the

Table II: Kinetics of the Transfer of Hemin from Liposomes to Proteins[a]

L	[Hm] (mM)	protein	concn range (mM)	obsd rate constants (s^{-1}) k_1^{obsd}	$k_{1'}^{obsd}$	k_2^{obsd}	estimated rapid/slow ratio[b]
A	0.006	Alb	0.006–0.24	2.6 ± 0.3	0.4 ± 0.1	$7 \times 10^{-3} \pm 1 \times 10^{-3}$	0.6 ± 0.3
A	0.005	Mb	0.005–0.1	2.1 ± 0.2		$8 \times 10^{-3} \pm 1 \times 10^{-3}$	2.0 ± 0.5
A	0.004	Hx	0.004–0.08	2.4 ± 0.1		$1.0 \times 10^{-2} \pm 1 \times 10^{-3}$	3.0 ± 0.5
B	0.006	Alb	0.006–0.12	1.5 ± 0.2	0.30 ± 0.07	$4.0 \times 10^{-3} \pm 5 \times 10^{-4}$	5 ± 2
B	0.003	Mb	0.003–0.03	1.1 ± 0.1		nm	10 ± 4
B	0.003	Hx	0.003–0.03	1.1 ± 0.1		nm	10 ± 3
A + Caf	0.005	Alb	0.005–0.1	2.8 ± 0.3	0.4 ± 0.1	$6 \times 10^{-3} \pm 1 \times 10^{-3}$	1.0 ± 0.4
A + Caf	0.003	Hx	0.003–0.015	2.8 ± 0.4		$8 \times 10^{-3} \pm 1 \times 10^{-3}$	2.0 ± 0.5
A, μ = 1 M	0.003	Hx	0.003–0.010	0.90 ± 0.04		$1.0 \times 10^{-2} \pm 2 \times 10^{-3}$	2.0 ± 0.4
C	0.005	Mb	0.005–0.05	0.4 ± 0.1		$8 \times 10^{-3} \pm 2 \times 10^{-3}$	1.5 ± 0.5
C	0.003	Hx	0.003–0.02	0.4 ± 0.1		$9 \times 10^{-3} \pm 2 \times 10^{-3}$	1.5 ± 0.5
D	0.003	Mb	0.003–0.03	0.2 ± 0.1		$8 \times 10^{-3} \pm 2 \times 10^{-3}$	3 ± 1
D	0.003	Hx	0.003–0.02	0.20 ± 0.05		$8 \times 10^{-3} \pm 1 \times 10^{-3}$	3 ± 1

[a] Abbreviations: Alb, albumin; Hx, hemopexin; Mb, apomyoglobin; Caf, caffeine; nm, not measurable; μ, ionic strength. [b] Ratio of rapid/slow refers to the relative contributions to the total absorbance change of the rapid and slow phases and is estimated from the ratio $(A_\infty - A_0)_{rapid}/(A_\infty - A_0)_{slow}$. Results are mean ± SEM.

hemin–caffeine complex is incorporated into the liposomes. The kinetics of the transfer of hemin from these liposomes to protein, reported in Table II, were similar to those without caffeine.

A preliminary examination of the ionic strength dependence of the kinetics was also performed. Hemin liposomes (A) were prepared while maintaining an ionic strength of 1.0 M with KCl; mixing with hemopexin at 1.0 M ionic strength lowered the value of k_1^{obsd}, as shown in Table II.

The effect of the method of preparation of hemin liposomes was also examined. In one case (A′), hemin liposomes were prepared as described above, except that the 7–10-min sonication step was omitted. In the other case (A″), hemin was introduced before the 25-min sonication step; i.e., hemin and buffer were added to the dried lipids, sonicated, centrifuged, and separated by gel filtration. Both A′ and A″ hemin liposomes had the same lipid–hemin composition as that of A. The kinetics of the interaction of these liposomes with hemopexin were examined, and the results (not shown) were similar to those given for A.

