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Interactions of heme with apomyoglobin and lipid bilayers

Light, William Richard, III, Ph.D.

Rice University, 1988
RICE UNIVERSITY

INTERACTIONS OF HEME WITH APOMYOGLOBIN AND LIPID BILAYERS

BY

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A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

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HOUSTON, TEXAS
DECEMBER, 1987
Interactions of Heme with Apomyoglobin and Lipid Bilayers
William Richard Light III

Thesis Abstract

CO-heme interactions with sperm whale apomyoglobin (apoMb) and model membrane systems were examined by spectroscopic and kinetic techniques. The binding of heme to apoMb appears to be a simple bimolecular process at relatively low apoprotein concentrations. The association rate constant, $3 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, was measured directly at 10°C. The circular dichroism spectra for newly reconstituted Mb was initially decreased when compared to native Mb. The half time for equilibration to the native state can range from several hours to days depending on the pH, ligand, and oxidation state. The rate constants for the association or dissociation of oxygen or carbon monoxide to newly formed Mb were identical to native Mb. Kinetic heterogeneity was observed in long-chain isonitrile binding. These results suggest that although rapid uptake of heme by the apoprotein can result in two orientations, there are no apparent changes in the physiological properties of myoglobin.

The effects of vesicle composition on heme binding to membranes were probed using thirty separate lipid mixtures. A sharp decrease in the rate of heme binding to liposomes was observed as the lipid vesicles changed from liquid-crystalline to gel phase. The addition of dicetyl phosphate, which has a negative charge at neutral pH, generally decreased the overall rate and affinity of the vesicles for heme binding. The rate and extent of heme uptake in unsaturated lecithins is unaffected by cholesterol content at levels up to 40% per mole. In contrast, the affinity of saturated dimyristoylphosphatidylcholine (DMPC) vesicles for heme dramatically decreased with increasing cholesterol content.
This effect appears to be related to the influence of cholesterol on the DMPC phase transition temperature \( T_m \). Although there is some variation, most lecithins with low \( T_m \) values have an overall equilibrium partition constant \( \approx 5 \times 10^5 \) and association and dissociation rate constants \( 3 \times 10^6 \text{ s}^{-1} \) and \( 7 \text{ s}^{-1} \), respectively, at 30° C.

Association, dissociation, column chromatography, and temperature dependence studies of CO-heme binding to liposomes indicate that the rate of heme transmembrane movement is slow and dependent on phosphatidylcholine acyl-chain length. The rate increases sharply at the phase transition temperature.
Acknowledgements

This work was made possible by the generosity, wisdom, and patience of my thesis advisor, Dr. John S. Olson. He not only donated the necessary equipment and materials but also provided timely insights and needed guidance. His ability to simplify complex schemes into a process that could be quantitatively analyzed was greatly appreciated and a source of inspiration.

This thesis was a scientific endeavor, but it was also a part of my formal education and development as a person. I have been very fortunate to have been helped by a lot of people. I would like to thank my parents for so many years of unselfish love and support. Their standards are high, but they set them by example. My brothers Ned and Bob not only kept my ego in check, they provided a sense of family. Peg and Clarence were true friends, they were there for the bad as well as the good times. My in-laws were amazingly patient and warm. Finally, my lovely wife Maureen, she deserved better than graduate student life but she never made me feel that way. Her contributions to my life and to this thesis are immeasurable.
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**LIST OF GENERAL SYMBOLS AND ABBREVIATIONS**

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<tr>
<td>ApoMb</td>
<td>Apomyoglobin</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>DT</td>
<td>Sodium dithionite</td>
</tr>
<tr>
<td>(H_b)</td>
<td>Bound heme</td>
</tr>
<tr>
<td>(H_f)</td>
<td>Free heme</td>
</tr>
<tr>
<td>(k_{obs})</td>
<td>Observed rate</td>
</tr>
<tr>
<td>Mb</td>
<td>Myoglobin</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>(O_2)</td>
<td>Oxygen</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>Y</td>
<td>Fractional saturation</td>
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GENERAL LIPID ABBREVIATIONS

Chol  
Cholesterol
DCP  
Dicetyl phosphate
DMPG  
Dimyristol phosphatidylglycerol
PC  
Phosphatidylcholine
PE  
Phosphatidylethanolamine

PHOSPHATIDYLCHOLINE ABBREVIATIONS.

ACYL CHAIN COMPOSITION, and PHASE TRANSITION TEMPERATURES.

<table>
<thead>
<tr>
<th></th>
<th>Chain 1</th>
<th>Chain 2</th>
<th>T_m °C</th>
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<tr>
<td>$cis \Delta^9$DMPC</td>
<td>Dimyristoleoyl PC (9-cis-tetradecenoic acid)</td>
<td>14:1</td>
<td>14:1</td>
</tr>
<tr>
<td>$cis \Delta^9$DPPC</td>
<td>Dipalmitoleoyl PC (9-cis-hexadecenoic acid)</td>
<td>16:1</td>
<td>16:1</td>
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<tr>
<td>DLPC</td>
<td>Dilauroyl PC</td>
<td>12:0</td>
<td>12:0</td>
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<td>DMPC</td>
<td>Dimyristol PC</td>
<td>14:0</td>
<td>14:0</td>
</tr>
<tr>
<td>DPDPC</td>
<td>Dipentadecanoyl PC</td>
<td>15:0</td>
<td>15:0</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitol PC</td>
<td>16:0</td>
<td>16:0</td>
</tr>
<tr>
<td>DTPC</td>
<td>Ditridecanoyl PC</td>
<td>13:0</td>
<td>13:0</td>
</tr>
<tr>
<td>EL</td>
<td>Egg lecithin PC</td>
<td>variable</td>
<td>variable</td>
</tr>
<tr>
<td>OSPC</td>
<td>1-Oleoyl-2-stearoyl PC</td>
<td>18:1</td>
<td>18:0</td>
</tr>
<tr>
<td>POPC</td>
<td>1-Palmitoyl-2-oleoyl PC</td>
<td>16:0</td>
<td>18:1</td>
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The following convention has been followed while naming vesicles of multiple lipid convention. The abbreviations of the components are separated by a slash, the major component (mol⁻¹) listed first. The stoichiometry follows, set off by brackets and the numbers separated by colon, for example, DMPC/Chol/DCP (5:4:1).

(2) Measured in this study (Chapter 4)
Chapter I.

Introduction to the Reaction of Heme with Lipids and Apoproteins

In all vertebrates and a number of invertebrates the transport and storage of oxygen is facilitated by hemoglobin and myoglobin. The functional cofactor of these and a variety of other oxygen binding proteins is protoheme, an iron complex with a conjugated structure made up of four pyrole rings. Gibson and Antonini (1960) examined the kinetics of the reaction of protoheme with apoproteins and later refined their technique with the use of monomeric CO-heme to demonstrate that the combination of the cofactor with apohemoglobin is extremely rapid and approximately monophasic (Gibson & Antonini, 1963). More recently, questions about the exact kinetic mechanism for the formation of holoprotein have been raised because of heterogeneity that is observed in both the binding reaction (Rose & Olson, 1983; Kawamura-Konishi & Suzuki, 1985) and in the final product (La Mar et al., 1984).

La Mar and co-workers have concluded from H NMR studies that heme initially inserts into a variety of natural apoproteins in two distinct orientations that differ by a 180° rotation about the heme α-γ meso axis (La Mar et al., 1978b; 1983; 1984). However, at equilibrium, only one orientation is seen by x-ray crystallography (Kuriyan et al., 1986). The time required for the "disordered" orientation to convert to the predominant crystallographic form is extremely long ($t_{1/2} > 8$ hours at neutral pH: La Mar et al., 1984). Studies by Livingston et al. (1984) suggested that the functional consequence of "heme orientational disorder" is heterogeneous oxygen binding, with the abnormal conformer having a much higher affinity for oxygen. Thus, the time required to reach equilibrium between the two orientations may have physiological implications.
Although in vitro combination of apoprotein and protoheme is simple to demonstrate, in vivo formation of holoprotein is complicated by the logistics of synthesis location. The site of apoprotein biosynthesis is the endoplasmic reticulum in the cytoplasm. The intermediate steps of heme biosynthesis also occur in the cytoplasm, but the terminal enzyme in the heme biosynthetic pathway, ferrochelatase, is located in the inner membrane of mitochondria (Granick and Beale, 1978; Taketani and Tokunaga, 1982; Dailey and Fleming, 1983). The active site, which catalyzes the incorporation of iron with protoporphyrin IX, is actually facing the mitochondrial matrix (Harbin and Flemmin, 1985). Thus, newly synthesized heme must pass through at least two membranes to reach the cytoplasm before it can combine with the apoprotein (Figure 1.1).

The translocation process is a crucial step in the extramitochondrial utilization of heme. A number of investigators have shown that heme, in one form or another, readily associates with lipid bilayers (Tipping et al., 1979; Ginsberg & Demel, 1983; Cannon et al., 1984; Rose et al., 1985). Most of these studies have used liposomes as model membranes. Phosphatidylycholines in aqueous dispersions will coalesce to form a bilayer with the polar head groups facing the water interfaces and the lipid side chains sequestered into the interior. The drive to separate the apolar lipid portion from the water forces the bilayer into a continuous spherical (liposomal) form with encapsulated aqueous medium. This geometry is convenient because the bilayer approximates that found in natural membranes and is easily maintained in solution. The aromatic portion of heme is thought to intercalate between the acyl-chains of neighboring lecithins, and the propionate groups appear to be "anchored" to the polar or hydrated region of the bilayer (Figure 1.2).

It has been suggested that ligandin and other glutathione S-transferases may be
Figure 1.1. A schematic of the proposed biosynthetic pathway of heme and its ultimate binding with apoprotein. Details for this scheme are presented in the text.
Figure 1.2. **Heme placement in a lipid bilayer of DMPC and cholesterol.** The bilayer representation is based on a model of a DMPC bilayer (Gaber et al., 1987), head group orientation (Gally et al., 1975; Seelig & Gally, 1976; and Yeagle et al., 1975), and deuterium NMR studies of the relative orientation of cholesterol in a DPPC liposome (Murai et al., 1986). The heme placement is based on considerations presented by Cannon et al. (1984) and Rose et al. (1985) as well as data presented in this study (Chapters 6 & 7).
involved in intracellular transport of porphyrin derivatives in liver tissue (Jakoby, 1978). However, this has never been tested directly, and Cannon et al., (1984) have questioned the theory's validity. They demonstrated that albumin, hemopexin, and apomyoglobin can extract oxidized heme fairly rapidly from single bilayer, phospholipid vesicles. This was also observed in a similar set of studies in this laboratory (Rose and Olson, 1979; Rose, 1982).

Although several investigations have explored the relationship between heme binding and lipid composition, the results have been conflicting (Tipping et al., 1979; Rose, 1982; Ginsberg & Demel, 1983; and Cannon et al., 1984). In particular, cholesterol has been reported to enhance the uptake of heme (Ginsberg & Demel, 1983), not have an effect on this process (Rose, 1982), or to decrease the equilibrium binding of heme (Cannon et al., 1984). Changes in a bilayer's relative fluidity have been noted as thermal transitions (Lentz et al., 1982) and this may play a role in the binding of heme. The rate of heme transmembrane movement, or the "flipping" of heme from one side of the membrane is still controversial (Cannon et al., 1984; Rose et al., 1985). This is a crucial parameter in considering a scheme of heme movement from the inner matrix of the mitochondria to the cytoplasm. Although other transmembrane processes have been shown to be very temperature sensitive in the region of the bilayer's phase transition temperature (De Kruijff & Van Zoellen, 1978), none of the parameters for heme binding or flipping have been studied systematically as a function of temperature.

Combined studies of heme uptake and release by membranes and incorporation into proteins were feasible because large absorbance changes in the Soret wavelength region (400-450 nm) occur when CO-heme partitioned from aqueous, to apolar or lipid, and to protein environments. The Soret absorption peak is sharp and well defined for each
condition (Figure 1.3a), and both the initial and final environments of the heme moiety can be determined by comparing the wavelength dependence of a particular reaction with the difference spectra of the various species shown in Figure 1.3b.

The association of CO-heme with apoMb was reinvestigated for three reasons. First, it was necessary to examine for heterogeneity in the binding reaction of CO-heme and apoMb in light of the recent studies by Kawamura-Konishi and Suzuki (1985) and LaMar and co-workers (1984). Second, accurate kinetic parameters were required for free CO-heme binding to apoMb to analyze the reaction of the apoprotein with membrane-bound heme. Third, in view of the physiological implications of heme orientational disorder, we sought to confirm both the existence of two heme orientations by circular dichroism and the unique ligand binding properties of the abnormal conformer.

The effects of lipid composition on heme binding to liposomes were examined because of the physiological roles membranes may play in both the biosynthesis and pathology of heme (Chau & Fitch, 1980). A wide variety of liposomes (30 separate compositions) were formed by the extrusion method (Mayer et al., 1986). Selected compositions were characterized both in the presence and absence of heme by U-V light scattering, scanning calorimetry, column chromatography, freeze-fracture electron microscopy, and cryo transmission electron microscopy (cryo-TEM). The association kinetics for CO-heme were followed directly by stopped-flow, rapid mixing techniques and the dissociation rate constants were determined by following the release of membrane-bound heme in the presence of apomyoglobin. To examine the effect of lipid phase transitions, kinetic studies were also done as a function of temperature. Finally, evidence was obtained for the transmembrane movement of heme, and the rate of this process was determined as a function of acyl-chain length.
Figure 1.3. The spectral characteristics of CO-heme in aqueous buffer, egg lecithin vesicles, or as COMb. A, the spectra of 2 μM CO-heme were measured in a 1 cm pathlength cuvette under conditions of only buffer, 100 μM egg lecithin, and 5 μM apoMb, and digitally recorded. B, difference spectra for free CO-heme binding to egg lecithin vesicles, or binding to apoMb, or membrane-bound CO-heme binding to apoMb were digitally created from the spectra presented in panel A. Note that similar absorbance changes can be expected for a 1μM CO-heme solution in the 2 cm pathlength of the stopped-flow apparatus used in the majority of the kinetic studies.
Chapter II.

The Kinetics of CO-Heme Binding to Apomyoglobin

Introduction

Measurements of the kinetics of heme binding to human apoMb or apohemoglobin (globin) were pioneered by Gibson and Antonini (1960). Several fundamental concepts were established by their studies. Only the monomeric form of heme reacts with the apoprotein; the association of heme with apoprotein is extremely rapid; and the reaction mechanism consists of the formation of a reversible intermediate complex and an essentially irreversible final step:

\[
\begin{align*}
\text{Gb} + \text{H}_f & \overset{K_d}{\rightleftharpoons} \text{Gb-H} \\
\text{Gb-H} & \overset{k_I}{\rightarrow} \text{Hb}
\end{align*}
\]

Gb, H_f, Gb-H, and Hb represent free apoprotein, unbound heme, a heme apoprotein complex, and fully reconstituted protein, respectively. \(K_d\) is the equilibrium dissociation constant for the formation of the intermediate complex species, Gb-H. The final step in the formation of fully reconstituted protein is a unimolecular, irreversible step governed by the rate constant \(k_I\). Equation 2.1 can be recognized as the general form for an irreversible bimolecular combination reaction and is usually simplified to a single step. However, both Gibson and Antonini (1960), and Rose and Olson (1983), reported evidence for a spectral intermediate representing Gb-H.

Most investigators agree on the reaction scheme as presented in Equation 2.1 for the formation of reconstituted protein, but the strict interpretation varies. Rose and Olson (1983) presented spectral evidence that the intermediate heme complex does not resemble
free heme or histidine bound heme but rather heme in a lipid environment. This implies that the first step is heme binding to a hydrophobic region of the apoprotein. The final step appears to be a conformational change involving apoprotein folding and iron-histidine bond formation (Rose & Olson, 1983). A point of confusion is a small, slow phase observed in kinetic traces of heme binding to apoproteins. Most investigators attributed this phase to incorrect or non-specific heme binding to the protein surface (Rose and Olson, 1983), or to hemin aggregation (Gibons and Antonini, 1960; Adams, 1977), but Kawamura-Konishi and Suzuki (1985) have suggested that it is the final step in the reaction scheme presented in equation 2.1. The assignment of the slow phase rate constant to $k_f$ would imply a drastically reduced rate constant for protein folding about the heme group, 1 s$^{-1}$ (Kawamura-Konishi & Suzuki, 1985) compared to the value of 500 s$^{-1}$ which Rose and Olson (1983) assigned to $k_f$.

Our reasons for reinvestigating the association of CO-heme with sperm whale apoMb are two-fold. First, if heme can bind to apoMb in one of two distinct orientations then it may be possible to observe differing kinetics for the two orientations, in particular, the anomalous slow phase described for this reaction may be a result of the two orientations. Second, apoMb has been reported to be more stable than globin and therefore it may be useful while studying the reaction of apoprotein with membrane-bound heme. We needed, therefore, to determine accurately the kinetic parameters for the reaction of free heme with sperm whale apoMb.

Materials and Methods

Buffer and Ligand Preparation:

The experiments were carried out in 0.05 M Tris, 0.05 M sodium chloride buffer at pH 8.0, 20° C as they were an extension of previous studies (Rose & Olson (1983), Rose
et al., (1985)) and Tris did not interfere with the phosphate analysis used in the liposome studies presented in the following chapters. CO solutions were prepared by equilibrating the buffer with 1 atm of pure gas (Matheson).

**Heme Preparation and Use:**

Free heme was prepared from hemin chloride (CalBiochem) and solutions were prepared just prior to the experiment by dissolving the solid hemin in 0.01 N NaOH. Undissolved heme was removed with a 5 μm Millipore filter, and concentrations were determined by the pyridine hemochrome method (Smith, 1975) or by measuring the absorbance at 407 nm of the reduced CO form using an extinction coefficient of 147 cm⁻¹ mM⁻¹ (Antonini and Brunori, 1971). Heme solutions were prepared from this stock by injecting the appropriate amount into a sealed syringe of buffer. The actual amount of stock required to achieve the desired final concentration was relatively low and had no measurable effect on the final pH of the buffer solution. Additionally, most experiments utilized the reduced CO form of heme. In these cases the buffer was equilibrated with CO prior to the hemin injection, and sodium dithionite was added to reduce the heme. 1 M stock dithionite solutions were prepared each day by first adding dry dithionite to a test tube, sealing it, flushing with nitrogen, and adding anaerobic buffer. Microliter quantities of the dithionite solution were injected into the sealed CO equilibrated buffer. If the course of the experiment was to continue for several hours and required subsequent heme additions, the heme was stored at 4°C and in the reduced CO form if possible. It was important that CO be present before the addition of the dithionite to the hemin solution; complex heme aggregation products are found if dithionite is added to the hemin before CO.
General Protein Handling

Lyophilized native sperm whale myoglobin (SWMb) and bovine serum albumin (BSA) were purchased from Sigma. The proteins were dissolved in the experimental buffer and filtered through a 5 μm millipore filter. SWMb concentration was determined spectrophotometrically using the reduced CO form and an extinction coefficient of 187 cm⁻¹ mM⁻¹ (Antonini and Brunori, 1971). The reduced form of SWMb was stored under CO. BSA concentration was measured at 280 nm with an extinction coefficient of 44 cm⁻¹ mM⁻¹. All protein handling and storage was done at 4°C unless otherwise noted.