The interaction of positively charged liposomes (C and D), both with and without cholesterol, with hemin and proteins was examined. The results for apomyoglobin and hemopexin are reported in Table II. The observed rate constants (k_1^{obsd}) were lower than those for negatively charged liposomes; the values were decreased by a factor of 2 in the presence of cholesterol. For albumin, the kinetics were more complex and the observed rates much slower: C + albumin, $2 \times 10^{-2} \pm 5 \times 10^{-3}$ and $2 \times 10^{-3} \pm 4 \times 10^{-4}$ s^{-1}; D + albumin, $1 \times 10^{-2} \pm 3 \times 10^{-3}$ and $2 \times 10^{-3} \pm 2 \times 10^{-4}$ s^{-1}. Preliminary results indicate that there may be some dependence on albumin concentration for these rate constants. The magnitude of the absorbance change during the slow phase for albumin was generally only 5–10% of that during the rapid phase.

Discussion

Hemin associates readily with liposomes of a variety of compositions. The observations reported here, and the fact that even neutral liposomes (E) incorporate hemin, indicate that the hemin is incorporated into the hydrophobic bilayer portion of the liposome, rather than located in the aqueous "core" of the liposome or bound electrostatically to the polar head-group region of the bilayer. These results support the findings of others (Ginsburg & Demel, 1983; Tipping et al., 1979; Makino et al., 1982), who have found that the interaction of hemin with phospholipids is largely hydrophobic with perhaps some electrostatic contribution (vide infra). The hemin is readily removed from liposomes A–F by the heme binding proteins albumin, hemopexin, and apomyoglobin. The results reported here are in contrast to those of Shviro et al. (1982), in which hemin is indicated to bind more strongly to phosphatidylserine liposomes than to apohemoglobin. This difference in results may be due to a subtle denaturation of apohemoglobin; the proteins used in this study may be less susceptible to denaturation than is apohemoglobin (Yip et al., 1972). In fact, Gaber et al. (1983) reported that hemoglobin can be entrapped in PC liposomes without hemin being transferred from globin to the phospholipid bilayer.

The dissociation constants for hemin–hemopexin and metmyoglobin are ca. 10^{-13} and 10^{-12} M, respectively (Hrkal et al., 1974; Gibson & Antonini, 1963). Albumin has a primary binding site for hemin, with $K_D \sim 10^{-8}$ M (Beaven et al., 1974; Adams & Berman, 1980), and several secondary binding sites for hemin of lower affinity (Parr & Pasternack, 1977). The results reported here show that the primary binding site of albumin removes hemin from liposomes, but the opposite is true for the secondary binding sites. This means that the binding strength of hemin to liposomes is between that of the primary and secondary sites of albumin. An accurate determination of the equilibrium constant is at present not possible because of the high tendency of hemin to aggregate.

The kinetics reported allow one to draw some conclusions regarding the nature of the hemin–liposome interaction and the mechanism of hemin efflux from liposomes in the presence of proteins. The independence of the observed rates on protein concentration indicates that the rate-limiting step of hemin–protein formation is the efflux of hemin from the liposome bilayer (vide infra). Upon efflux of hemin from the liposomes, this free hemin, monomeric or perhaps dimeric, is immediately trapped by protein before more slowly reacting hemin aggregates can be formed.

The occurrence of two phases of observed rate constants $k_1^{obsd} \sim 1$ s^{-1} and $k_2^{obsd} \sim 10^{-2}$ s^{-1} means that there are either two sequential processes or two parallel processes giving rise to the two effects. That the second possibility is the correct one is shown by the fact that decreasing the ratio of protein to hemin to 0.4 eliminates the slow (k_2^{obsd}) phase. Were there two sequential processes, with the rapid formation of an intermediate followed by a slow conversion to product hemin protein, product formation would still require the slow phase even with an excess of hemin. For two parallel processes, however, with an excess of hemin, all of the protein binding sites would be filled by the rapid process, leaving none to participate in the slow process.