Apomyoglobin Preparation:

Apoprotein was prepared from SWMb using the standard ethyl methyl ketone procedure (Teale, F.W.J., 1959). The myoglobin was first dissolved in a minimal amount of deionized water. The covalent linkage between the proximal histidine of the protein and the iron of the heme group was hydrolyzed by lowering the pH to 2.0 by dropwise addition of 0.1 N HCl to a continuously stirring Mb solution. The free heme was then extracted by the addition of methyl ethyl ketone. After vigorous shaking, and phase separation, the lower aqueous layer containing the apoprotein was recovered. Residual methyl ethyl ketone was removed from the apomyoglobin phase by exhaustive dialysis against water.

The following steps were added due to the sensitivity of the binding experiments to inactive protein. To enhance the yield, it was necessary to aid protein refolding by dialyzing into a low salt solution of 0.0016 M sodium bicarbonate at pH 7. Irreversibly denatured protein was precipitated by dialyzing in the experimental buffers, which contained much higher salt concentrations, and removed by centrifugation. Concentrations were determined by UV absorption at 280 nm using an extinction coefficient of 15.2 cm⁻¹ mM⁻¹. The coefficient was determined with the use of the Bio-Rad Protein assay
(Bradford, 1976) and using SWMb of known concentration for the standard curve. The value is in good agreement with those of 14.9 (Gibson, 1964), 15.6 (Adams, 1977), and 15.9 (Harrison and Blout, 1965). Heme contamination in the apoprotein samples was monitored in the Soret region and was always less than 1% on a per mole basis, and samples were used within a week after preparation. Before use, the apomyoglobin stock solution was filtered and its concentration redetermined.

**Myoglobin Reconstitution Conditions:**

While examining the heme uptake reaction by apoMb, separate μM solutions of heme and apoMb were prepared in separate syringes containing CO-equilibrated, Tris buffer, pH 8.0, and dithionite. These conditions kept the heme both reduced and monomeric during the course of the association reaction. The effects of viscosity were examined by varying the percentage, by volume, of ethylene glycol in the Tris buffer. The final mixture of buffer and ethylene glycol was equilibrated with CO.

**Kinetic Determinations:**

Spectral measurements were made in the 400-450 nm region to take advantage of the sensitivity of the Soret peak to the heme environment and the large extinction coefficients observed at these wavelengths. Rapid mixing experiments, in which the physically separated reaction components are mechanically mixed and the subsequent reactions are observed spectrophotometrically, were carried out with a Gibson-Durrum stopped-flow spectrophotometer as previously described (Reisberg and Olson, 1980). The major limitation of this type of device is the time between the mixing of the solutions and the first measurement. If the reaction being studied is extremely fast, then most of the absorbance changes are complete before observation begins. The 'dead-time' of the
Gibson stopped-flow device is 0.002 seconds and restricts data collection to observed rates of 400 s\(^{-1}\) or less.

The stopped-flow apparatus was computer interfaced to an OLIS microcomputer data collection system allowing multiple traces for each experiment to be averaged as needed to obtain the final time course. To aid resolution of multiphasic processes, individual traces could be collected at two separate time scales. The traces were then fitted to a generalized exponential equation by a non-linear least-squares fitting routine to obtain the observed rate (Bevington, 1969). This routine is capable of analyzing both the rate and the total absorbance contribution for up to five parallel exponentials in one trace.

**Results and Discussion**

*Reaction of ApoMb with CO-heme:*

Sample time courses for the association of CO-heme with apoMb are shown in Figure 2.1 and deviate from single exponential behavior. At least two processes were observed, a fast phase and a slow phase. It can be seen from Figure 2.2 that the rate of the fast phase at 10° C was linearly dependent on the apoMb concentration. The apparent bimolecular rate constant for the fast phase, as determined from the slope in Figure 2.2 and was 2 x 10\(^7\) M\(^{-1}\) s\(^{-1}\). The rate of the slow phase was 2 s\(^{-1}\) at 10° C, pH 8.0 and was not dependent on the apoMb concentration. Other investigators (Rose & Olson, 1982; and Kawamura-Konishi & Suzuki, 1985) using apohemoglobin instead of apoMb also
Figure 2.1. Time courses for the reaction of apomyoglobin with CO-heme.

The association of 0.5 μM CO-heme with 5 μM apoMb was followed in the stopped-flow apparatus at 408 nm, pH 8.0, 0.05 M Tris, 0.05 M NaCl. Note the two time scales. The gray area represents the dead-time of the apparatus in which a fast reaction can be occurring but cannot be measured. The open circles, closed squares, closed circles, and open squares represent data collected at 5, 10, 15, and 20° C, respectively. The insert is the absorption spectra of CO-heme and newly reconstituted sperm whale COMb, 0.5 μM heme content, 2 cm pathlength (the same observation conditions as found in the stopped-flow). The arrowed line indicates the absorbance difference between the two spectra at 408 nm, the observation wavelength of the stopped-flow trace.
Figure 2.2. The concentration dependence of apomyoglobin on the observed rate of CO-heme uptake at 10°C. The association of 1.5 μM CO-heme with apoMb was followed in the stopped-flow apparatus at 407 nm, pH 8.0, 0.05 M Tris, and 0.05 M NaCl; the observed rates were determined by exponential fitting. Two phases were observed at all apoMb concentrations, the closed circles represent the rates of the initial fast phase and the open circles represent the rates of the slower phase.
observed a slow phase that was independent of apoprotein concentration. A rate constant of 0.5 s$^{-1}$ at 10° C, pH 7.0 was measured for the small slow phase (<10% of the total change) when CO-heme was used as the ligand (Rose & Olson, 1982). A larger slow phase, 50% of the total change, was observed when a hemin/caffeine complex was reacted with apoGb, but the rate constant was similar, 1 s$^{-1}$ at 15° C, pH 7.2, (Kawamura-Konishi & Suzuki, 1985).

At 20° C, the relative magnitudes of the observed absorbance changes for the two phases would appear to be roughly equal. However, the total observed absorbance change at 20° C is much less then the expected absorbance change. The expected absorbance change is derived from the difference of the spectra of both free CO-heme and COMb (Figure 2.1; insert). The rate of the fast phase is so high that the majority of the absorbance change is lost in the dead-time of the stopped-flow apparatus. This was confirmed by reducing the rate of the fast phase by lowering the reaction temperature. The magnitude of the absorbance change for the fast phase increased dramatically with decreasing temperature; the absorbance change of the slow phase did not (Figure 2.1). Thus, the slow phase observed while reacting apoMb with CO-heme represents less than 10% of the total absorbance change, even at 20° C. This result is identical to that reported by Rose and Olson (1982) but contradicts the observations of Kawamura-Konishi & Suzuki, 1985.

The decrease in total observed absorbance change with temperature (Figure 2.1) also complicated accurate determination of bimolecular rate constants for the fast phase at higher temperatures. When a significant portion of the absorbance change is lost during the dead-time of the stopped-flow apparatus, the observed rate is based on only a small portion of the reaction. Thus, estimates for the bimolecular reaction rate constants at 20 (≈ 7 x 10$^7$ M$^{-1}$ s$^{-1}$) and 30° C (≈ 1 x 10$^8$ M$^{-1}$ s$^{-1}$) were determined by extrapolation from rate constants measured at temperatures around 10° C (Figure 2.3).
Figure 2.3. The temperature dependence of CO-heme binding to 
apomyoglobin. The binding reaction of 1 μM CO-heme with 5 μM apoMb was 
measured at 408 nm in the stopped-flow apparatus and the observed rates were plotted as a 
function of temperature in an Arrhenius plot. The closed circles represent the measured 
data points and the straight line is a least-squares fit to the data.
The two binding phases observed for the binding of heme to apoMb are spectrally distinct. The wavelength dependence for the calculated absorbance changes of the two phases showed positive peaks at 408 and 414 nm and negative peaks at 425 and 427 nm for the fast and slow phases, respectively (Figure 2.4A). The existence of two spectrally distinct reacting species is highlighted by the observed time course at 418 nm shown in Figure 2.4B. The absorbance change of the fast phase is negative while the absorbance change of the slow phase is positive. This would be predicted from Figure 2.4A.

The wavelength dependence of the fast phase is equivalent to the difference spectra between free CO-heme and native COMb. These spectral changes, and the rate dependence for the reaction of CO-heme and apoMb seen in Figure 2.2, strongly indicate the fast phase represents the combination of CO-heme with apoMb to form the fully reconstituted protein. The wavelength dependence of the slow phase, on the other hand, is closer to a difference spectra for membrane-bound CO-heme reacting with apoMb to form reconstituted protein. If the slow phase does represent the final step in native protein formation (Kawamura-Konishi & Suzuki, 1985), then it can be predicted from Rose and Olson (1983) that this step would represent heme in a hydrophobic environment forming an iron-histidine bond and the resultant wavelength dependence would be similar to that actually observed. This is initially an attractive match: the slow phase would be both a first-order process and independent of apoMb concentration at high protein concentration.
Figure 2.4. Absorbance changes of the two phases observed with the binding of CO-heme to apoMb. 1.5 μM CO-heme was mixed with 3 μM apoMb (final concentrations) at pH 8.0, 10° C in the stopped-flow apparatus. A, the resultant time courses for the recombination reaction were fit to a two exponential expression and the calculated absorbance changes of the fast phase (closed circles) and the slow phase (open circle) were plotted as a function of the observation wavelength. Note the two different absorbance scales, and that the faster absorbance change is ~ 10 times greater than the slower change. B, a sample time course at 418nm, the closed circles represent the observed data points. Note the two different time scales.
However, then the difference spectrum for the fast phase should be similar to that of CO-heme binding to a liposome. The observed difference spectra were closer to that of CO-heme binding to form native Mb. Furthermore, given that the membrane-bound CO-heme peak is midway between free CO-heme and COMb, one would also expect the relative amplitudes of the two phases to be equal at 424nm; and this is obviously not true. Thus, it seems unlikely that the slow phase seen here represents the final step in myoglobin formation.

The relative sizes of the absorbance changes for the two phases did not vary greatly over a four-fold change in apoprotein concentration (Figure 2.5A), or for a 10-fold change in CO-heme concentration (Figure 2.5B). The independence of both the rate and absorbance change of the slow phase with apoprotein concentration indicates that the slow phase does not originate from differing apoprotein species. If, for example, one form of apoMb bound heme more quickly than another, then under limiting protein conditions (excess heme) one would expect to see two different bimolecular phases. However, at high protein concentrations (excess protein), the more rapidly reacting conformer would bind all the available heme and only a single phase would be observed. Thus, the slow phase does not appear to originate from a heterogeneous apoMb population.
Figure 2.5. **Heme and protein concentration dependence of the observed time courses for CO-heme binding to apoMb.** The association of CO-heme and apoMb in 0.05 M NaCl, 0.05 M Tris, pH 8.0, and 20° C was followed at two separate wavelengths, 407 and 423 nm, with the stopped-flow apparatus. *A*, the CO-heme concentration was maintained at 2 μM while the apoMb concentration was varied from 5 μM (*open circles*), to 10 μM (*closed triangles*), and 20 μM (*open squares*). *B*, the apoMb concentration was maintained at 26 μM while the CO-heme concentration was varied from 0.5 μM (*closed squares*), to 2.0 μM (*open circles*), and 5.0 μM (*closed triangles*).
**CO-heme in Ethylene Glycol:**

It has been shown that hemin association with globin is characterized by heterogeneous binding curves thought to be due in part to the slow, first order dissociation of hemin aggregates (Gibson & Antonini, 1960; and Adams, 1977). CO-heme, however, has been reported to be monomeric at low concentrations in simple buffers (Smith, 1959). Furthermore, CO-heme aggregation does not seem likely because of two other experimental observations. First, aggregation does not explain the difference between the separate wavelength dependencies of the two phases observed for CO-heme binding to apoMb (Figure 2.4A). Second, varying the heme concentration should have influenced the relative concentration of aggregates versus monomers and hence the relative magnitudes of the two phases for Mb reconstitution (Figure. 2.5B). However, to be complete, we decided to determine more directly whether or not CO-heme aggregates.

If CO-heme is perfectly monomeric, then in accordance with the Beer-Lambert law, the absorbance of the complex should be proportional to the concentration of heme in solution. If, on the other hand, CO-heme formed aggregates and these exhibited different absorbance characteristics then there should be a deviation from the Beer-Lambert law at higher heme concentrations. In Figure 2.6, the absorbance of heme in CO-equilibrated Tris buffer, and in CO-equilibrated Tris buffer containing 20% ethylene glycol was plotted versus concentration. 20% ethylene glycol was added to aid in the solubilization of CO-heme by lowering the solvent dielectric constant. There is a definite linear dependence of the absorption of CO-heme on concentration in 20% ethylene glycol. There is a slight deviation from linearity at high CO-heme levels in Tris buffer alone. However, linear behavior is observed for CO-heme in Tris buffer at the low CO-heme concentration levels routinely utilized in this study (≤ 5.0 μM).
Figure 2.6. Absorbance of CO-heme as a function of heme concentration.

The closed circles represent CO-heme in the standard Tris buffer (0.05 M Tris, 0.05 M NaCl, pH 8.0) and the open circles is the titration of Tris buffer and 20 % (by volume) ethylene glycol with CO-heme. The straight line is a least-squares fit to the first three open circles.
Further proof of the relative monomeric nature of CO-heme is shown in Figure 2.7 in which the spectra of both CO-heme and free heme are plotted as a function of percent ethylene glycol. The peak height and wavelength of the CO-heme spectrum vary only slightly from 0 to 50% ethylene glycol (Figure 2.7A), but the spectrum of free heme, fully reduced but not liganded, varies greatly from 0 to 70% ethylene glycol (Figure 2.7B). Thus, it appears that free heme does exist as aggregates but again, CO-heme does not at concentrations below 5-10 μM. The role of the CO molecule in keeping heme monomeric is not completely clear. Although CO can bind at both sides of the heme molecule, only one CO is bound in aqueous solutions. If heme stacking is hindered sterically by the bound CO molecule, it would seem that dimerization of the heme molecules could still occur at the unliganded sides. However, since CO binding is a dynamic process, in the presence of high levels of CO the unoccupied side of heme is only available for dimerization for a short period of time. Furthermore, the strength of the π-π interactions that give rise to heme stacking is relatively weak and hence any dimers that do form may by rapidly disrupted by CO molecules binding to the iron.

The slight deviations from perfectly monomeric behavior of CO-heme seen in Figures 2.6 and 2.7 may be the result of transient dimer formation at high porphyrin concentrations. However, it is unlikely that the binding of CO-heme to apoMb is retarded by such interactions. If the formation of CO-heme dimers were responsible for the slow phase, the addition of ethylene-glycol should reduce, if not totally abolish, the slow absorbance change. A series of stopped-flow studies at 20° C were carried out to test if the addition of ethylene glycol would affect the slow phase. In all cases, the time courses exhibited two phases and were similar to those obtained in the absence of ethylene glycol (Figure 2.8A).
Figure 2.7. Absorbance spectra of heme in the presence of ethylene glycol.

A stock amount of heme was added to cuvettes of different ethylene glycol concentrations, reported as percentage (by volume) and the spectra measured in the Soret region. The ethylene glycol levels are indicated in the individual panels. $A$, the absorbance of $3.5 \mu M$ CO-heme. $B$, the absorbance of $2 \mu M$, fully reduced, but not liganded, heme.
Figure 2.8. The influence of ethylene glycol on the binding of CO-heme to apoMb. The percentage of ethylene glycol in the Tris buffer solutions was varied from 0 to 50 % (by volume) and the reaction of 1 μM CO-heme with 10 μM apoMb was followed with the stopped-flow apparatus at 20°C. The time courses were characterized by at least two phases and were therefore fit to two exponentials to obtain the calculated observed rate and absorbance changes of the slow phase are presented here as a function of percentage ethylene glycol. The average value is plotted with the standard deviation as error bars when data from 3 separate experiments was available. The closed and open circles are the changes observed at 407 and 423 nm, respectively.
In addition, the rate of the slow phase did not vary significantly with increasing ethylene glycol concentration. The lack of any changes in rate or amplitude for the slow phase over the entire ethylene glycol range also indicates that the dissociation of CO-heme aggregates is not the cause of the slow phase.

Discussion

The binding of heme by apoMb appears to be a simple bimolecular process at relatively low apoMb concentrations (Figure 2.2). The association rate constant was difficult to determine accurately, even at 10° C, where it is 3 x 10^7 M^{-1} s^{-1}. Rate constants at high temperatures could not be determined directly because the majority of the reaction occurred during the dead-time of the stopped-flow apparatus (Figure 2.1). Extrapolation from rate constants determined at temperatures around 10° C (Figure 2.3), however, indicates that the rate constants are ≈6 and ≈9 x 10^7 M^{-1} s^{-1} at 20 and 30° C, respectively.

The origin of the small, slow, first order process observed during the binding of CO-heme to apoMb is not clear. The slow phase is clearly not a result of CO-heme aggregation or protein heterogeneity, nor does it represent the final step in the combination of CO-heme with globin as proposed by Kawamura-Konishi and Suzuki (1985). It may represent a small proportion of the CO-heme molecule that bind in the pocket in an "upside-down orientation and cannot bind to histidine until the CO has dissociated. This idea would fit with La Mar's observations of heme orientational disorder (see next chapter). In any event, CO-heme binding to apoMb is complete within 1 second and apoMb does appear to be stable for extended time at 20° C. These two considerations make apoMb well suited for later investigations on the reaction of apoprotein with membrane-bound heme.
Chapter III.

Heme Orientational Disorder in Sperm Whale Myoglobin

Introduction

The actual orientation of the asymmetric heme group in myoglobin and hemoglobin has recently been questioned. Only one heme orientation has been observed in x-ray crystallographic studies (Kuriyan et al., 1986). Over the past decade, however, La Mar and co-workers have published a large number of papers which indicate that protoheme can insert into a variety of natural apoproteins in either one of two distinct orientations that differ by a 180° rotation about the heme α-γ meso axis (Figure 3.1: sperm whale apomyoglobin: La Mar et al., 1978b; 1983; 1984; yellowfin tuna apomyoglobin; Levy et al., 1985; monomeric insect hemoglobin from Chironomus thummi thummi: La Mar et al., 1978a; and human apohemoglobin: La Mar et al., 1985). The majority of the studies, however, were done with sperm whale apomyoglobin and the following points are based on those studies. The most compelling evidence for "heme orientational disorder" came from assignments of the H NMR heme methyl resonances using selective deuterium labeling (La Mar et al., 1983) and were confirmed by a careful examination of nuclear Overhauser effects between amino acid side chain protons and those on the 8- and 5-methyl groups of the heme (Lecomte et al., 1985). The apoprotein does not appear to discriminate between heme in either of the two orientations during insertion, and, as a result, newly reconstituted sperm whale myoglobin initially contains a 50:50 mixture of the two conformations. However, at equilibrium the conformer observed exclusively by x-ray crystallography predominates (> 90 %). The reorientation process is very slow (t₁/₂ > 8 h) at neutral pH, is limited by the rate of heme dissociation from the holoprotein, and is
Figure 3.1. The two proposed heme orientations in newly reconstituted sperm whale myoglobin. The \( \alpha-\gamma \) meso axis is indicated by the dotted lines and the 180\(^{\circ} \) rotation indicated by the arrows.
dependent on the coordination structure of the iron atom (La Mar et al., 1984).

The functional consequences of the two heme orientations are less clear. In all previous studies of the reconstitution of myoglobin there have not been any reports of heterogeneous ligand binding behavior by the newly formed holoprotein (for a review see Ascoli et al., 1981 and references contained therein). However, in most cases the samples were stored for extended periods of time prior to analysis, and heme reorientation could have occurred before the ligand-binding measurements were made. Livingston et al., (1984) attempted to resolve this question directly by comparing the oxygen equilibrium curve of newly reconstituted sperm whale myoglobin with that of native protein. They measured a Hill coefficient of 0.85 and a 2- to 3-fold smaller P50 for a freshly reconstituted myoglobin which contained a 55:45 mixture of the two heme orientations as measured by NMR techniques. Quantitative analysis of the equilibrium curve for the reconstituted sample indicated that the abnormal conformer exhibited a 10-fold higher affinity for O2 than the normal or native heme conformation. Livingston et al., (1984) also measured the O2 dissociation rate constant by stopped-flow, rapid mixing techniques. They reported that the observed dissociation rate constant for the reconstituted sample was equal to that measured for native myoglobin. Taken together, these results suggested that the unstable heme orientation should exhibit an approximately 10-fold higher association rate constant for O2 binding that that of the native conformation.

In view of the uniqueness of Livingston et al.'s (1984) oxygen binding results with reconstituted myoglobin and the ramifications of that study and others of La Mar and co-workers, we carried out a careful and systematic study of the ligand-binding properties of newly reconstituted sperm whale myoglobin. Association and dissociation rate constants for O2, CO, and alkyl isonitrile binding to reconstituted and native myoglobin
were measured directly by stopped-flow mixing, conventional flash photolysis, and laser photolysis techniques. The results were analyzed quantitatively in terms of the number of observed kinetic components and the magnitude of the corresponding rate constants. We sought to confirm rotational heterogeneity in the heme pocket by measuring the CD spectra of native and freshly reconstituted myoglobin in the ferric azide and ferrous CO forms.

Materials and Methods
Buffer and Ligand Preparation:

The majority of the experiments were carried out in 0.05 M Tris, 0.05 M sodium chloride buffer at pH 8.0, 20° C. In myoglobin reconstitution studies designed to simulate the conditions described in the oxygen equilibrium investigation of Livingston et.al. (1984), 50 mM sodium phosphate buffer, pH 7.4 was used. All buffer solutions were made anaerobic by bubbling with nitrogen. Stock CO and NO solutions were prepared by equilibrating the buffer with 1 atm of pure gas (Matheson). Oxygenated solutions were air equilibrated. Isonitriles were synthesized and characterized by others in the laboratory following the procedures of Reisberg and Olson (1980). Isonitrile solutions were prepared by injection of the neat liquid into sealed syringes of nitrogen equilibrated buffer. Vigorous shaking for 5-10 minutes was required to fully disperse the long chain isonitriles into solution. Final ligand concentrations were obtained by injecting appropriate amounts of the stock into anaerobic buffer solution in sealed syringes.

Myoglobin Reconstitution Conditions:

Two sets of mixing conditions were employed in the functional studies of newly reconstituted protein. When dithionite was present to keep the system reduced and CO was
available to keep the heme monomeric, hemin and apoMb could be combined in low quantities (µM) directly in the sealed vessel to form reduced Mb. For the flash photolysis studies, this was accomplished in a stoppered cuvette, and for the stopped-flow investigations, a sealed syringe with buffer was utilized. This allowed kinetic measurements to be made very quickly after reconstitution. In order to simulate more closely the higher concentrations employed in H NMR investigations, a stock solution of 0.5 mM MetMb was prepared by combining hemin and apoMb, and then dilutions were made into appropriate buffers for kinetic measurements at various time intervals after reconstitution.

Since dithionite cannot be present while measuring the association rate constant for O₂ binding, it was necessary to reduce an aliquot of the concentrated MetMb stock and then quickly pass it through a small Sephadex G-25 column to remove excess reducing agent and its reaction products. This was repeated for each reconstitution time point. Except where noted specifically, the apomb/heme ratio was never less than one during reconstitution, and generally there was a two-fold excess of apoMb to prevent non-specific heme binding to the apoprotein.

**Kinetic Determinations:**

Spectral measurements were made in the Soret region. Ligand-binding kinetics were measured by one of three techniques depending on the speed of the reaction and photolability of the iron-ligand complex. Rapid mixing experiments were carried out with the stopped-flow spectrophotometer described in Chapter 2. The dead-time of the apparatus was sufficiently short to allow accurate determinations of the association and dissociation rate constants for CO and isonitrile binding to Mb and the dissociation rate
constant for the apomyoglobin complex. Alkyl isocyanide and O₂ dissociation from myoglobin were measured by displacing the ligand with an excess of CO; CO dissociation was observed by displacing with NO. The dissociation rate for O₂ was also measured by employing sodium dithionite as an O₂ scavenger. Although CO binding to newly reconstituted SWMb could be measured by rapid mixing techniques, photolysis techniques were used in most cases. A conventional flash photolysis apparatus equipped with dual photographic strobe units was used for most of the CO experiments (Reisberg and Olson, 1980, McKinnie and Olson, 1981).

O₂ association rate constants were measured using a pulsed dye laser photolysis system (Phaser-R model 2100 B using Rhodamine 575 dye) which is almost identical to that described by Sawicki and Morris (1981). The laser photolysis apparatus is capable of completely photolyzing oxymyoglobin in 1 μs and the rate of recombination can be measured at high oxygen concentrations where the observed rate approaches 1000 s⁻¹. The principle behind this type of on-rate determination is simple, the light pulse photodissociates the ligand and after the flash rebinding is observed.

All three apparatus, stopped-flow, flash, and laser photolysis were interfaced to an OLIS microcomputer data collection system. Modifications were made in the data analysis routines (Bevington, 1969) to allow for any parameter to be fixed by the user so that only the actual parameters of interest were fitted.

**CD Spectral Measurements:**

CD spectra were recorded on a Jasco J500C spectropolarimeter with the supervision of Dr. Graham Palmer of Rice University. The spectropolarimeter was linked to a microcomputer for data processing and calibrated with a standardized solution of
camphor-sulfonic acid. The pathlength of the cell was 1 cm; the scanning parameters were: scan rate, 20 nm/min; time constant, 2 s; number of scans to be averaged, 4 to 8; and spectral band width, 2nm.

Results and Discussion

CD Spectra of Newly Reconstituted Myoglobin:

In agreement with previous reports, we found that the visible absorption spectra of newly reconstituted CO-myoglobin and azide metmyoglobin were identical to those of the native protein. The same was true for the magnetic circular dichroism spectra of the two protein preparations. In contrast, freshly reconstituted myoglobin exhibited considerably less circular dichroism in the Soret wavelength region than the native protein (Figure 3.2). The similarity of the MCD spectra of native and newly reconstituted myoglobin indicates that the oxidation or ligand state of the heme is not changed during reconstitution and that the heme is bound in the heme pocket. The differences in the CD spectra under the same conditions must be attributed to a difference in the local environment of the heme in the newly reconstituted holoprotein. Rather than assuming that the tertiary structure is different, a simple interpretation of the differences in CD spectra is that the heme is bound in the two distinct orientations described by LaMar and co-workers and that the CD spectrum of the reconstituted population is the sum of the spectra of the two heme conformers.

The heme reorientation reaction for CO-myoglobin is extremely slow and difficult to demonstrate (Jue et al., 1983). Even for aquometmyoglobin, equilibration of the conformers is quite slow at neutral pH (t_{1/2} > 12 h), but at pH 5, heme reorientation occurs in less than 5 hours and is readily measured (LaMar et al., 1984). This is demonstrated in
Figure 3.2. Circular dichroism spectra of native and reconstituted sperm whale myoglobin. *Solid lines* represent observed spectra and *dotted lines* are computed spectra for the abnormal conformer as outlined in the text. 

*A*, the ferrous CO-myoglobin forms, pH 8.0, 20°C. The reconstituted sample was prepared by mixing reduced CO-heme with an anaerobic solution of globin (final heme concentration, 9 μM).

*B*, time dependence of the CD spectrum of reconstituted aquometmyoglobin at pH 5.0, 20°C. Aliquots of protein were removed from the reconstitution mixture, sodium azide added, and the spectrum of the azide form measured. The final hemin concentration was 9 μM.
Figure 3.2b. A 2-fold excess of apoprotein was mixed with ferric heme, and aliquots were removed at various times, reacted with 10 mM sodium azide, and the circular dichroism spectrum obtained. The addition of azide prevented any further reorientation and, in effect, fixed the relative amounts of the normal and abnormal heme conformers (LaMar et al., 1984). Under these conditions, there was a monotonic increase in the circular dichroism signal with time of incubation before the addition of azide. After 4 hours, the spectrum of the reconstituted sample was identical to that of the native preparation. At either pH 7 or 8, the CD spectrum of newly reconstituted azide metmyoglobin was identical to that of the 1-min sample shown in Figure 3.2b, but the re-equilibration process was at least 5-10 times slower.

An exact structural interpretation of the results in Figure 3.2 is difficult and beyond the scope of this work. The CD spectrum for the abnormal conformer was computed by adopting LaMar and co-workers theory of two distinct heme orientations and then assuming that the native protein is one component and that the newly reconstituted myoglobin contains a 50:50 mixture of both orientational conformers (dotted curves, Figure 3.2). Working under these assumptions, half of the spectral contribution of the native control was subtracted from the spectral signal of newly reconstituted myoglobin to generate a theoretical CD spectrum for the abnormal heme conformer. The most striking result of these calculations is the lack of circular dichroism in the Soret region for the abnormal heme orientation. For this conformation, there appears to be almost complete cancellation of the rotational strengths arising from interactions between the allowed \( \pi-\pi^* \) transitions of aromatic amino residues and those of the heme group. In the native protein the net rotational strength of these interactions is roughly +0.3 Debye-magnetons (Hsu and Woody, 1971). Thus there must be differences between the interatomic distances and/or
orientations of the heme group and certain aromatic side chains in the two conformations present in newly reconstituted myoglobin.

*Oxygen and Carbon Monoxide Binding*:

Time courses for CO and O₂ association with newly reconstituted myoglobin are shown in Figures 3.3 and 3.4. In the case of CO binding, the reaction was followed by both conventional flash photolysis and stopped-flow, rapid mixing techniques. Identical results were obtained by the two methods, and under pseudo-first order conditions, a single exponential time course was observed for reconstituted myoglobin. The computed bimolecular rate constant for CO binding did not vary with time after reconstitution (Figure 3.5a) and was equal to that of the original native myoglobin sample.

CO dissociation was measured in the stopped-flow apparatus by displacing the bound ligand with NO. Since the NO association rate is on the order of 200-fold greater than the corresponding rate constant for CO binding, the observed displacement rate is equal to the rate constant for CO dissociation at equimolar concentrations of the two gases (Olson, 1981). Again, newly formed myoglobin exhibited monophasic time courses (Figure 3.3B) which were invariant with time after reconstitution (Figure 3.5B). The observed CO dissociation rate constant was equal to that observed for native myoglobin samples.

Oxygen dissociation was measured by reacting MbO₂ with carbon monoxide. In this case, sets of time courses were measured at varying ratios of [O₂]/[CO] in order to determine the oxygen dissociation rate constant, k, and the ratio of the O₂ and CO association rate constants, k'/l' (Olson, 1981). The observed rate is given by $k_{obs} = k / (1 + k' [O₂]/ l' [CO])$. As in the case of CO dissociation, newly formed myoglobin exhibited
Figure 3.3. Time courses for the reaction of newly reconstituted myoglobin with either carbon monoxide or oxygen at pH 8.0, 0.05 M Tris, 0.05 M NaCl, and 20° C. Closed circles, observed data; solid lines, single exponential fits to the observed time courses. A, CO association reaction, 15 μM myoglobin containing 1000 μM CO was photolyzed in the conventional flash photolysis apparatus, 1-cm pathlength, 422nm. B, CO dissociation reaction, 10 μM myoglobin containing 100 μM CO was mixed with 1900 μM NO in the stopped-flow apparatus, 2-cm pathlength, 422 nm. C, O₂ dissociation, 15 μM myoglobin containing 40 μM O₂ was mixed with 100 μM CO, 2-cm cm pathlength, 422 nm.
Figure 3.4. Theoretical and experimental time courses for \( O_2 \) association with newly reconstituted sperm whale myoglobin at pH 8.0, 0.05 M Tris, 0.05 M NaCl, and 20° C. Closed circles represent the observed data points while the solid line represents a single exponential fit. The dotted line was calculated assuming a 10-fold increase in the association rate of the abnormal heme conformer as outlined in the text. The heme and \( O_2 \) concentrations were 10 and 73 \( \mu \)M, respectively. The reaction was carried out in the laser photolysis apparatus with a 2-mm pathlength and observed at 436 nm.
Figure 3.5. **Time dependence of reconstituted sperm whale myoglobin binding kinetics at pH 8.0, 0.05 M Tris, 0.05 M NaCl, and 20° C.** *Solid circles* are the measured rates and *lines* represent least squares analysis of those points. *A*, CO association rates as measured by stopped-flow and flash photolysis. *B*, CO dissociation measured by replacement with NO. *C*, O₂ association rates determined with laser photolysis. *D*, O₂ dissociation rates determined by reaction with dithionite or CO displacement. In all cases the heme to globin ratio was < 1.0. The data points represent the average of at least three independent experiments. In the case of CO association, the data were taken from reconstitution experiments in 0.05 M phosphate, pH 7.4, in 0.05 M Tris, 0.05 M NaCl, pH 8.0, and at high and low protein concentrations. The small standard deviations indicate that the exact conditions have little effect on the observed rates.
B.

CO DISSOCIATION

TIME (hr)

k s⁻¹

0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0
C.

OXYGEN ASSOCIATION

TIME (hr)

\( k' \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1} \)
D.

OXYGEN DISSOCIATION

TIME (hr)

k s⁻¹
monophasic O$_2$ dissociation curves; the fitted values of $k$ and $k'/l'$ were invariant with time after reconstitution; and the observed values were virtually identical to those measured for the native protein. Biphasic oxygen dissociation curves were observed when oxymyoglobin samples were mixed with sodium dithionite in either the absence or presence of CO. The slower phase represented the reduction of metmyoglobin which had been produced by autoxidation and was more prevalent in the reconstituted sample (= 5-30% at the zero time point). The faster phase exhibited a rate equal to the oxygen dissociation rate constant evaluated from the CO displacement reactions.

Laser photolysis techniques were used to measure the reaction of O$_2$ with newly reconstituted deoxymyoglobin because of the large value of the bimolecular rate constant (1.4 x 10$^7$ M$^{-1}$ s$^{-1}$, Table 3.1). Three oxygen concentration, 73,135, and 260 µM, were used in an attempt to observe heterogeneous time courses and to ensure that any fast kinetic phases could be detected. In all cases, monophasic time courses were observed, and the calculated bimolecular rate was in agreement with that measured for the native protein (Figure 3.4). Again, the rate observed for the reconstituted sample was invariant with time and independent of the reaction conditions.

Quantitative Analysis of the Time Courses:

The equilibrium and dissociation rate data reported by Livingston et al. (1984) predict that the O$_2$ association rate constant for the abnormal heme orientation conformer is ~10-fold greater than that for the normal, more stable conformer. Since La Mar et al. (1984) have demonstrated that there is an ~50:50 mixture of the two conformers in the initial reconstituted sample, O$_2$ binding to newly formed myoglobin was expected to exhibit two phases with roughly identical amplitudes and bimolecular rates equal to
Table 3.1
Kinetic and Equilibrium Parameters for Ligand Binding to Sperm Whale Myoglobin at 20°C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kd (nM)</td>
<td>4.7</td>
</tr>
<tr>
<td>K_m (nM)</td>
<td>5.2</td>
</tr>
<tr>
<td>V_max (μM/min)</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Legend:
- Kd: Dissociation constant
- K_m: Michaelis constant
- V_max: Maximum velocity
$\sim 1.5 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ (abnormal orientation) and $1.5 \times 10^7 \text{M}^{-1}\text{s}^{-1}$ (normal orientation). Livingston et al.'s kinetic model was used to compute the dotted theoretical curve in Figure 3.4. As shown, the observed time course for O$_2$ binding does not exhibit a rapid phase and can be successfully fitted to a single exponential expression. This is demonstrated more clearly in Figure 3.6A where the deviations between the observed absorbance changes and those calculated from the fitted, single exponential expressions are plotted versus time. The solid line Figure 3.6A represents the deviations which would have been observed if Livingston's et al.'s (1984) predictions were correct. Although there may be a systematic pattern for the experimental data, the magnitude of these deviations is $< 1\%$ of the total absorbance change (i.e. $\pm 0.002$ absorbance units). In contrast, if the unstable heme conformer did exhibit a 10-fold greater rate of O$_2$ binding, the deviations from single exponential behavior would be on the order of $\pm 10$-20$\%$ of the normalized absorbance change ($\approx \pm 0.040$ absorbance units).