That there are two parallel processes indicates that there are two different populations of hemin within the liposomes giving rise to the observed rate constants k_1^{obsd} and k_2^{obsd}. This raises the question of what these two types of hemin can be; there are several possibilites outlined below: (1) Hemin is well-known to occur in different aggregation states; thus, the rapid phase could arise from monomeric hemin and the slow phase from dimeric hemin. The fact that the presence of caffeine, which monomerizes hemin, does not eliminate the slow phase argues against this possibility. (2) The rapid phase could arise from unilamellar liposomes and the slow phase from multilamellar liposomes, which could have hemin buried deep within the inner lamellae. The method used for liposome preparation has been shown, however, to produce unilamellar liposomes (Barrow & Lentz, 1980). The hemin liposomes used appear to be a homogeneous population on Sepharose 4B chromatography, and centrifugation at 200000g for 1 h immediately before mixing with albumin did not eliminate the slow phase of hemin–albumin formation. (3) One phase could result from hemin bound hydrophobically within the bilayer and the other phase from hemin bound electrostatically to the outer hydrophilic portion of the bilayer. This is unlikely, because raising the ionic strength to 1 M neither decreases the amount of hemin that can be bound to liposome nor eliminates either phase of hemin–protein formation. (4) One phase could arise from hydrophobically bound hemin and the other from hemin entrapped in the aqueous "core" of the liposome. The kinetics were not significantly dependent on the method of preparation of hemin liposomes (i.e., A vs. A′ vs. A″). Liposomes prepared by the A″ method would be expected to have an increased amount of inner aqueous hemin, whereas those prepared by the A′ method would have an increased amount of hydrophobically bound hemin; thus, this possibility also seems unlikely. (5) A more likely explanation for the two forms of hemin is related to the orientation of hydrophobically bound hemin within the bilayer. Judging from the effects of cholesterol content and of the charge of the

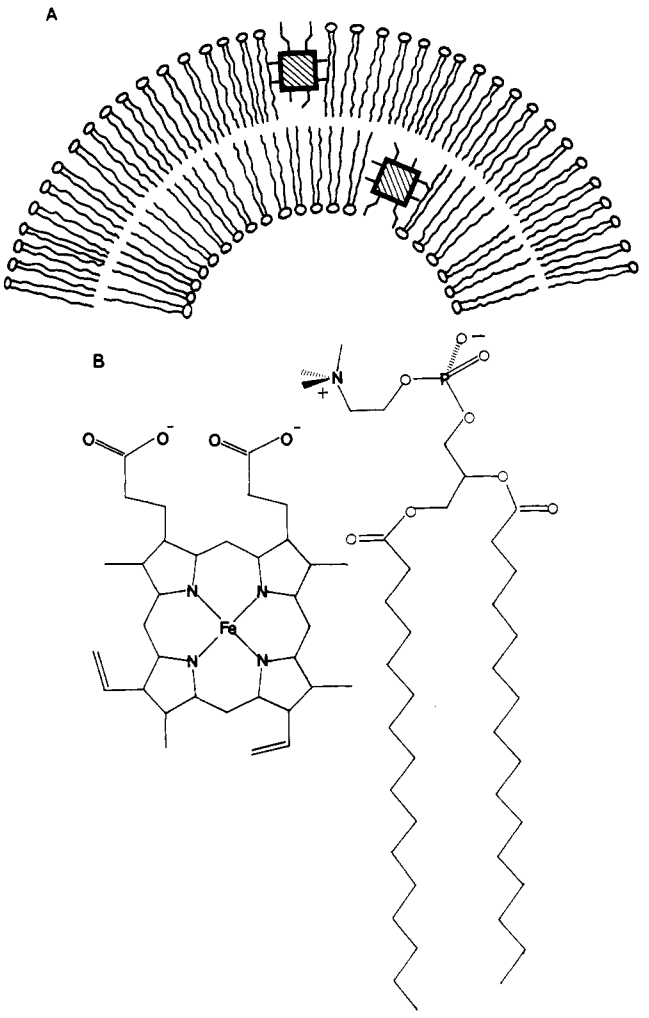

FIGURE 1: (A) Possible inner and outer orientations of hemin in the liposome bilayer which could account for the kinetic results obtained. (B) Detailed structures of phosphatidylcholine and hemin in the bilayer, showing possible electrostatic interaction between the negative charge of hemin and the positive charge of the choline group.