Heterogeneous ligand binding time courses were obtained when excess heme was present in the reconstitution mixture. Figure 3.7 presents data for CO binding to newly reconstituted myoglobin at a heme to globin ratio of 4:1. The observed time course was clearly not monophasic, and quantitative analysis indicated the presence of kinetic phases which exhibit rates both greater and smaller than that observed for CO binding to native myoglobin. The faster phase appears to represent CO binding to heme absorbed to the surface of the protein molecule. The chemical nature of the slower reacting species is unclear but may represent heme entrapped in the interior of the myoglobin. Monophasic ligand-binding curves were only obtained when the heme/globin ratio was $\leq 1$. In most of our experiments, a 2-fold excess of globin was present in the reconstitution mixture to prevent nonspecific binding of heme.
Figure 3.6. Residual plots for ligand binding to newly reconstituted sperm whale myoglobin (4 μM apomyoglobin and 2 μM heme). The time scales have been normalized to the half-times of the individual reactions. The ordinate is the percentage of the total absorbance change. A, closed circles are the observed deviations of the data points from a single exponential fit for a typical O2 association time course while the solid curve represents a theoretical set of residuals computed for the case of a 10-fold greater association rate course for the abnormal heme conformer. B, the closed circles, closed triangles, open circles, open squares, and closed squares are residual patterns computed from time courses for methyl, ethyl, n-propyl, n-butyl, and n-hexyl isonitrile binding to newly reconstituted myoglobin. Note the scale in panel B is three times smaller than that in panel A. The isonitrile reactions were observed at 431 nm in the stopped-flow apparatus.
Figure 3.7. CO association to sperm whale myoglobin reconstituted with a 4-fold excess of heme. Final concentrations were 3, 12, and 1000 μM for myoglobin, heme, and CO, respectively. A, the observed time course for the binding of CO measured by conventional flash photolysis techniques using a 1-cm cell and observing at 422nm. Solid circles are the data points and the solid line represents the single exponential fit. B, residual plot of the same data.
Dependence on Ligand Size:

The results in Figures 3.3-3.6 demonstrate that the two heme orientational conformers in sperm whale myoglobin exhibit identical O₂ and CO binding properties. Thus, the structural perturbations caused by changing the positions of the methyl and vinyl groups in the innermost portion of the heme pocket appear to exert no influence on the strength of the proximal histidine-iron bond nor on interactions between distal amino acid residues and the bound gas molecules. A systematic examination of alkyl isocyanide binding was carried out to see if these methyl/vinyl positional changes caused structural effects which are further away from the iron atom but still in the vicinity of the distal ligand-binding site. A summary of the results is shown in Figure 3.6B and Table 3.1.

When the time courses for isonitrile binding were fitted to a single exponential expression, the observed rates for newly reconstituted myoglobin were equal to or slightly greater than the corresponding values for the native protein. The deviations from monophasic behavior increased with increasing ligand size (Figure 3.6B), but, even in the most extreme case, the reaction with n-hexyl isocyanide, the maximum deviations were ≤10% of the total change (±0.015). A similar phenomenon was observed for the isonitrile dissociation time courses: monophasic behavior was observed for smaller compounds and biphasic traces were obtained with the larger ligands. When the data for the longer isonitriles were fitted to a two-exponential expression, the observed deviations were randomly distributed, and a substantial reduction in the root mean square residual was obtained. However, the exponents and amplitudes of the two phases were poorly defined primarily because the difference in rates was less than or equal to a factor of 3.

La Mar and co-workers' model was adopted in order to estimate more accurately the rate constants for isonitrile binding to the abnormal heme conformation. The time courses
for binding to the newly formed myoglobin were assumed to be comprised of two phases with equal amplitudes. The rate of one of the phases was fixed to that observed for the control sample which was assumed to represent the kinetic behavior of the normal heme conformer. This analysis was applied to the results for all of the ligands examined and is summarized in Table 3.1.

For O₂, CO, and methyl isocyanide, the rate and equilibrium constants computed for the "abnormal" heme orientation are, within the limits of the standard deviations, identical to those for the native orientation. For larger isonitriles, the abnormal orientational conformer exhibits 1.5 to 2.5 times greater association rate constants. In the case of isonitrile dissociation, the abnormal heme conformer exhibits 2- to 3-fold lower dissociation rate constants for the larger isonitriles when compared to those of the native conformation. As a result, the affinity of the abnormal conformer for the larger isonitriles may be as much as 3- to 7-fold greater than that of the native protein, although the propagation of errors prevents an accurate determination of this ratio (Table 3.1).

Discussion

The existence of two distinct heme conformational states in newly reconstituted myoglobin was first proposed by La Mar and co-workers (La Mar et al., 1978b) and is supported by the CD spectral changes shown in Figure 3.9. Similar CD results were observed independently by Aojula et al. (1986). Although an exact structural assignment is not possible from the CD data alone, the pH and oxidation state dependence of the time course for regaining the native CD spectra agrees well with the effects seen for heme reorientation by NMR techniques (La Mar et al., 1984). Thus, it appears that the CD spectrum of a newly reconstituted Mb sample can be used as an accurate measure of heme
disorder and that the reconstitution conditions used in our study do result in a heterogeneous Mb population.

The conformers present in samples of newly reconstituted myoglobins, however, are equivalent with regard to their oxygen, carbon monoxide, and methyl isocyanide-binding properties. This suggests that the methyl/vinyl interchange which occurs at the heme-binding site causes little perturbation of the protein structure in the immediate vicinity of the iron atom. The proximal and distal effects on the rate and extent of ligand binding for these small molecules are the same, regardless of heme rotation about the α-γ meso axis. Only in the cases of the longer isonitriles are changes in the rate and equilibrium constants observed, and even in these cases, the differences in the activation free energy changes between the stable and unstable heme conformers are small. Thus, some perturbation of the protein structure occurs, but these changes appear to be at least 5-6 Å away from the iron atom in the heme (Mims et al., 1983a). A two-dimensional H-NMR study of the structural consequences of heme isomerism in monomeric Hb of *Glycera dibranchiata* also indicated that the few changes in amino acid tertiary structure that may exist are removed from the actual ligand binding pocket (Cooke and Wright, 1987).

Our oxygen binding data do conflict with the oxygen affinity study of La Mar and co-workers which suggested that the O₂ on-rate of the abnormal conformer is substantially greater than the association rate constant found for native Mb (Livingston et al., 1984). Recently, however, another kinetic study also found no evidence for oxygen binding heterogeneity in newly reconstituted Mb and it was suggested that the complicated binding behavior observed by La Mar and co-workers could be the result of a partial oxidation of the newly reconstituted sample (Aojula, et al., 1987). Although complex equilibrium behavior has subsequently been reported for the oxygen off-rate in monomeric insect
hemoglobin by co-workers of La Mar (Gersonde et al., 1986), they speculated that the conditions that led to binding heterogeneity in insect Hb did not exist in Mb and that, in contrast to the earlier report, the binding characteristics of newly reconstituted Mb should be normal.
Chapter IV

An Equilibrium Study of Heme Binding to Complex Lipid Mixtures

Introduction

One of the first investigations to demonstrate heme binding to liposomes was conducted by Tipping and co-workers (1979). The spectral shift in the Soret region when hemin partitions into a bilayer was used to measure equilibrium binding to phosphatidylcholine (PC) or PC/Chol (1:1) vesicles. Although their measurements were complicated by hemin aggregation, they concluded that there was a limiting stoichiometry of approximately 1 hemin per 4 phospholipids for both lipid systems. The presence of cholesterol (Chol) did decrease the apparent affinity constant by a factor of almost 3. Liposomes with bromosulphophthalein also exhibited decreased affinity for hemin which Tipping et al. (1979) attributed to charge repulsion between the ionized propionic acid groups of hemin and bromosulphophthalein. They concluded from analysis of the spectra produced by the binding of hemin to liposomes containing both phosphatidylcholine and ethanolamine that the binding of hemin involves head-group and acyl chain interactions.

One of the most widely used lipid systems for modeling membrane behavior is egg lecithin (EL) liposomes. It was the obvious starting point for studying CO-heme interactions in this lab (Rose et al., 1985). Natural membranes, however, are far more complex. At least 1500 lipids have been identified in vivo. Some of the more common lipids found in biological membranes include phosphatidylcholine, phosphatidylethanolamine, cholesterol, sphingomyelin, and cardiolipin. Phosphatidylcholines are usually present in large amounts. The acyl chain composition can vary widely in PC's, but mammalian forms consist primarily of 16:0, 18:0, 18:1, 18:2, 18:4, 20:4, 20:5, and 20:6
fatty acids. In animals, the fatty acyl chain esterified to the sn 1 position of a PC will often be fully saturated, while the sn 2 position chain is usually unsaturated, however, it is not uncommon for both chains to be fully saturated or unsaturated (Barton and Gunston, 1975). The lipids that make up a membrane vary greatly from organism to organism and even from cellular organelle to organelle. For example, almost half of the lipid content in plasma membranes is cholesterol, whereas endoplasmic reticulum membranes contain only 10% cholesterol and mitochondrial membranes contain even less (Yeagle, 1985). Even in the mitochondrion, the cholesterol content varies; the outer membrane contains a small but significant amount, whereas the inner membrane appears to contain none (Daum, 1985).

Although membranes of varying lipid compositions have the same general bilayer structure, there are substantial differences in the relative fluidity of the hydrocarbon phases. Even for a specific lipid composition, the bilayer viscosity can change dramatically as a function of temperature. The most commonly measured characteristic of bilayers is specific heat capacity. Vesicles of pure phospholipids undergo at least one characteristic thermal transition in which a large amount of heat is absorbed over a narrow temperature range. When the derivative of heat absorbed versus temperature is plotted, the extra absorbed heat during the phase change is seen as a peak. The temperature at which the peak occurs is often reported as the midpoint of the transitional change and is called the phase transition temperature, \( T_m \). The major change is analogous to the chain-melting transition of soaps going from solids to liquids, and involves the acyl chains of the phospholipids. Below the \( T_m \), the acyl chains are extremely ordered in a gel-like state and are considered "solid" because chain interactions and packing are maximized. Above the \( T_m \), the chains are in the liquid crystalline phase and are considered "fluid". Other transitions have been noted in some phospholipids and are thought to involve changes in orientation of head groups or the
glycerol moiety (Gally et al., 1975).

The addition of cholesterol to phospholipid membranes (sphingomyelin: Oldfield & Chapman, 1972; DPPC; DMPC: Mabrey et al., 1978, and mixed chains: Davis and Keough, 1983) has a dramatic effect on the phase transition of the bilayer, and sometimes totally represses the normal phase change as measured by calorimetry. This led Oldfield and Chapman (1972) to conclude that cholesterol perturbs a membrane in such a way to induce liquid-crystalline like behavior in lipids that would normally be in a gel phase state. In general, cholesterol is considered to decrease the order of a bilayer below the $T_m$, and increase the order above the $T_m$ (Pink et al., 1981). In native erythrocyte membranes, increasing the concentration of cholesterol was also found to decrease the membrane fluidity at physiological temperatures (Vanderkooi et al., 1974; Cooper et al., 1978).

The similarity of the four fused rings of cholesterol and the ring system of heme is small. However, both structures are very rigid molecules and have been proposed to intercalate between the acyl chains. It is possible that heme could have an effect on the $T_m$ of the lipid structures. The gel to liquid crystalline phase transition of DMPC vesicles, both in the presence and absence of hemin, was measured by differential scanning calorimetry to test this idea.

Ginsberg and Demel (1983) measured an increase in the surface pressure of a phospholipid monolayer as the result of heme insertion. Similar types of studies have been performed to study phospholipid incorporation into surface monolayers (Pattus, F. et al., 1978; Schindler, 1979; Jähnig, 1984). Ginsberg and Demel found that the pressure change due to hemin addition was dependent on the lipid composition. Negatively charged phospholipids decreased the relative change in surface pressure. They concluded that this decrease was the result of charge repulsion between the propionic acid groups of hemin and
the phospholipid head groups, and that these unfavorable interactions limit the amount of heme which can intercalate into the monolayer. Fully saturated DPPC exhibited a larger change in surface pressure with the addition of hemin than unsaturated lecithins. The presence of cholesterol to the membrane also resulted in an increase in surface pressure with the addition of the same amount of hemin.

Ginsberg and Demel (1983) concluded that intercalation of heme into a monolayer is dependent on two factors, surface potential, and the relative degree of the hydrophobicity of the lipid core. Based on the second conclusion they stated that cholesterol actually facilitated the uptake of hemin and allowed for a greater number of heme groups to be dissolved in the membrane. Finally, they drew a parallel between their results on varying hemin intercalation and the relative toxicity of hemin in vivo with different membrane systems.

Our study of heme binding to membranes was focused on several key lipid types. Phosphatidylcholines were used as the primary liposome component for three reasons. First, lecithins are abundant in natural membranes, and form stable liposomes with a variety of other lipids. Second, the physical properties of phosphatidylcholines have been well characterized. Third, these compounds are readily available commercially in a variety of forms so that parameters like acyl chain saturation and length can be easily manipulated. Cholesterol was also examined. This sterol is naturally abundant, miscible in liposomes composed of phosphatidylcholines, and thought to play a role in altering the general permeability characteristics of membranes and their phase transition temperatures (Oldfield & Chapman, 1972; Mabrey et al., 1978; Davis & Keough, 1983). Finally, dicetyl phosphate (DCP) and dimyristyl phosphatidylglycerol (DMPG) were used to modulate the charge of the membrane.
Unfortunately, the ethanolic injection method described in Rose et al. (1983) for the production of egg lecithin liposomes is not well suited for the rapid production of a wide variety of lipid vesicles for analytical studies. A version of the extrusion method was tested and found to be much more versatile and convenient. Once the method's suitability had been determined, the phase transition temperatures of DMPC vesicles in the presence of DCP or heme were measured by differential scanning calorimetry, and the binding of heme to a variety of DMPC vesicles was qualitatively examined by column chromatography.

Materials and Methods

Liposome Preparation, Extrusion Method:

Dicetyl phosphate and cholesterol were purchased from Sigma. All other lipids were purchased from Avanti Polar-Lipids, Inc. The purity of these compounds was checked by TLC on silica gel chromatography plates. A series of liposomes of defined size but varying composition were prepared by rapid extrusion (Hope et al., 1985; Mayer et al., 1986). The lipid components were weighed and mixed in a minimum amount of chloroform/methanol 2:1 in a round bottom flask. A rotovap at 36°C was used to remove the chloroform/methanol and to deposit the lipid mixture in a thin white film along the sides of the flask. Large multilamellar vesicles were prepared by adding the standard tris buffer, 0.05 M NaCl, 0.05 M Tris, pH 8.0, to the dry lipid and vortexing. A typical total lipid concentration was 25 mM. The aqueous lipid dispersion was then forced through polycarbonate filters (Nuclepore Corp.) using the Extruder™ (Lipex Biomembranes Inc.). 2-8 ml of sample was put into the central cavity of the apparatus through a quick-release valve and then pressure was applied via a standard compressed gas cylinder of nitrogen. The only exit for the sample is through the polycarbonate filters located at the bottom of the
chamber. Pressures of up to 800 lb/in² were employed, but at these high pressures two stacked filters were required to prevent tearing. The cycle was repeated for a total of at least 8 passes to insure uniformity of the sample.

Multilamellar vesicles are still present in the effluent from filtration with pore sizes larger than 200nm (Mayer et al., 1986), so a final pore size of 100 nm was routinely used to insure that only unilamellar liposomes were present. To prevent the filters from clogging, it was necessary to start extruding at a larger pore size, generally 200 nm, and subsequently decrease the pore size by changing the filters. Lipid mixtures containing cholesterol had to be extruded first at even greater pore sizes. All manipulations, including the original dispersion step, were carried out at a temperature greater than the phase transition temperature for that particular liposome. This was accomplished by immersing the Extruder™ into a water bath and regulating the temperature.

Phosphate Determination:

The phospholipid content of the unilamellar vesicles was assayed by the method of Chen, Toribara, and Warner (1956). Samples were diluted to 1 ml final volume with deionized water, four drops of concentrated sulfuric acid added, and the mixture heated until thick white sulfur trioxide fumes appeared. To the remaining dark liquid 3 drops of 60% perchloric acid was added and the heating renewed until all traces of color and fumes were gone. The final ashed sample solution was diluted with deionized water. An equal volume of assay reagent was added to the ashed sample solutions and the final mixture was incubated at 37° C for 1.5 hours prior to reading its absorbance at 820 nm. The concentration was determined by comparison of the unknown samples with a linear graph constructed at the same time from known phosphate standards. The assay reagent was
mixed just prior to use and consisted of 2 parts water, 1 part 6N sulfuric acid, 1 part 2.5% ammonium molybdate, and one part 10% ascorbic acid. The ascorbic acid could be stored for a maximum of 6 weeks at 4°C.

*Differential Scanning Calorimetry:*

The gel to liquid crystalline phase transition of lipid bilayers was measured with a Microcal MC-2 differential scanning calorimeter that was interfaced to an IBM PC with the DA-2 data acquisition and analysis system (Microcal). The scan rate was 30°C per hour with a 15 second filter constant and 16 x sensitivity setting. Concentrated liposome suspensions (5-20 mM phosphate) were used. The data were analyzed in terms of phosphate content, peak height, location, width, and area. These studies were done under the guidance of Dr. Martha Mims at the Baylor College of Medicine, Department of Medicine.

*Electron Microscopy:*

The structure and size range of vesicles prepared by extrusion were examined with the assistance of Dr. Ron Price of the Naval Research Laboratory by freeze-fracture and cryotransmission electron microscopy. The electron micrographs were taken on a transmission electron microscope (a Carl Zeiss EM10-CA with a CryoStage). For freeze fracture the samples were transferred to Balzers (Hudson, NH) copper specimen plates, blotted to remove excess fluid, equilibrated at room temperature, and then quickly frozen by plunging into a nitrogen slurry. The slurry is prepared by exposing liquid nitrogen to a vacuum until the nitrogen crystallizes. After freezing, the samples were placed in a BAF 400D freeze fracture device, fractured, and then replicated at -100°C and 10^-6 Torr. The
replicas were formed with 2nm Pt-C and 20nm of carbon film. The replicas were then floated off into distilled water, exposed to sodium hypochlorite for 3 hours, rinsed in distilled water, cleaned with 20% ethanol for an hour, and then transferred to Butvar B-98 coated grids for examination.

Cryo-TEM was performed by putting a minimal amount of sample directly on a Butvar B-98 coated grid, blotting off the excess fluid, and then rapidly freezing the specimen by plunging the grid into a nitrogen slurry. The sample was then transferred to a CryoStage in liquid nitrogen and placed in the electron microscope, taking care not to allow the sample temperature to rise above the devitrification temperature (140° K). The observation temperature was typically below 115° K and low electron doses were used to preserve the specimen's structure. The presence of vitreous instead of hexagonal ice was checked by electron diffraction. The presence of any internal structures in the liposomes was probed for by altering the focus through the liposome.