liposomes, it appears that the observed rates k_1 and k_2 are affected by changes in both the hydrophobic and the polar portions of the bilayer. The results of other workers cited above similarly suggest that liposome-bound hemin has both hydrophobic and electrostatic components. It has been suggested that hemin intercalates into the hydrophobic lipid bilayer but that the two carboxylate groups protrude into the polar region to interact with the lipid head groups (Ginsburg & Demel, 1983; Evers et al., 1978); possibly even the iron could be near the surface, with the vinyl group side of the porphyrin ring buried in the bilayer (Tipping et al., 1979). Thus, there could be one orientation of hemin in the outer monolayer with its carboxylates oriented toward the outside of the liposome, and the other similarly oriented in the inner bilayer (Figure 1). The former orientation would give rise to the rapid phase, and the latter (inner) orientation would give rise to the slow phase. This requires a "flip-flop" of hemin, analogous to phospholipid flip-flop, known to have a half-life of hours to days (De Kruijff et al., 1978). Chlorophyll, whose structure is similar to that of hemin, is reported to have a flip-flop $t_{1/2}$ of 4 min in phospholipid bilayers (Birrell et al., 1980). This finding compares well with the observed rate k_2^{obsd} $\sim 10^{-2}$ s^{-1} ($t_{1/2} \sim 1$ min) for negatively charged liposomes.

The kinetic results, therefore, can be explained by the participation of two orientations of hemin, one (HmL_i) in the inner side of the bilayer and the other (HmL_o) in the outer side of the bilayer. The kinetic scheme for the efflux of the latter species is shown in Scheme I. According to the Scheme I

$$HmL_o \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Hm + L$$

$$Hm + P \xrightarrow{k_3} HmP$$

steady-state approximation, the rate law for this scheme would be

$$\text{rate} = \frac{d[HmP]}{dt} = \frac{k_1k_3[HmL_o][P]}{k_{-1}[L] + k_3[P]} \quad (1)$$

If $k_3[P] \gg k_{-1}[L]$, then this expression reduces to

$$\text{rate} = k_1[HmL_o] \quad (2)$$

This would mean that the observed rate (k_1^{obsd}) would be independent of protein concentration and depend only on k_1; the value of k_1 would therefore be ca. 2 s^{-1} for negatively charged liposomes. That $k_3[P] \gg k_{-1}[L]$ is supported by the known values of the rate constants for the binding of monomeric hemin to proteins: 2×10^5 M^{-1} s^{-1} for albumin in 40% Me_2SO, 2×10^6 M^{-1} s^{-1} for hemopexin in 40% Me_2SO, and 6×10^5 M^{-1} s^{-1} for apomyoglobin in phosphate buffer (Adams & Berman, 1980; Pasternack et al., 1983; Adams, 1977).

For the other species, HmL_i, an additional step, the flip-flop, would be required before efflux. The kinetic scheme for the process, occurring in parallel to the k_1-dependent process in Scheme I, is shown in Scheme II. According to the Scheme II

$$HmL_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HmL_o$$

$$HmL_o \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Hm + L$$

$$Hm + P \xrightarrow{k_3} HmP$$

steady-state approximation, the rate law for this scheme would be

$$\text{rate} = \frac{d[HmP]}{dt} = \frac{k_1k_2k_3[HmL_i][P]}{k_1k_3[P] + k_{-2}k_3[P] + k_{-1}k_{-2}[L]} \quad (3)$$

If $k_1k_3[P] + k_{-2}k_3[P] \gg k_{-1}k_{-2}[L]$, then eq 3 reduces to

$$\text{rate} = \frac{k_1k_2[HmL_i]}{k_1 + k_{-2}} \quad (4)$$

Furthermore, if $k_1 \gg k_{-2}$, this reduces to

$$\text{rate} = k_2[HmL_i] \quad (5)$$

Like k_1^{obsd}, k_2^{obsd} would be independent of protein concentration and dependent only on k_2, giving a value for k_2 of ca. 10^{-2} s^{-1} for negatively charged liposomes. The reverse flip-flop rate constant, k_{-2}, would be expected to be the same or somewhat smaller than k_2, thus validating the assumption that $k_1 \gg k_{-2}$.

In the above schemes, k_{-3}, the dissociation rate constant for HmP, is neglected. These values can be estimated from the reported K_D and k_3 values to be in the range of 10^{-3}–10^{-6} s^{-1} (Adams & Berman, 1980; Pasternack et al., 1983; Adams, 1977) and can thus be neglected in Schemes I and II.

For albumin, an additional phase (observed rate $k_{1'} \sim 0.4$ s^{-1}) is observed as part of the rapid process. This probably arises from a conformational change of albumin immediately following the formation of the initial hemin–albumin complex. This result is consistent with previous observations on the combination of albumin with hemin in 40% Me_2SO, in which

the rate constant for the conformational change was measured to be 6.3 s^{-1} (Adams & Berman, 1980). The conformational change has been interpreted to be an internalization of the hemin into the primary binding site. The difference of a factor of 10 in the values of the rate constant between this study and that of Adams & Berman (1980) is probably attributable to the different media employed: phosphate buffer vs. 40% Me_2SO. Moehring et al. (1983) recently proposed the existence of two stable conformations of albumin in aqueous solution; however, it is unlikely that this could give rise to the k_1 and $k_{1'}$ phases, since both of these observed rate constants were independent of protein concentration.

While the observed rate constant k_1^{obsd} had a dependence on ionic strength, there was only a slight dependence for k_2^{obsd}. Since the flip-flop rate would be expected to be controlled primarily by the hydrophobic character of the bilayer, k_2^{obsd} would have little dependence on ionic strength. The observed ionic strength dependence of k_1^{obsd} for negatively charged liposomes (Table II) is consistent with the fact that hemin is also negatively charged. It is interesting to note that the ionic strength has a similar effect on metalloporphyrin efflux from mitochondria: raising the ionic strength by addition of K^+ decreases the efflux (Husby et al., 1980).

With the exception of the interaction of positively charged liposomes and albumin, the kinetics appear to have little dependence on the protein acceptor but are highly dependent on the lipid composition. Cholesterol retards k_1^{obsd} by about a factor of 2 for both positively and negatively charged liposomes and also increases the ratio of the absorbance changes during the fast and slow phases. This would mean that the presence of cholesterol destabilizes the orientation of hemin that gives rise to the slow phase. Cholesterol imparts a greater rigidity to unsaturated phospholipid bilayers; it may be that the inner monolayer, with its greater curvature relative to the outer monolayer, can accommodate hemin less readily when cholesterol is present.

The ratio of the absorbance changes of the observed fast and slow phases (Table II) would be expected to be independent of the protein. This does not appear to be the case for albumin + HmL A. This inconsistency could be an artifact arising from the presence of the $k_{1'}$ phase, which could make the magnitude of the slow effect appear artificially large.

The kinetics of hemin efflux from positively charged liposomes (C and D) are more complex and slower than those from negatively charged liposomes. For apomyoglobin and hemopexin, the greater electrostatic attraction between the positively charged lipids and negatively charged hemin probably accounts for the slower rates observed. The very slow rates observed for albumin were an unexpected result. This result may be due to the reassociation of hemin with liposomes becoming competitive with the binding of hemin with albumin, thus invalidating the assumptions made for the simplified rate laws and giving a complex dependence of observed rates on albumin concentration. More work must be done to investigate this finding. It should be noted that this result is similar to the finding by Husby et al. (1980) that the rate of efflux of a metalloporphyrin from mitochondria was accelerated in the presence of globin or hemopexin but not albumin.