*Column Chromatography:*

Molecular sieve chromatography was used to separate liposomes from unbound hemin in order to determine if heme binds to a particular lipid composition. A 1.5 x 30 cm column with Sephadex G-200 was maintained at 4° C. Fractions were collected at a rate of 10 ml/hr by a LKB 2100 series fraction collector system and both the absorbance and transmission were continuously monitored at 280 nm. The column was visually monitored and the spectra of samples that appeared to contain hemin were measured between 350 and 450 nm. Qualitatively, the presence of hemin was obvious by its green color which is quite different from the white appearance of liposomes. Free hemin has a broad absorbance between 350 and 400 nm but there is a shoulder at 390 nm that can be used for
quantification. The contribution of any lipid must be subtracted out. The absorbance of the lipid can be estimated from the wavelength dependence of light scattering (Figure 4.1) and using the absorbance at 450, where the contribution from hemin is minor (Figure 4.5), as a baseline.

Results

Liposome Characterization:

The size of the vesicles produced by extrusion is related only to the pore size of the filter and appears to be independent of lipid composition (Hope et al., 1985). An important parameter is the number of extrusion cycles required for the process to be complete. The dependence of vesicle size on the pass number through the filter was determined by monitoring the absorbance of the liposome solution at 280 nm. The lipids themselves have no intrinsic absorbance at 280 nm, the observed signal is due to light scattering of the vesicles. This is shown in Figure 4.1 in which the predicted wavelength dependence of light scattering, 1/wavelength^4, is contrasted to the observed liposome "spectrum". The magnitude of the absorbance due to light scattering is proportional to the size of the particle and hence a change in absorbance can be related to a change in size by normalizing to the total lipid phosphate concentration.

As shown in Figure 4.2, a lipid suspension was passed through filters of decreasing pore sizes (200, 100, 50, and 30 nm) and the logarithm of the absorbance of the effluent was plotted versus pass number to show the stepwise decrease in the vesicle size. A mathematical relation is not meant to be implied by the choice of a ln plot, but rather it was used to emphasize the change during extrusion with the lower pore sizes. There is roughly a 40-fold difference in absorbance from the first extrusion at a pore size of 200 nm
Figure 4.1. **Absorbance spectrum of egg lecithin liposomes.** The vesicles were prepared by extrusion of egg lecithin through a 100 nm polycarbonate filter at 30° C. The points represent the measured values while the solid line represents a theoretical spectrum based on the predicted wavelength dependence of light scattering, $1/\text{wavelength}^4$. 
Figure 4.2. **Absorbance of Egg Lecithin Liposomes as a Function of Extrusion.** The natural log of the absorbance at 280 nm of an egg lecithin aqueous dispersion, 20 mM lipid phosphate, after each extrusion through a polycarbonate filter of defined size. The extrusions were repeated at least 8 times for each filter size for a succession of decreasing filter sizes. The lines are simply curves drawn through the points to indicate the trend in absorbance change.
to the final extrusion at 30 nm for a given lipid phosphate concentration. Qualitatively, the liposomal solution goes from a dense milky white to a clear opalescent color. There is a large apparent decrease in vesicle size in the first six extrusions for each pore size with little or no change thereafter. In all experiments presented, at least 8 passes through each filter was used.

Liposome Characterization by Electron Microscopy:

Large multilamellar vesicles are the classic model for liposome studies but they are inappropriate for heme binding studies. Kinetic investigations would be complicated by the existence of multiple diffusion barriers. Although a number of studies have indicated that the vesicles produced by extrusion are unilamellar (Olson et al., 1979; Dearden et al., 1982; Hope et al., 1985; and Mayer et al., 1986), this was checked with electron microscopy techniques. No evidence was found for internal liposomal structures in DMPC, DMPC/DCP (9:1), or DMPC/Chol (6:4) vesicles when the focus of the electron microscope was moved through liposomes that had been rapidly frozen with a nitrogen slurry. If multilamellar structures had been present they would have been seen as bilayers within bilayers. DMPC/Chol (6:4) vesicles did have a noted tendency to aggregate together whereas the DMPC/DCP (9:1) liposomes were almost never seen touching. The addition of hemin to DMPC vesicles at high heme to phosphate ratios (1:2) appeared to promote the formation of smaller structures as well as a tendency for these vesicles to aggregate.

DMPC vesicles, both in the absence and presence of low levels of hemin, were examined by freeze fracture techniques. In freeze fracture, as the name implies, the samples are rapidly frozen and then "cracked open". A fracture line will often develop
along a portion of the structure to be examined and will be highlighted when examined with an electron microscope. As shown in Figure 4.3, the vesicles appear to be relatively homogenous with an average diameter of 100 nm. Again, there is no evidence of internal structures, the fractures are clear depressions or bulges without any signs of a secondary fracture. The DMPC vesicles were subjected to a freeze etch procedure designed to highlight any features that may be particularly deep and the vesicles still appeared to be simple spheres. Thus, it would appear that vesicles prepared with a 100 nm pore size filter by the extrusion method are unilamellar and on the order of 100 nm in diameter.
Figure 4.3. Freeze fracture electron micrographs of liposomes prepared by the extrusion method. DMPC/DCP (9:1) liposomes, 4 mM lipid phosphate concentration, were incubated with 1 mM hemin at 35°C and then prepared for electron microscopy by the freeze fracture procedure as outlined in the text. A, the vesicles were magnified 25,000 times (45nm/mm in the picture). B, the vesicles were magnified 63,000 times (18nm/mm in the picture).
Effect of Heme on Liposome Phase Transitions:

A possible consequence of heme binding to lipid membranes is an alteration in the cooperative bilayer structure and the ability for acyl chains to interact. To test this, DMPC liposomes prepared by extrusion through a 100 nm pore size polycarbonate filter were examined by scanning calorimetry both in the presence and absence of hemin. The results were analyzed as the differential of heat absorbed versus temperature, and the area beneath a peak is related to the enthalpy change, $\Delta H$, of the transition.

The main transition temperature for DMPC liposomes was 23.8°C (Figure 4.4). Our measured $T_m$ agrees well with literature values of 23.9, 23.6, and 23.8°C (Mabrey and Stuttevant, 1976; Chen et al., 1980; Huang et al., 1982, respectively). It should be pointed out that these values are significantly higher than those obtained for DMPC liposomes made by sonication (18°C, Melchoir & Steim, 1976). The phase transition of small unilamellar vesicles is 5°C lower than that of their larger counterparts and the transition itself occurs over a broader temperature range. Since the $T_m$ is thought to be an indication of the ordering of the acyl chains, this difference between sonicated and extruded vesicles of the same lipid composition undoubtedly reflects a difference in chain packing induced by the considerable degree of curvature in the much smaller sonicated particles (Mabrey & Stuttevant, 1978; and Brouillette et al., 1982). The relative broadening of the $T_m$ is also indicative of the difference in packing between the inner and outer leaflets of the bilayer in sonicated vesicles. When the radius of a spherical liposome begins to approach the thickness of the bilayer, then the degree of curvature on the inside must be much greater than that on the outside. In the interior of the liposome the head groups will be closely
Figure 4.4. **The heat capacity as a function of temperature for several DMPC liposome compositions.** The phase transition temperatures of the DMPC liposomes were measured by differential scanning calorimetry. The points represent the measured values for DMPC (closed circles), DMPC/DCP (9:1) (open circles), and DMPC/Hemin (9:1) liposomes (open triangles). The lines simply connect the points.
packed, but as the distance increases from the center of the particle, the packing of the fatty acyl chains of the inner lipid layer will be decreased because of the increase in curvature. The reverse is true for the head groups and acyl chains along the exterior of the vesicle: the polar region is more loosely packed compared to the hydrocarbon phase.

The $T_m$ value of 23.8°C is an indirect indication that the size of these DMPC vesicles is greater than that obtained by sonication. The sharpness of the peak is a good indication that most of the lipids are packed in the same state (i.e. the liposomes are large and of similar size), and the inner and outer bilayers exhibit equivalent physical structures. The small shoulder at lower temperatures (22.5°C-23.8°C) indicates that some acyl size heterogeneity may exist in the preparation. The enthalpy change, 5.7 kcal/mol, also agrees well with literature values of 5.44, 6.26, and 5.4 kcal/mol (Mabrey and Stutevant, 1976; Mason and Huang, 1981; Huang et al., 1982, respectively).

The measured $T_m$ for DMPC/DCP (90:10) liposomes was 26.3°C (Figure 4.4). Only one peak was observed, indicating that the lipids are miscible, and the enthalpy change of the two liposome populations (DMPC and DMPC/DCP (90:10)) are similar, 5.7 kcal/mole versus 6.7 kcal/mole, respectively. Taken together, these results suggest that the peak shift comes from the cooperative interactions between the two lipids and not from two separate lipid pools.

For a similar amount of heme (10%) in DMPC liposomes, the $T_m$ was only slightly affected (23.3°C), and there was little change in $\Delta H$ (5.6). Direct evidence of hemin miscibility with DMPC liposomes is presented in Figure 4.6. Furthermore, if the DMPC liposome affinity for hemin and CO-heme are at all similar, then all of the hemin should be bound at the high lipid phosphate levels employed (Table 5.1). The lack of any significant change underscores the difference between the heme/lipid interaction and the lipid/lipid
interaction. The lack of any change also indicates that the basic lipid bilayer structure is not radically altered by low levels of heme and that kinetic and partitioning analyses based on this assumption are valid.

*Chromatographic Analysis of Hemin Binding to Liposomes:*

In our initial experiments, CO-heme was titrated with DMPC/Chol/DCP (50:40:10) liposomes to compare with the results presented by Rose *et al.* (1985) for egg lecithin vesicles. This lipid mixture was chosen because it was being used in our and other laboratories to produce artificial red blood cells (Gaber *et al.*, 1983; and Hysell *et al.*, 1983). The addition of DMPC/Chol/DCP (50:40:10) liposomes to sealed cuvettes containing micromolar quantities of CO-heme failed to alter the heme absorbance peak position in the Soret region. Despite repeated attempts with fresh lipid, buffer, and heme, a peak shift was never observed. The lack of an absorbance change was quite unexpected because all previous investigations of heme interactions with membranes had been accompanied by large spectral shifts, regardless of lipid composition (Tipping *et al.*, 1979; Rose, 1982; and Cannon *et al.*, 1984).

There were at least two possible explanations for the lack of a spectral change between free CO-heme and CO-heme in the presence of DMPC/Chol/DCP (50:40:10) vesicles. Either the liposomes do not bind heme, or, heme does interact with the bilayer but in a unique manner that does not give rise to a spectral shift. Given the wide variety of lipid compositions in natural membranes, and the general toxicity of heme, there are important physiological implications if these and other liposomes do not bind heme. If there is uptake by the membrane, but no absorbance change, then the spectral methods used in determining the kinetics and thermodynamics of heme binding must be reviewed.
Column chromatography was used to resolve this issue. Hemin was used because of the
difficulty of keeping the heme reduced in a column environment and to compare the results
with earlier studies using ferric protoporphyrin (Tipping et al., 1979; and Cannon et al.,
1984). Egg lecithin vesicles were used as a standard membrane because of its
demonstrated ability to bind heme.

The absorbance changes of hemin in the Soret wavelength region were examined by
split beam spectroscopy in both normal cuvette cells, and double sectored cells. The
spectrum of the liposomes being investigated was first measured. Then a known amount
of free hemin was added and the measurement repeated. When a spectral shift
accompanied hemin binding the observed final spectrum was not a linear combination of
the spectral contributions of free hemin and the liposomes. As shown in Figure 4.5a, the
addition of egg lecithin liposomes to hemin produced a spectral shift which was similar to
that reported by other investigators (Tipping et al., 1979). A similar study with
DMPC/Chol/DCP (5:4:1) liposomes showed that the spectrum of hemin was unchanged by
the addition of vesicles and that the spectra of the mixtures could be simulated by
combining the spectral properties of free hemin and the liposomes (Figure 4.5b). These
results confirm those reported earlier with CO-Heme.

The spectral difference observed with EL liposomes required direct contact of the
hemin and liposomes, and was not caused by simple light scattering by the liposomes. The
spectrum obtained when EL liposomes and hemin were isolated in a double sectored
cuvette, was in fact identical to the sum of the lipid and hemin spectra, but when the two
components were mixed the spectrum changed. A similar study with hemin and
DMPC/Chol/DCP (5:4:1) liposomes revealed no difference when the components were
separated by the glass partition or mixed together.
Figure 4.5. **Absorbance spectra of hemin.** The absorbance of 5 μM hemin, both free in a buffer solution (*closed circles*) or in a buffer with lipid vesicles (*open circles*), was measured by an Amino DW-2A. The *solid lines* represent the theoretical spectra calculated solely on the sum of the separate spectral contributions of the hemin and the liposomes (not shown). Two types of lipid vesicles are shown; 280 μM egg lecithin vesicles (A), and 50 μM (by phosphate) DMPC/Chol/DCP (5:4:1) (B).
If hemin does not bind to DMPC/Chol/DCP (5:4:1) vesicles, then it should be possible to separate the two components by column chromatography. However, if heme is bound by the liposomes then the heme and liposomes should co-elute when applied to the column together. Hemin binding to liposomes containing egg lecithin, DMPC alone, and DMPC, cholesterol and DCP mixtures was examined by this technique and the results are presented in Figure 4.6. Fractions were collected from a Sephadex G-200 column at 4 °C and analyzed for liposome content by measuring absorbance at 280 nm (light scattering). It should be noted that while hemin does absorb weakly at 280 nm, this is not a complicating factor since the heme content is determined separately at 390 nm. In Figure 4.6a, the absorbance at 280 nm versus elution volume is plotted for a number of lipid compositions which were mixed with 10% hemin, on a per molar basis. A set of control experiments with egg lecithin liposomes without hemin and free hemin without lipid are also plotted. The hemin content alone is plotted in Figure 4.6b. It can be seen that while the egg lecithin vesicles eluted quite early, presumably in the void volume of the column, movement of free hemin was greatly retarded.

Hemin in the presence of egg lecithin vesicles was observed to co-elute with the liposomes, and no free hemin was observed. In agreement with earlier work, hemin is tightly bound by egg lecithin liposomes (Tipping et al., 1979; and Cannon et al., 1984). It is important to note that all the liposomes tested were observed to elute at the same fraction number. Hemin, however, did not always co-elute with the liposome fractions. When hemin and DMPC/Chol/DCP (50:40:10) liposomes were passed through the Sephadex G-200 column, it was clear that the hemin was completely excluded from the liposomes. All of the lipid material was collected with no trace of heme and all of the hemin was eluted where free hemin fractions were expected. Thus, it appears that hemin and, presumably,
Figure 4.6. **Separation of Liposome-bound and unbound Hemin by Column Chromatography.** 5 different lipid compositions prepared by extrusion through a 100nm filter were mixed with 10%, on a molar basis, hemin, and applied individually to a Sephadex G-200 column at 4° C. The hemin to phospholipid ratio was kept constant for all samples but the actual phospholipid concentration ranged from 10 to 20 mM. The points represent the measured absorbance values for DMPC/Chol/DCP 5:4:1 (*closed triangles*), DMPC/Chol 6:4 (*open squares*), DMPC/DCP 9:1 (*closed squares*), DMPC (*open diamonds*), and EL vesicles (*closed diamonds*). The *open* and *closed circles* are control values for EL with no hemin and hemin with no lipid. Two separate wavelengths were monitored as described in the text. *A*, absorbance at 280 nm. *B*, absorbance due to hemin at 390 nm.
CO-heme do not bind to DMPC/Chol/DCP (50:40:10) liposomes.

In order to determine which lipid component was responsible for the apparent failure of DMPC/Chol/DCP (50:40:10) vesicles to bind heme, the vesicle composition was varied systematically, and the ability of hemin to bind to these preparations was tested (Figures 4.6 a&b). A listing of the compositions studied, and a summary of the results are presented in Table 4.1. Intermediate behavior was observed when vesicles containing DMPC were tested; some hemin was detected in both the lipid and free hemin fractions (Figure 4.6b). This implies that although DMPC liposomes can bind heme, the affinity is reduced with respect to egg lecithin liposomes and not all of the hemin is bound. Although considerable binding was observed, the affinity was even lower in DMPC vesicles that contained DCP. No hemin was found to bind to vesicles of DMPC/Chol (6:4), and thus, it would appear that cholesterol is primarily responsible for the total exclusion of hemin from the DMPC/Chol/DCP (5:4:1) vesicles.

Discussion

Liposome preparation by the extrusion method is both rapid and adaptable for a variety of lipid mixtures. Relatively homogenous liposome populations of large, unilamellar vesicles were prepared with this method and were ideal for studying the effects of lipid composition on heme binding to liposomes. The addition of low levels of hemin does not significantly alter the phase transition temperature of DMPC vesicles, which suggests that the bilayer structure is minimally altered in the presence of bound heme. The lack of a change in the $T_m$ also eliminates a potential complicating factor in studying the temperature dependence of heme binding to vesicles.
TABLE 4.1
HEME BINDING BY LIPOSOMES AS
DETERMINED BY COLUMN CHROMATOGRAPHY

<table>
<thead>
<tr>
<th>LIPOSOME COMPOSITION</th>
<th>FRACTION BOUND*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL</td>
<td>100 %</td>
</tr>
<tr>
<td>DMPC</td>
<td>80 %</td>
</tr>
<tr>
<td>DMPC/DCP (9:1)</td>
<td>65 %</td>
</tr>
<tr>
<td>DMPC/CHOL (6:4)</td>
<td>0 %</td>
</tr>
<tr>
<td>DMPC/CHOL/DCP (5:4:1)</td>
<td>0 %</td>
</tr>
</tbody>
</table>

* Determined by comparison of the calculated areas beneath the peaks in Figure 4.6b.
Several liposome compositions were tested for their ability to bind hemin by spectroscopy and column chromatography. The exclusion of hemin from DMPC/Chol (6:4) and DMPC/Chol/DCP (5:4:1) vesicles, and the apparent reduced affinity of hemin for DMPC vesicles as contrasted to egg lecithin vesicles, suggest several things about the nature of heme partitioning into liposomes. At 4 °C, liposomes of DMPC are in the gel state. At the same temperature, egg lecithin liposomes are in the liquid-crystal state. The degree of order and chain viscosity will be higher in DMPC vesicles than in EL liposomes. If heme partitioning proceeds by the insertion of the porphyrin ring between the chains of adjacent lipid molecules, then it seems reasonable that increased order in the hydrocarbon phase would result in a lower affinity for heme.