The kinetic schemes depicted above have not dealt with the possibility that the proteins would be able to penetrate the liposome bilayer to catalyze the removal of hemin. If such a process were possible, it could be described by the reaction below, which would occur in parallel to the steps in Schemes I and II:

$$\mathrm{HmL} + \mathrm{P} \xrightarrow{k_4} \mathrm{HmP} + \mathrm{L}$$

The simplified rate laws would become as indicated in eq 6 and 7 for the fast and slow processes, respectively:

$$\text{rate} = k_1[\mathrm{HmL_o}] + k_4[\mathrm{HmL}][\mathrm{P}] \quad (6)$$

$$\text{rate} = k_2[\mathrm{HmL_i}] + k_4[\mathrm{HmL}][\mathrm{P}] \quad (7)$$

The independence of the observed rates on protein concentration indicates that if this penetration can occur, then $k_1 \gg k_4[\mathrm{P}]$ and $k_2 \gg k_4[\mathrm{P}]$. Thus, on the time scale examined, the proteins did not enhance the rate of efflux, and penetration was not detectable. This fact does not exclude the possibility of their penetration into the lipid bilayer over a longer period of time.

In conclusion, the results reported here provide a model system for investigating the intracellular transport of heme, in particular that in the liver cell. Hemin would associate readily with the phospholipid bilayer of the mitochondrial and plasma membranes. The heme binding proteins of the liver cytosol (e.g., ligandin) have affinities for heme similar to those of albumin and thus would be expected to readily accept hemin from these biological membranes. The results reported here provide an interesting comparison to those of Boyer et al. (1983), who showed that liver cytosolic glutathione *S*-transferases (which bind hemin) are not able to penetrate liposome bilayers to remove the lipid-soluble substrates sulfobromophthalein and 1-chloro-2,4-dinitrobenzene. Thus, a plausible mechanism for transport of newly synthesized hemin out of mitochondria would be the partitioning of hemin into the outer mitochondrial membrane by hydrophobic association with the phospholipid bilayer, followed by efflux of hemin and uptake by one of the cytosolic heme binding proteins. The results reported here indicate that penetration by these proteins into the membrane may not be necessary for the efficient transfer of hemin to the protein carrier. Similarly, there is evidence for the transfer of hemin across the plasma membrane by a process which is independent of the heme–hemopexin receptor protein (Smith & Morgan, 1981). The fact that the rate-limiting step of hemin efflux from liposomes in the presence of proteins is the dissociation of hemin may have relevance to hemin transport across both the plasma and the mitochondrial membranes.

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A Relationship between Nuclear Poly(adenosine diphosphate ribosylation) and Acetylation Posttranslational Modifications. 1. Nucleosome Studies†

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ABSTRACT: The chromatin-associated enzyme poly(ADP-Rib) polymerase catalyzes the posttranslational modifications of histones. Antibody to poly(adenosine diphosphate ribose) [poly(ADP-Rib)] has been coupled to Sepharose, and the resulting immunoadsorbant was used to fractionate, specifically, oligonucleosomes derived from cells pulse labeled for the acetylation modifications of chromatin by incubation with [^{3}H]acetate followed by treatment with sodium butyrate. Generally, about 50% of the histone H3 and H4 mass becomes acetylated under these conditions. Pulse-labeled acetylated regions of chromatin were selectively retained by the anti-poly(ADP-Rib)–Sepharose column due to the presence of endogenous poly(ADP-Rib) components. The data suggest that certain histone molecules might be mutally poly(ADP-ribosylated) and acetylated, and this phenomenon was further explored at the protein level in the accompanying paper [Wong, M., & Smulson, M. (1984) *Biochemistry* (following paper in this issue)].

Acetylation, phosphorylation, and poly(ADP-ribosylation) of histones are postsynthetic covalent modifications that may be involved in the modulation of chromatin structure and function. Major questions, still unanswered, are the temporal and topographic relationships between these modifications and how they work in concert to regulate various biological functions. Recently, we developed a technique that is capable of elucidating how these modifications may be structurally related. Antibody to poly(ADP-Rib)[1] was coupled to Sepharose, and the resultant immunoabsorbant was used to fractionate either subpopulations of poly(ADP-Rib) acceptor proteins (Wong et al., 1983a) or domains of polynucleosomes (Malik et al., 1983) undergoing this specific nuclear protein modification. By employment of immunofractionation with

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[1] Abbreviations: ADP-Rib, adenosine diphosphate ribose; NAD, nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; KSCN, potassium thiocyanate; Cl_3CCOOH, trichloroacetic acid.

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