The total exclusion of heme from DMPC liposomes containing 40% cholesterol is a relatively unique observation among the studies that have looked at the binding of heme to cholesterol-containing lipid interfaces (Ginsberg & Demel, 1983; Cannon et al., 1984; and Rose et al., 1985). There is considerable confusion in the literature, however, on the exact effect of cholesterol on heme binding. Cholesterol has been reported to enhance the uptake of heme (Ginsberg & Demel, 1983), to have little effect on this process (Rose, 1982), or to decrease the equilibrium binding of heme (Cannon et al., 1984). Our results suggest that the addition of cholesterol to DMPC vesicles completely inhibits hemin binding, at least at 4° C.

Evidence for enhanced liposome uptake of heme by cholesterol or fully saturated DPPC monolayers (Ginsberg & Demel, 1983) can be reconciled by examining the assay technique. Most of the investigations on heme binding to lipid interfaces used spectroscopic techniques, Ginsberg and Demel, measured an increase in the surface pressure of a phospholipid monolayer as the result of heme insertion. They measured a
greater pressure increase when cholesterol or DPPC were present. Their interpretation was that more heme was binding when cholesterol or DPPC were present. Another explanation is that the monolayer is more rigid in the presence of cholesterol or the saturated phospholipids. The consequence of increased order is a decrease in lateral compressibility, or free space, and thus heme binding could cause a relatively larger pressure change. A strict interpretation of their data may not be possible because a change in pressure may not be proportional to the extent or amount of heme intercalation but rather may indicate the relative amount of free space available between the phospholipids.
Chapter V

A Survey of Heme Binding to Complex Lipid Mixtures

Introduction

Cannon and co-workers (1984) examined the efflux of hemin from sonicated phospholipid vesicles by measuring the rate of heme transfer from liposomes to apomyoglobin, serum albumin, or hemopexin. Their work was complicated by the tendency of hemin to aggregate. They did establish that the observed dissociation rate was not dependent on protein concentration. Rather, the rate-limiting step appeared to be heme dissociation from the membrane. The kinetics of heme efflux were affected by liposome composition. Heme dissociation from vesicles formed with the addition of stearylamine, which imparts a positive charge to the liposome, was slower than from those without stearylamine. The use of cholesterol appeared to retard the initial rate of heme dissociation by a factor of two.

Similar equilibrium and kinetic studies were conducted in our laboratory by Rose and co-workers (Rose & Olson, 1979; Rose, 1982; and Rose et al., 1985). CO-heme was used almost exclusively in order to avoid the complications of hemin aggregation. The liposomes were prepared by the ethanolic injection method of Batzri and Korn (1973). The exact conditions employed favored the production of small unilamellar vesicles with a diameter of 15-20 nm as determined by electron microscopy (Rose, 1982). Whereas Cannon and co-workers (1984) analyzed their results in terms of the membranes having a fixed number of independent heme-binding sites, Rose and co-workers (1985) developed a more general model of partitioning of the porphyrin between buffer and the lipid matrix.

The equilibrium between aqueous and membrane-bound heme was described by a
partition constant, $K_p$:

$$K_p = \frac{H_m}{H_a} \quad \text{(5.1)}$$

where $H_m$ and $H_a$ are the heme concentrations in the membranes and aqueous phases, respectively. The fractional amount of heme bound in the lipid phase is obtained from equation 5.1 and a consideration of the volume fractions of the two phases and the total solution concentration of heme, $H_t$:

$$H_t = H_b + H_f \quad \text{(5.2)}$$

$$H_t = H_m \cdot V_p C_p + H_a (1 - V_p C_p) \quad \text{(5.3)}$$

$H_b$ and $H_f$ are the total solution concentrations of lipid-bound and free aqueous heme, respectively. $V_p$ and $C_p$ are the partial molar volume of phosphatidylcholine and its concentration in moles of phosphate per liter. $V_p C_p$ and $1 - V_p C_p$ are the volume fractions of the lipid and aqueous phases, respectively. The fractional amount of lipid-bound heme is defined experimentally by $y = H_b / (H_b + H_f)$, which in terms of Equations 5.1 and 5.2 is given by:

$$y = \frac{H_m V_p C_p}{H_m V_p C_p + (1 - V_p C_p) H_a} = \frac{K_p V_p C_p}{K_p V_p C_p + 1 - V_p C_p} \quad \text{(5.4)}$$

The kinetics of heme binding to liposomes was described by:
\[ \frac{dH_m}{dt} = k_i H_a - k_{-i} H_m \]

where \( k_i, k_{-i} \) are unimolecular rate constants describing heme partitioning into and out of the membrane phase (Rose et al., 1985). Converting \( H_a \) and \( H_m \) to the corresponding total suspension concentrations, \( H_b \) and \( H_f \), the final differential equation becomes:

\[ \frac{dH_b}{dt} = \frac{k_i V_p C_p H_t}{1 - V_p C_p} - \left( \frac{k_i V_p C_p}{1 - V_p C_p} + k_{-i} \right) H_b \]

5.5

This is a simple linear, first order differential equation and predicts that heme binding will exhibit a simple exponential time course with an observed rate equal to \( (k_i V_p C_p / (1 - V_p C_p)) + k_{-i} \). Since \( V_p C_p \) is always \( \leq 0.0001 \), the observed rate is proportional to lipid-phosphate concentration and a plot of \( k_{obs} \) vs \( C_p \) should exhibit a slope (i.e. an apparent bimolecular rate, \( k' \)) equal to \( k_i V_p \), and an intercept (\( k \)) equal to the heme dissociation rate constant, \( k_{-i} \).

In our previous work, association kinetics were determined by rapid-mixing of heme and egg lecithin liposomes in a stopped-flow device and following the absorbance change as heme partitioned from the aqueous environment into the lipid environment. At high lipid to heme ratios, the rate of heme binding was linearly dependent on the phospholipid concentration. A value of \( 1.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \) at \( 10^\circ \text{ C} \) was calculated for the apparent association bimolecular rate constant from a simple plot of \( k_{obs} \) versus phosphate lipid concentration (i.e. \( = k_i V_p \) in Equation 5.5). Only a limited study of the dependence of heme uptake on vesicle composition was carried out. The addition of 12.5% stearylamine to the egg lecithin vesicles increased the rate of uptake by a factor of 2 to 3.
The rate increase seen with stearylamine was suggested to be the result of favorable electrostatic interactions between the propionate side chains of heme and the positive charge on the fatty acid. The further addition of cholesterol to the stearylamine/egg lecithin vesicles had little effect. The addition of just 20% cholesterol to the egg lecithin liposomes did decrease the rate of heme uptake but the effect was small and not analyzed quantitatively (Rose et al., 1985).

In view of the dramatic effects of lipid composition on the equilibrium binding of hemin and CO-heme to liposomes shown in Chapter 4 we felt that a systematic kinetic investigation was warranted. The kinetics of CO-heme binding were determined by rapid-mixing, stopped-flow spectrophotometry for a wide variety of liposomes prepared by the extrusion method. Since the role of the structural state of the lipid, a critical parameter in most membrane phenomena, had not been previously addressed, the temperature dependence of the rate of heme binding was investigated using several of saturated and unsaturated phosphatidylcholines.

Materials and Methods

Liposomes were prepared and handled as described in Chapter 4. Kinetic measurements were made with a Gibson-Durrum stopped-flow spectrophotometer that was thermostatically-controlled by a water-bath. Long term measurements (> 1 min) at 30 °C in the stopped-flow device were commonly complicated by the formation of gas bubbles in the observation cuvette. The decrease in CO solubility with increasing temperature was particularly troublesome when both reactant syringes contained buffer equilibrated with 1 atmosphere of the gas at room temperature (~25°C). When possible, degassed, evacuated buffer was used in one of the mixing syringes. This simple precaution eliminated the
formation of gas bubbles.

**Results and Discussion**

**Kinetics of CO-heme binding to Liposomes:**

The effects of lipid composition on the kinetics of heme binding were examined by measuring the association and dissociation rate constants with a wide variety of vesicles. The time courses (Figure 5.1) are similar to those previously described for the binding of CO-heme to simple egg lecithin vesicles (Rose *et al.*, 1985). Unlike most of the studies of Rose and co-workers (1985), some of these newer measurements were complicated by an additional, and sometimes substantial, slow phase. The spectral changes for both phases were identical to those shown in Chapter 1 for the movement of heme into a lipid bilayer (Figure 1.3). The initial phase exhibited bimolecular kinetics with the observed rate being linearly dependent on phosphate lipid concentration. Accurate determinations of both the observed rates and relative absorbance changes were obtained for all the lipid mixtures studied. The slow phase was unimolecular and appears to represent transmembrane movement of CO-heme molecules. This conclusion is discussed in detail in Chapter 6.

This chapter describes results for the initial heme uptake phase and its dependence on lipid composition.

To simplify analysis, and for direct comparison with the results reported by others, the rate parameters for heme uptake by liposomes are presented as bimolecular association rate constants on a per lipid phosphate basis \( k' = k_1 V_p \) (Equation 5.5) and unimolecular dissociation rate constants \( k = k_d \) (Equation 5.5). The apparent association rate constant, \( k' \), was determined by measuring the rate of uptake as a function of phosphate concentration. In this type of experiment, \( k_{obs} = k' [\text{lipid phosphate}] + k \). A plot of \( k_{obs} \)
vs $[P_i]$ will be linear with a slope equal to $k'$ and a y-intercept equal to $k$. Sample data for
several lipid compositions are shown in Figure 5.2. A listing of all the lipid compositions
investigated and a summary of the results of the kinetic analyses are presented in Table 5.1.
The listing is organized primarily by acyl chain length.

**Effects of Temperature**

Large scale conformational changes in lipid bilayers are well documented at phase
transition temperatures. The actual $T_m$ for any given lipid is a complex function of several
packing parameters. For saturated phospholipids with equivalent chains, every
2-methylene carbon unit in the acyl chains increases the $T_m$ about 14-17° C. In a
mixed-chain saturated phospholipid the longer chain determines the $T_m$, but the chain at
carbon 1 of the glycerol backbone has a greater influence. Unsaturated chains decrease the
$T_m$ by a substantial amount. For example the phase transition temperatures of
distearoylphosphatidylcholine (C1 18:0, C2 18:0), OSCP (C1 18:1, C2 18:0), and
dioleoylphosphatidylcholine (C1 18:1, C2 18:1), are 55, 11 (Small, D.M., 1986), and -22°
C (Ladbrooke & Chapman, 1969), respectively. The type of double bond is important, a
cis bond has a larger effect on lowering the phase transition temperature than a trans double
bond. The position of the double bond, furthermore, is important. A double bond in the
center of the acyl chain has the largest influence on lowering the $T_m$ and this effect
diminishes with increased distance of the double bond from the center (Barton and
Figure 5.1. Sample time courses for the reaction of 1 $\mu$M CO-heme with 300 $\mu$M egg lecithin liposomes. The association reaction was followed at 30° C in the stopped-flow apparatus at 408 (closed squares) and 420 nm (open circles).
Figure 5.2. Observed association rates of CO-heme with liposomes as a function of lipid phosphate concentration. The reactions of 1 μM CO-heme with liposomes of various lipid compositions were followed at both 408 and 420 nm with the stopped-flow apparatus. The average of the fitted observed rates were plotted as a function of total lipid phosphate concentration to determine the apparent bimolecular association rate as described in the text. The lipid compositions presented here are EL/Chol (6:4) (open diamonds), cis Δ⁹ DPPC (closed squares), cis Δ⁹ DPPC/DCP (9:1) (open circles), POPC/DMPG (85:15) (closed triangles), and DPPC/DCP (9:1) (closed circles). The lines are the least-squares fit to the points.
Table 5.1
Kinetic Constants for the Major Phase Observed with the Association and Dissociation of CO-Heme to Various Phosphatidylcholine Liposome Mixtures

<table>
<thead>
<tr>
<th>Liposome Composition</th>
<th>Temp °C</th>
<th>$k'$ x 10^{-6} M^{-1} s^{-1}$</th>
<th>$k$ s^{-1}</th>
<th>$k'/k$ μM^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC</td>
<td>20</td>
<td>0.62</td>
<td>2.7</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.91</td>
<td>5.1</td>
<td>0.18</td>
</tr>
<tr>
<td>DTPC</td>
<td>30</td>
<td>1.1</td>
<td>22</td>
<td>0.050</td>
</tr>
<tr>
<td>DMPC</td>
<td>33</td>
<td>0.63</td>
<td>20</td>
<td>0.032</td>
</tr>
<tr>
<td>DMPC/DCP (95:5)</td>
<td>30</td>
<td>0.68</td>
<td>24</td>
<td>0.028</td>
</tr>
<tr>
<td>(90:10)</td>
<td>30</td>
<td>0.61</td>
<td>25</td>
<td>0.024</td>
</tr>
<tr>
<td>(85:15)</td>
<td>30</td>
<td>0.71</td>
<td>38</td>
<td>0.019</td>
</tr>
<tr>
<td>DMPC/Chol/DCP (8:1:1)</td>
<td>30</td>
<td>0.40</td>
<td>33</td>
<td>0.012</td>
</tr>
<tr>
<td>(7:2:1)</td>
<td>30</td>
<td>0.30</td>
<td>41</td>
<td>0.0073</td>
</tr>
<tr>
<td>(5:4:1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis Δ⁹ DMPC</td>
<td>30</td>
<td>1.2</td>
<td>23</td>
<td>0.052</td>
</tr>
<tr>
<td>cis Δ⁹ DMPC/DCP (9:1)</td>
<td>20</td>
<td>0.60</td>
<td>23</td>
<td>0.026</td>
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<tr>
<td></td>
<td>30</td>
<td>0.98</td>
<td>48</td>
<td>0.020</td>
</tr>
<tr>
<td>DPDPC</td>
<td>30</td>
<td>0.24</td>
<td>11</td>
<td>0.022</td>
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<tr>
<td>DPPC/DCP (9:1)</td>
<td>30</td>
<td>0.078</td>
<td>33</td>
<td>0.0024</td>
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<tr>
<td>cis Δ⁹ DPPC</td>
<td>30</td>
<td>2.0</td>
<td>15</td>
<td>0.13</td>
</tr>
<tr>
<td>cis Δ⁹ DPPC/Chol (8:2)</td>
<td>30</td>
<td>2.6</td>
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<tr>
<td>cis Δ⁹ DPPC/Chol (6:4)</td>
<td>30</td>
<td>1.9</td>
<td>13</td>
<td>0.15</td>
</tr>
<tr>
<td>cis Δ⁹ DPPC/DCP (95:5)</td>
<td>30</td>
<td>1.3</td>
<td>18</td>
<td>0.072</td>
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<tr>
<td>cis Δ⁹ DPPC/DCP (9:1)</td>
<td>20</td>
<td>0.82</td>
<td>20</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.3</td>
<td>26</td>
<td>0.050</td>
</tr>
<tr>
<td>cis Δ⁹ DPPC/DCP (85:15)</td>
<td>30</td>
<td>0.91</td>
<td>35</td>
<td>0.026</td>
</tr>
<tr>
<td>POPC</td>
<td>20</td>
<td>0.61</td>
<td>8.7</td>
<td>0.070</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.1</td>
<td>14</td>
<td>0.079</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.3</td>
<td>12</td>
<td>0.11</td>
</tr>
</tbody>
</table>
### Table 5.1 Contd.
Kinetic Constants for the Major Phase Observed with the Association and Dissociation of CO-Heme to Various Phosphatidylcholine Liposome Mixtures

<table>
<thead>
<tr>
<th>Liposome Composition</th>
<th>Temp °C</th>
<th>$k'$ x 10^{-6} M^{-1} s^{-1}$</th>
<th>$k$ s^{-1}</th>
<th>$k'/k$ μM^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC/Chol (8:2)</td>
<td>30</td>
<td>1.4</td>
<td>15</td>
<td>0.093</td>
</tr>
<tr>
<td>POPC/Chol (6:4)</td>
<td>30</td>
<td>2.0</td>
<td>24</td>
<td>0.083</td>
</tr>
<tr>
<td>POPC/DCP (95:5)</td>
<td>30</td>
<td>1.1</td>
<td>23</td>
<td>0.048</td>
</tr>
<tr>
<td>POPC/DCP (9:1)</td>
<td>20</td>
<td>0.58</td>
<td>20</td>
<td>0.029</td>
</tr>
<tr>
<td>POPC/DCP (85:15)</td>
<td>30</td>
<td>1.1</td>
<td>38</td>
<td>0.029</td>
</tr>
<tr>
<td>POPC/DMPG (95:5)</td>
<td>30</td>
<td>1.2</td>
<td>11</td>
<td>0.11</td>
</tr>
<tr>
<td>POPC/DMPG (85:15)</td>
<td>30</td>
<td>0.81</td>
<td>24</td>
<td>0.034</td>
</tr>
<tr>
<td>EL</td>
<td>20</td>
<td>1.1</td>
<td>6.6</td>
<td>0.17</td>
</tr>
<tr>
<td>EL/Chol (6:4)</td>
<td>20</td>
<td>1.8</td>
<td>7.3</td>
<td>0.25</td>
</tr>
<tr>
<td>OSPC/DCP (9:1)</td>
<td>20</td>
<td>0.50</td>
<td>15</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.88</td>
<td>25</td>
<td>0.035</td>
</tr>
</tbody>
</table>
The temperature dependence of CO-Heme binding to a variety of liposomes was measured to determine the activation energies ($E_a$) and to examine for the effects of lipid phase transitions. The rates of most reactions decrease as the temperature is lowered in a manner described by the Arrhenius equation:

$$k = A \, e^{-E_a/RT}$$

A is known as the pre-exponential factor. Equation 5.6 is normally rearranged to a linear form that is easily plotted:

$$\ln k = \ln A - \frac{E_a}{RT}$$

Deviations from linearity in an Arrhenius plot usually indicate a highly cooperative conformational change in one of the reactant molecules. An example is the change in rate as enzymes begin to denature at high temperature (Dixon & Webb, 1964).

An Arrhenius plot of the temperature dependence of CO-Heme binding to cis $\Delta^9$ DMPC/DCP (9:1) vesicles is essentially linear between 20 and 35 °C, and a straight line fit to the points is adequate (Figure 5.3). DLPC, DTPC, POPC, POPC/Chol (8:2 and 6:4), EL, EL/Chol (6:4), and OSPC/DCP (9:1) liposomes all behaved similarly. The lack of any deviation from linearity is not surprising because the $T_m$ values of these phosphatidylcholines are below the observation ranges (the $T_m$'s are in the List of Abbreviations). The calculated activation energies for these lipids are listed in Table 5.2, and are all about 7 Kcal/mol.
In the same range, the temperature dependence of CO-heme binding to DMPC/DCP (95:5) liposomes exhibited a sigmoidal plot with a steep change centered about the \( T_m \) at \( \approx 26^\circ C \) (figure 5.3). This deviation from normal Arrhenius behavior is in sharp contrast with the linear behavior measured with \( \text{cis} \> \Delta^9 \) DMPC/DCP (9:1) (Figure 5.3) liposomes, which exhibits a \( T_m \) well below the measurement temperatures used here. This sigmoidal temperature dependence is a general characteristic of all DMPC-containing vesicles; the same patterns were found in DMPC/DCP (9:1), DMPC/Chol/DCP (8:1:1), and DMPC/Chol/DCP (7:2:1) liposomes and was also found for DTPC and DPDPC liposomes. The inflection point of the curve varies linearly with the phase transition temperature for the particular lipid system being studied. At temperatures higher than the \( T_m \), the slope of the Arrhenius plot is comparable to that found with the other unsaturated liposomes. As the temperature approaches the \( T_m \), however, the slope changes dramatically and approaches an \( E_a \) value of 100 ± 20 kcal/mole, and then levels off when the temperature decreases past the \( T_m \). The effect of cholesterol on DMPC/DCP vesicles is quite dramatic at temperatures below the \( T_m \); however at temperatures above the \( T_m \) the association rates appear to approach those of DMPC/DCP (9:1) and \( \text{cis} \> \Delta^9 \) DMPC/DCP (9:1). This indicates that the inhibitory effect of cholesterol is pronounced only when the membranes are in the gel state. As will be seen in Chapter 7, cholesterol has little or no effect on CO-heme binding when the measurements are carried out at 30° C with membranes having \( T_m \) values \( \leq 15^\circ C \) (Table 7.1).
Figure 5.3. **Temperature dependence of CO-heme binding to DMPC-containing liposomes.** The reactions were followed in a temperature controlled stopped-flow apparatus. Arrhenius plots of the observed association rates of 1 μM CO-Heme with: 125 μM DMPC/DCP (95:5) (open squares) or 95 μM cis Δ⁹ DMPC /DCP (9:1) (closed circles).
**TABLE 5.2**

**ACTIVATION ENERGIES FOR THE BINDING**

**OF CO-HEME TO VARIOUS LIPOSOMES COMPOSITIONS**

**ABOVE THEIR PHASE TRANSITION TEMPERATURES**

<table>
<thead>
<tr>
<th>Lipid Composition</th>
<th>$E_a$ kcal/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC</td>
<td>7.8</td>
</tr>
<tr>
<td>DTPC</td>
<td>7.8</td>
</tr>
<tr>
<td>cis $\Delta^9$ DMPC / DCP (9:1)</td>
<td>8.1</td>
</tr>
<tr>
<td>DPDPC</td>
<td>7.7</td>
</tr>
<tr>
<td>POPC</td>
<td>4.8</td>
</tr>
<tr>
<td>POPC/Chol (8:2)</td>
<td>5.8</td>
</tr>
<tr>
<td>POPC/Chol (6:4)</td>
<td>5.3</td>
</tr>
<tr>
<td>EL</td>
<td>7.8</td>
</tr>
<tr>
<td>EL/Chol (6:4)</td>
<td>8.0</td>
</tr>
<tr>
<td>OSPC/DCP (9:1)</td>
<td>7.2</td>
</tr>
</tbody>
</table>
Discussion

The dramatic and totally unexpected result presented in Chapter 4, total exclusion of CO-heme by DMPC liposomes with the addition of 40% (per molar) cholesterol, prompted us to screen a number of different lipid compositions by kinetic techniques. Thirty separate compositions were studied but total exclusion of heme was only observed in the original liposomes that prompted this investigation (DMPC/Chol/DCP (5:4:1) vesicles). Most of the observed effects are relatively small and a detailed analysis of the data in Table 5.1 will be presented in Chapter 7 along with direct measurements of the CO-heme dissociation rate constants in the presence of apoMb. However, some general observations on the DMPC/Chol system seem appropriate. In Figure 5.4 it can be seen that raising the levels of cholesterol in DMPC liposomes decreased the relative affinity of the vesicles for heme \((k'/k)\) until none was observed to bind. This effect appears to be the result of both a decreased association rate and an increased dissociation rate. DMPC vesicles also exhibit a remarkable temperature dependence for CO-heme binding near the phase transition temperature. Only moderate differences between cis \(\Delta^9\) DMPC and DMPC vesicles are observed above the \(T_m\), but in the range surrounding the \(T_m\) the observed rate for DMPC membranes drops dramatically by almost a factor of 10 as the temperature drops from 30 to 20\(^\circ\) C (Figure 5.3). This undoubtedly explains the extremely poor binding noted for DPPC/DCP (9:1) \((T_m = 45^\circ\) C) and DPDPC \((T_m = 35^\circ\) C) liposomes at 30\(^\circ\) C, especially when contrasted to the high affinity and association rate constant exhibited by cis \(\Delta^9\) DPPC liposomes \((T_m = -36^\circ\) C).

It is possible that the decrease in the association rate constant for the binding of CO-heme to DMPC/Chol/DCP liposomes compared to DMPC/DCP vesicles is the result of a cholesterol induced broadening of the phase transition peak so that at 30\(^\circ\) C the membrane
is not a liquid crystal (Table 5.1). Precise measurements of the width of the Arrhenius transition were complicated by the reduced extent of heme binding, but the rate decreased much more dramatically at temperatures below the $T_m$ with DMPC/Chol/DCP (8:1:1) liposomes and broadening was apparent with DMPC/Chol/DCP (7:2:1) vesicles. Other investigators have observed similar behavior on the phase transition temperature with the addition of cholesterol (Mabrey & Sturtevant, 1978; and Presti & Chan, 1982).

Although the unusual temperature dependence of the association process for DMPC vesicles is difficult to explain in terms of either conventional ligand binding systems or partitioning equilibrium it is not without precedence in investigations involving lipid bilayers. The sigmoidal curve is a general characteristic of changes in a bilayer at the phase transition as seen by several types of measurements. Lentz et al. (1982) measured the temperature-dependent changes in microviscosity in small, sonicated vesicles of DMPC by following changes in the fluorescence of the membrane-bound probe, 1,6-diphenyl-1,3,5-hexatriene. These results are shown in Figure 5.5. The similarity of the CO-heme binding curve in Figure 5.3 and the microviscosity data in Figure 5.5 supports a model of heme intercalation between the acyl chains. If the rate limiting step in heme binding to a bilayer is the insertion process then it would be expected that an increase in viscosity due to the phase transitions would slow down the overall association step.
Figure 5.4. The effect of raising the cholesterol levels in DMPC liposomes on the binding parameters with CO-heme. Liposomes of four separate lipid compositions were prepared by extrusion, DMPC, DMPC/Chol/DCP (8:1:1), DMPC/Chol/DCP (7:2:1), and DMPC/Chol/DCP (5:4:1), and the association and dissociation rate constants with CO-heme were determined from a simple plot of $k_{obs}$ versus phosphate lipid concentration ($k_{obs} = k [P] + k'$). The measurements were done at 30°C, except that those with DMPC vesicles were done at 33°C. The relative affinities, $k'$, dissociation rate constants, $k'$, and association rate constants, $k$, are indicated by the closed circles, closed squares, and open squares, respectively.
Figure 5.5. Temperature dependence of acyl-chain viscosity in DMPC vesicles. Arrhenius plots of the chain viscosity in sonicated DMPC vesicles (closed circles, redrawn from Lentz et al., 1982).
Chapter VI
Evidence for Transbilayer Movement of Heme

Introduction

The initial binding of CO-heme to liposomes was described in Chapter 5 as a single step that is influenced by both vesicle composition and the state of the hydrocarbon phase. However, under some conditions slower, secondary absorbance changes were observed in association experiments and appeared to represent the additional binding of CO-heme to membranes. Other investigators have also reported heterogeneous time courses for the reaction of heme with lipid vesicles. Cannon and co-workers (1984) reported two phases with widely different rates, 2 and 0.01 s⁻¹, while measuring the efflux of hemin from lipid vesicles in the presence of apoheme proteins. These investigators concluded that the fast phase was the result of heme dissociating from the outer portion of the bilayer and that the slow phase represented transmembrane movement of hemin from the inner half to the outer portion of the bilayer.

In our previous work with small egg lecithin vesicles, slow absorbance changes were observed in simple uptake experiments (Rose et al., 1985). However, we concluded that the slow phase was not due to heme 'flipping' across the membrane, but rather, the result of endogenous bases (ethanolamine) slowly chelating the heme iron atom. This conclusion was based on several observations. Liposomes prepared directly with hemin were reacted with excess apoglobin, and the mixture was placed on a Sephadex G-200 column. All of the heme which was originally bound to the liposomes, presumably in both the inner and outer portions of the bilayer, eluted as newly formed hemoglobin (Rose et al., 1985). This indicated that transbilayer movement of hemin had occurred within the time
required to load the column and separate the protein from the liposomes (at least 5-10 minutes). If the small slow phase observed in rapid kinetic association studies was due to heme flipping, then the relative magnitude of the absorbance change should have decreased with increasing phospholipid to heme ratios (see Equation 6.7). In fact, the slow phase observed by Rose et al. (1985) exhibited an amplitude which increased slightly with increasing phospholipid concentration.

Clear evidence for iron chelation by endogenous bases was seen when stearylamine was added to small egg lecithin vesicles. The resultant liposomes exhibited two distinct phases in heme uptake experiments. The rapid phase was similar in rate and amplitude to that observed with just egg lecithin vesicles. The slow phase was very pronounced and its rate and amplitude were proportional to stearylamine concentration. Spectral analysis indicated that the slow phase was due to a further red shift in the Soret peak. This shift is similar to the red shift caused by the binding of the histidine to heme (i.e. CO-hemoglobin or CO-myoglobin formation).

An estimation of the rate of heme crossing the bilayer is critical for the development of a complete model of its interactions with membranes. The results of Cannon and co-workers (1984) suggested that the rate of heme transbilayer movement is relatively slow and rate-limiting for the dissociation of 30-50% of the bound heme molecules. In contrast, Rose and co-workers (1985) suggested an opposite conclusion: heme dissociation from a bilayer was slower than transbilayer movement. In an attempt to resolve the apparent discrepancy between the results of Cannon et al. (1984) and Rose et al. (1985), we have carefully re-examined the slow phases observed in heme uptake and release experiments. The temperature dependences of the observed slow phases were compared with behavior described by other workers for the transmembrane movement of lipid molecules. Finally,
the pivotal column chromatography experiments described in Rose et al. (1985) were repeated with a variety of liposomes. In these new experiments, purified synthetic lecithins were examined to reduce the possibility of heme binding to endogenous chelating ligands.

Methods

Kinetic Measurements:

Extremely long term kinetic measurements were not always practical with our single beam stopped-flow instrument. An IBM 9430 spectrophotometer with automated data collection routines was used when following extremely slow reactions \((t_{1/2} \geq 10 \text{ min})\). Absorbances at twenty discrete wavelengths were collected simultaneously at various intervals and then plotted as time resolved spectra. In this manner, either changes at a particular wavelength could be monitored and the traces fitted as described in Chapter 2 to obtain rate constants, or the whole spectrum could be examined qualitatively. The efflux of heme from liposomes into the aqueous surroundings was measured directly by following the incorporation of CO-heme into reconstituted myoglobin (Cannon et al., 1984; and Rose et al., 1985). All other kinetic and equilibrium measurements were performed as described in Chapter 5. Lipid preparation and handling were described in Chapter 4.

Column Chromatography:

Molecular chromatography was used to separate liposomes from apoMb and Mb. This allowed a determination of the fraction of the lipid-bound heme that was available for rapid uptake by the apoMb. Separations were carried out with a 2 x 20 cm Sephadex G-200 column at both 4 and 24°C and under aerobic as well as anaerobic conditions. CO-equilibrated buffer containing sodium dithionite was used to maintain anaerobic
conditions. Every effort was made to minimize the time between mixing apoMb with the heme containing liposomes, loading the mixture on the column, and separating the protein from the lipid material. Fractions were collected at a rate of 30 ml/hr. Lipid content of the effluent was analyzed by phosphate assay. The heme contents of the liposome containing fractions was determined as described in Chapter 4. The heme content of the myoglobin fractions were calculated using extinction coefficients of 157 cm$^{-1}$ mM$^{-1}$ at 409nm and 187 cm$^{-1}$ mM$^{-1}$ at 423nm for metMb and COMb, respectively (Antonini & Brunori, 1971).

*Complications in the Reaction of Heme with Liposomes in Stopped-Flow Experiments:*

Two complications resulted from the use of high concentrations of lipid material: heme oxidation due to residual oxygen and vesicle aggregation. Large amounts of lipid were not required in our early egg lecithin studies because of the relatively high affinity of these liposomes for heme. Unfortunately, many of the lipid compositions studied in Chapter 5 were found to have a substantially reduced affinity for heme, and a large amount of lipid material had to be used to measure accurately the observed association and dissociation rate constant.

In stopped-flow experiments, slow, wavelength independent, changes were observed when high lipid concentrations were mixed with CO-heme. Solutions premixed with heme and then mixed with buffer exhibited similar behavior. A number of reports indicate that liposomes can fuse together to form larger vesicles (Schullery *et al.*, 1980; Gibson & Strauss, 1984; and Morris *et al.*, 1985). The exact mechanism for the fusion process is poorly understood. Vesicle exposure to temperature fluctuations at, and subsequent storage below, the phase transition temperature may be one factor that promotes fusion or aggregation (Gibson & Strauss, 1984). We observed both fusion and
aggregation of vesicles with a high phase transition temperature. DLPC, DMPC, and DPDPC vesicles would precipitate out of solution overnight if left in the refrigerator. cis Δ⁹ DMPC vesicles were stable under similar conditions because of their much lower phase transition temperature. Although the time course for vesicle fusion is thought to be outside of the range of most of the measurements presented here, the demonstrated ability of these liposomes to fuse indicates the possibility of large absorbance changes due to alterations in the light scattering properties of the lipid suspension. Aggregation would explain the three experimental observations which occur at high liposome concentrations: (1) a large, wavelength independent absorbance increase, (2) a similar change even if heme was premixed with the vesicles, and (3) an increasing amplitude with increasing lipid phosphate content. To reduce these complications, experiments were carried out at reduced liposome and heme concentrations. However, the lipid phosphate concentration had to be high enough for significant heme binding. A further precaution was the addition of DCP or DMPG to many lipid mixtures. Electrostatic repulsion between negatively charged liposomes was sufficient to keep DMPC/DCP (9:1) vesicles from aggregating overnight at 4°C, even though the T_m of these vesicles is 23°C.

Heme oxidation was aggravated indirectly by high lipid concentrations. Liposomes can not be subjected to the usual method of oxygen removal by bubbling with nitrogen. At high final lipid concentrations, a significant amount of aerobic stock lipid solution was added to the anaerobic buffer. Sodium dithionite was used as an oxygen scavenger. Unfortunately, dithionite is only available to the oxygen surrounding the liposomes because this cation passes through the liposome membrane extremely slowly. This was determined by measuring the rate of reduction of vesicle entrapped MetMb. For complete oxygen removal, it was necessary to incubate the liposomes with dithionite for
5-10 minutes. Failure to do so resulted in the oxidation of CO-heme after it had intercalated into the membrane.

Heme oxidation by liposomal oxygen was demonstrated with DMPC liposomes. When a solution CO-Heme and dithionite was mixed with an aerobic solution of liposomes there is a slow conversion of CO-heme to hemin. At high concentrations of dithionite, the rate of oxygen scavenging is extremely rapid so that the heme is initially protected in buffer. A fast spectral change was observed (Figure 6.1a) and exhibited the expected wavelength dependence and rate for CO-heme binding to the liposomes. This was followed by a large, slow absorbance change which occurred after the CO-heme had been bound. The difference spectrum for this second reaction resembled that expected for the oxidation of CO-heme to hemin in liposomes. The addition of a large excess of dithionite (1 mM) to the lipid material prior to mixing reduced markedly the amplitude of the slow phase and changed its spectral characteristics (Figure 6.1b). The residual slow phase was not altered by further additions of dithionite or longer preincubation times and appears to represent additional binding of CO-heme to the membranes. Thus, to remove oxidation problems humidified nitrogen gas was blown over the stock liposome preparations; the vesicles were diluted into anaerobic buffer containing 1 mM dithionite, and this solution was preincubated for several minutes prior to mixing with CO-heme.
Figure 6.1  **The binding of CO-heme to DMPC liposomes under potentially oxidizing conditions.** The association of 2 μM CO-heme and 25 μM phospholipid was measured in the stopped-flow apparatus at 34° C. Prior to mixing, the DMPC solution was aerobic, but the CO-heme buffer contained 1 atm of CO and 1mM DT. *A*, the wavelength dependence of both the fast phase (*closed circles*) and the slow phase (*open circles*) observed with CO-heme binding to DMPC liposomes that contained residual amounts of oxygen. *B*, kinetic traces of the binding reaction followed at 420 nm, oxygen contaminated liposomes (*open circles*) and the same liposomes incubated in 1 mM DT (*close squares*).
Results

**Heterogeneous Time Courses in the Association Reaction of Heme and Liposomes:**

Heterogeneous binding behavior was observed in the reaction of CO-heme with low concentrations of DMPC/DCP (95:5) vesicles even after the elimination of complications due to vesicle aggregation or oxygen contamination. There were at least two phases in the time courses for the association reaction (Figure 6.2a). The slow phase had exactly the same wavelength dependence as the fast phase (Figure 6.2b), and this similarity strongly indicates that both phases represent the binding of CO-heme to lipid material. Identical behavior was observed with 8 different lipid compositions of DMPC or cis Δ⁹ DMPC as well as liposomes composed of DLPC, DTPC, and DPDPc. All of these phosphatidylcholines have relatively short acyl-chains (≤ 15 carbons). Consistent, heterogeneous binding behavior was difficult to demonstrate with longer acyl-chain lecitins on the same time scale. The two most obvious explanations for the second phase are: (1) there are two pools of aqueous CO-heme that react differently with the membranes, or (2) CO-heme partitions into two distinct lipid phases at widely different rates.

The first possibility, two separate free heme pools, is reminiscent of the problems early investigators had with aggregates while studying hemin association with apohemoglobin (Gibson & Antonini, 1960). A hemin solution consists of monomers, dimers, and more complex aggregates, but CO-heme has been described as being a monomer (Smith, 1959). Experimental evidence was presented in Chapter 2 which supports the earlier observation that CO-heme is monomeric. If the dissociation of CO-heme aggregates caused the slow phase, then the ratio of the fast to slow amplitudes should depend only on the initial free heme concentration. As shown in Figure 6.3a, the
Figure 6.2  The reaction of 1 μM CO-heme with 50 μM DMPC/DCP (95:5)
liposomes. The association reaction was followed in the stopped-flow apparatus. A, the
observed time courses observed at 420 nm (closed circles) and 408 nm (open circles).
Note the two time scales. B, the wavelength dependence of the absorbance changes due to
the observed fast (closed circles) and slow (open circles) phases.
fractional amount of slow phase decreased with increasing phosphate concentration. Thus, it seems doubtful that there are two kinetically distinct pools of CO-heme in the buffer.

Evidence for two distinct lipid pools can be inferred from the data in Figure 6.3a. If the two lipid populations had different rates of heme binding, then as the lipid concentration increased all of the heme would be preferentially bound by the liposome population with the higher association rate and the slow phase would disappear. This matches the experimental observation. However, a simple model based on two lipid pools with different affinities for heme is not correct because the rate for the fast phase showed a linear dependence on lipid phosphate concentration, whereas the rate for the second phase had little dependence on lipid concentration (Figure 6.3b). Thus, the slower kinetic event appears to be a simple first order process, while the faster event is clearly bimolecular. This difference in reaction order underscores the difference between the two phases. Although the two phases both represent heme binding to the liposome, the rates are influenced by distinct physical events.

There are two obvious lipid pools if the structure of a bilayer is considered. In a vesicle, there is an outer leaflet of lipid molecules arranged with their polar head groups orientated toward the bulk aqueous surroundings, and a similar inner leaflet composed of lipid molecules with their polar head groups facing the inner aqueous core. The interior of a membrane is composed of acyl chains from both layers. Aqueous free heme can only bind to the outer side of the bilayer. When the outer portion of the bilayer is saturated with heme, subsequent binding will occur only after movement of heme from the outer to the inner portion of the bilayer with a net change in orientation of the charged propionate groups. If the rate of heme transbilayer movement is faster than the initial binding process (Rose et al., 1985), then this distinction between the two sides of the bilayer is
Figure 6.3 The phosphate dependence of CO-heme binding to DMPC/DCP (95:5) liposomes. The reaction of 2 µM CO-heme with DMPC/DCP (95:5) liposomes at 30 °C was observed at 420 nm in the stopped-flow apparatus. 

A, the measured fractional amount of the slow phase absorbance change as a function of phosphate concentration (open circles) and the theoretical fractional amount based on Equation 6.7 in the discussion (line, calculated assuming $K_pV_p = 0.03 \mu M^{-1}$). B, phosphate dependence of the observed rates of the fast phase (close circles) and the slow phase (rates X 1000, open circles).
unnecessary, only one lipid pool needs to be considered, and a monophasic time course is expected. If the rate of heme crossing from one side of the membrane to the other is slow (Cannon et al., 1984), then two separate lipid pools must be considered. Heme binding to the outer bilayer and heme flip-flop across the membrane, are distinct physical events and are expected to exhibit second order and first order reaction mechanisms, respectively. Thus, the results in Figures 6.2 and 6.3 suggest that transmembrane movement is slow and distinct from the initial binding process.

Heterogeneous Time Courses for the Dissociation of Heme from Liposomes:

If the slow first order process observed for heme binding to DMPC liposomes is heme flipping, then a similar process should be observed when measuring the dissociation of CO-heme from liposomes. As shown in Figure 6.4a, heterogeneous time courses were observed when short-chain phosphatidylcholines containing bound CO-heme were mixed with apomyoglobin. The two phases exhibited similar wavelength dependences (Figure 6.4b). The rates of the first phases were similar to the heme dissociation rate constants measured for the lipid compositions in Chapter 5 (data presented in Chapter 7), and the rates of the slow phases matched the slow first-order processes measured during the association reaction of heme with the same set of liposomes (Table 6.1). A complete analysis of these results will be presented in the Discussion section of this chapter but it is important to note that in general, the rates for the slow phases decreased with increasing acyl-chain length of the lecithins being studied (Figure 6.5). This implies that Rose et al. (1985) may have failed to observe slow phases for long acyl-chain egg lecithin vesicles, not because the rates were too fast, but rather because they were too slow for stopped-flow, rapid mixing experiments. This was tested in a spectrophotometer by long
Figure 6.4  The reaction of 12 μM apoMb with 100 μM cis Δ⁹ DMPC/DCP (9:1) liposomes that had been preincubated with 1 μM CO-heme. The dissociation reaction was followed in the stopped-flow apparatus. A, the observed time courses at 424 nm (closed circles) and 408 nm (open circles). Note the two time scales. B, the wavelength dependence of the absorbance changes due to the observed fast (closed diamonds), slow (open circles) phases, and total changes (closed squares).
<table>
<thead>
<tr>
<th>Liposome Composition</th>
<th>Temp °C</th>
<th>$k_{\text{slow}}$ s$^{-1}$ Association (1)</th>
<th>$k_{\text{slow}}$ s$^{-1}$ Dissociation (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC</td>
<td>20</td>
<td>0.36</td>
<td>0.087</td>
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<tr>
<td></td>
<td>30</td>
<td>0.52</td>
<td>0.19</td>
</tr>
<tr>
<td>DTPC</td>
<td>30</td>
<td>0.40</td>
<td>0.083</td>
</tr>
<tr>
<td>cis $\Delta^9$ DMPC</td>
<td>30</td>
<td>0.040</td>
<td>0.038</td>
</tr>
<tr>
<td>cis $\Delta^9$ DMPC/DCP (9:1)</td>
<td>20</td>
<td>0.022</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.13</td>
<td>0.048</td>
</tr>
<tr>
<td>DMPC</td>
<td>34</td>
<td>0.15</td>
<td>n.m.</td>
</tr>
<tr>
<td>DMPC/DCP (95:5)</td>
<td>30</td>
<td>0.11</td>
<td>n.m.</td>
</tr>
<tr>
<td>(90:10)</td>
<td>30</td>
<td>0.11</td>
<td>n.m.</td>
</tr>
<tr>
<td>(85:15)</td>
<td>30</td>
<td>0.13</td>
<td>n.m.</td>
</tr>
<tr>
<td>DPDPC</td>
<td>30</td>
<td>0.044</td>
<td>0.057</td>
</tr>
<tr>
<td>cis $\Delta^9$DPPC/DCP (9:1)</td>
<td>30</td>
<td>n.o.</td>
<td>0.0055 (2)</td>
</tr>
<tr>
<td>POPC/DCP (9:1)</td>
<td>30</td>
<td>n.o.</td>
<td>0.0025 (2)</td>
</tr>
<tr>
<td>EL</td>
<td>30</td>
<td>n.o.</td>
<td>0.00083 (2)</td>
</tr>
<tr>
<td>OSPC/DCP (9:1)</td>
<td>30</td>
<td>n.o.</td>
<td>0.00085 (2)</td>
</tr>
</tbody>
</table>

n.m.: Not measured
n.o.: Not observed

(1) Rates of the slow phases measured in association (CO-heme and liposomes) or dissociation (liposome-bound CO-heme + apoMb) experiments.

(2) These rates were measured with a spectrophotometer as described in the Methods section.
Figure 6.5. Rates of the slow phases as a function of acyl chain length at 30°

C. The first-order reaction rates of the slow phases seen in both the association and
dissociation of heme from various liposomes were determined by both stopped-flow and
timed spectroscopic studies. These rates are listed in Table 6.1 and were plotted as a
function of the acyl chain length of the phosphatidylcholine tested. The curves were drawn
through the points using an exponential function; and the actual data points are represented
by the circles. A, rates of the slow phase found in the dissociation reaction. B, rates of the
slow phase found in the association reaction.
KOBS OF THE SLOW PHASE SEEN DURING THE DISSOCIATION REACTION

n-acyl CHAIN LENGTH (carbons)
B. KOBS OF THE SLOW PHASE SEEN DURING THE ASSOCIATION REACTION

n-acyl CHAIN LENGTH (carbons)
term measurements of heme efflux from various lecithins by apoMb uptake. An extremely slow phase was observed for all of the long acyl-chain lecithins tested. Time resolved spectra had an isobestic point and indicated that COMb was being formed (Figure 6.6a). When CO-heme and ApoMb were mixed prior to the addition of liposomes, or liposomes were mixed with ApoMb prior to the addition of CO-heme, no long term absorbance changes were noted. These controls strongly indicated that the extremely slow absorbance changes observed were due to heme slowly dissociating from the liposomes and not from interactions between reconstituted myoglobin and the membranes. A reaction time course was constructed and a rate determined by plotting the change at a single wavelength as a function of time (Figure 6.6b). The rates for several long-chain phosphatidylcholines, were determined by this method, and are listed in Table 6.1.
Figure 6.6. Time resolved spectra for the dissociation of CO-heme from egg lecithin vesicles. A, the spectral changes were observed after the addition of ApoMb to a cuvette of premixed 2 μM CO-heme and egg lecithin vesicles (100 μM phosphate) and plotted as a function of time. B, a reaction time course was constructed by plotting the change (delta absorbance = absorbance_t - absorbance_∞) at 424 nm as a function of time. The closed circles represent the measured data points and the solid line represents an exponential fit to the data.
The Temperature Dependence of the Slow Phase for Heme Binding to Liposomes:

In Chapter 5, a marked non-Arrhenius temperature dependence was reported for the association of CO-heme with liposomes in the range surrounding the vesicle's phase transition temperature (Figure 5.3). The temperature dependence of the slow phase also deviated from a straight line, but in a different manner. The observed rate increased with decreasing temperature near but above the \( T_m \) reached a maximum at the \( T_m \), and then decreased dramatically with decreasing temperature. This is reflected as a peak in the Arrhenius plot (Figure 6.7a). The shape of this Arrhenius plot was observed for all DMPC-containing vesicles that had a phase transition in this region. The width of the Arrhenius peak closely matches the width of the calorimetry peak of DMPC/DCP (9:1) in Figure 4.3. Similar non-Arrhenius behavior was observed for the slow phase in CO-heme uptake by DTPC and DPDPC liposomes which have phase transitions at 15 and 34° C, respectively (Figure 6.7b). There is also a small shoulder in the DTPC plot which was not seen with other lipid mixtures. Lewis et al. (1987) reported similar thermal anomalies with DTPC. The nature of this "extra" transition in DTPC vesicles is unknown. When cis \( \Delta^9 \) DMPC, which has a low \( T_m \), was used instead of DMPC simple Arrhenius behavior was observed (Figure 6.7a). Thus, the unusual temperature dependence of the slow phase appears to be directly related to the lipid phase transition. Finally, similar temperature effects were observed for the slow phase of heme efflux in the presence of excess apoMb.

The peak found in the Arrhenius plots of the slow rate for heme uptake has precedence in the study of liposome behavior. While examining the rate of DMPC transmembrane movement by NMR spectroscopy, investigators found a profound effect of temperature in the phase transition region (De Kruijff & Van Zoelen, 1978). The rate for phospholipid flipping increased markedly at the gel-to-liquid crystalline transition. An
Arrhenius plot of their data is shown in Figure 6.8, and is very similar to that shown in Figure 6.7 for the slow rate of heme uptake by DMPC vesicles. The only major difference in the two Arrhenius plots is the temperature of the peak of the anomalous rate increase, 19°C vs 25°C. The DMPC vesicles used in the NMR study of De Kruijff and Van Zoelen (1978) were prepared by sonication and, as described in Chapter 4, these smaller vesicles have a $T_m$ (18°C, Melchoir & Steim, 1976) that is significantly lower than the larger liposomes prepared in this study. A $T_m$ of 25°C for our vesicles is reasonable for liposomes composed of DMPC/DCP (95:5) considering that DMPC and DMPC/DCP (90:10) vesicles produced in this lab had measured phase transition temperatures of 23.8 and 26.3°C, respectively. Marked rate increases at the phase transition temperature have also been observed in bilayer permeability studies (Papahadjopoulos et al., 1973), and these phenomena were explained by an increase in lateral compressibility due to the coexistence of gel and liquid crystalline phase lipid. Thus, the anomalous temperature dependence of the slow process in CO-heme uptake suggests strongly that this kinetic phase represents transmembrane flipping of the porphyrin molecule.

Since linear Arrhenius plots were observed for liposomes with low $T_m$ values, apparent activation energies for the slow rate in CO-heme uptake experiments could be measured fairly accurately in the 15 - 30°C range. The $E_a$ values for this slow process varied widely depending on lipid composition: 6.0, 22, and 30 Kcal for DLPC, cis Δ⁹ DMPC/DCP, and DPDPC, respectively. This correlates with the decrease in the overall rate constant of the slow phase increasing with acyl chain length. The measured activation energy required for transmembrane migration of DMPC in a DMPC vesicle is 23.7 kcal/mole (De Kruijff & Van Zoelen, 1978).
Figure 6.7. The Temperature Dependence of the Slow Phase in Heme

**Binding to Liposomes.** Arrhenius plots of the observed rates of the slow phases observed at 420 nm when binding 1 μM CO-heme to several liposomes. A, the closed squares and open circles are the measured values of the slow phase with 125 μM DMPC/DCP (95:5) and 95 μM cis Δ⁹ DMPC/DCP (9:1) liposomes, respectively. The straight line is a least-squares analysis to the measured values of cis Δ⁹ DMPC/DCP (9:1) liposomes. B, the open diamonds and closed circles are the measured values of the slow phase with 100 μM DTPC and DPDPC liposomes, respectively. The curves are computer drawn with a cubic-spline algorithm.
Figure 6.8. The temperature dependence of DMPC transmembrane movement.

The Arrhenius plot was drawn from NMR data on the rate of DMPC flip-flop in sonicated DMPC vesicles. The data points are represented by the open circles, and the observed rates are per hour (redrawn from De Kruijff & Van Zoelen, 1978).
Chromatographic Analysis of Heme Transmembrane Movement:

The experimental observations presented in this chapter are consistent with the model first suggested by Cannon et al. (1984) in which heme transmembrane movement is slow. They are in apparent contradiction with the chromatography results of Rose et al. (1985). In those experiments, heme bound to egg lecithin was mixed with an excess of apoHb and loaded onto a Sephadex G-200 column. All of the heme was bound to the apoHb before the protein could be separated from the liposomes. It was concluded that the rate of heme transmembrane movement from the inner to the outer portion of the membrane must be rapid for all of the heme to be available to the apoprotein. However, if the measured rates for the slow phases seen in our more recent association and dissociation experiments are an indication of the rate of heme transmembrane movement, then the half-time for heme flipping across an egg lecithin membrane should be approximately 10 minutes. Thus, we felt that it should be possible to repeat the column chromatography experiment of Rose et al. (1985), and separate the liposomes from the apoMb before all the heme had time to cross the membrane and form reconstituted myoglobin.

In Figure 6.9a it can be seen that when a mixture of CO-heme bound initially to egg lecithin and apoMb was loaded onto a Sephadex G-200 column, all of the lipid material (open diamonds) eluted as a single peak but the heme eluted in two peaks. The first heme peak co-migrated with the lipid material as membrane-bound hemin. It was also clear from spectrophotometric measurements that the second peak was newly reconstituted COMb (Figure 6.9b). Thus, not all of the lecithin-bound CO-heme was available for binding by apoMb during the time it took to separate the liposomes from the protein. Although the total amount of recovered heme, as determined by the areas under the peaks, stayed constant for the two experiments in Figure 6.9a the heme distribution between the lipid and
protein material changed. At room temperature less than 30% of the total heme was retained by the liposomes, the rest formed Mb, but when the column and buffer were cooled to 4°C, 40% of the total heme remained membrane-bound. As shown in Figure 6.7, the rate of heme transmembrane movement slows down at lower temperatures and this decrease would be expected to result in less heme being available for binding by apoMb. Thus, the observations in this set of chromatography experiments indicate that CO-heme crosses the membrane slowly.

A similar set of experiments were done with hemin and vesicles composed of POPC and DTPC (Figure 6.9c). Slow phases were observed for both of these lipids in heme dissociation reactions (Table 6.1). The measured rate constant for flipping in DTPC is 0.083 s⁻¹ and indicates a half time of 8.3 seconds which is much quicker then the predicted half time heme for transmembrane movement in POPC vesicles (= 5 minutes) or the time that it takes to separate the liposomes from the protein on the column (= 5 minutes). All of the DTPC-bound heme, but only a portion of the POPC-bound heme, should be available for binding by apoMb during the course of the separation if the previously measured slow phases are an accurate measure of the rates of heme flipping for the two lipid compositions. Hemin did elute as both membrane bound and MetMb when applied with POPC vesicles and apoMb, but only the metMb form was observed when heme was applied with DTPC vesicles. Thus, it would appear that transmembrane flipping is relatively fast in the short-acyl chain lecithin vesicles of DTPC but slow in both POPC and egg lecithin vesicles.
Figure 6.9. Separation of Newly Reconstituted Myoglobin and Lipid Vesicles on Sephadex G-200. 10 mM lipid phosphate vesicles in 0.05 M NaCl, 0.05 M Tris, pH 8.0 were equilibrated with 125 μM heme, mixed rapidly with a 2-fold excess of apoMb, and then quickly loaded onto a Sephadex G-200 column. A, the eluting buffer and column had been equilibrated with CO and sodium dithionite was present to keep the heme reduced during the mixing with the egg lecithin vesicles and apoMb. The open diamonds represent the measured phosphate concentrations of the eluted fractions of the 24° C experiment, and the measured heme concentrations when the experiment was carried out at 4° C and 24° C are represented by open circles and closed circles, respectively. It is important to note that CO-heme bound to the liposomes oxidized during passage through the column since dithionite was retained by the gel and residual oxygen was present B, Samples of observed spectra for lipid-bound hemin (column fraction 12) and newly formed COMb (column fraction 24) collected during the experiment carried out at 4° C. C, hemin was mixed with DTPC vesicles and POPC vesicles at 24° C and the hemin content assayed as described in Chapter 4, represented by the open and closed circles, respectively. Note that unlike the other lipid mixtures, DTPC vesicles did not retain any heme after the addition of apoMb and only a metMb peak was measured after the components were separated by the column.
A.

LIPOSOMES

MYOGLOBIN

\[ \mu \text{M HEME, mM PHOSPHATE} \]

COLUMN FRACTION (ml)
Discussion

A number of experimental observations indicate that heme transmembrane movement is slow. Two phases with an identical wavelength dependence were observed while measuring both the association and dissociation of CO-heme with short acyl-chain phosphatidylcholine liposomes (Figures 6.2 & 6.4, respectively). When measuring the association reaction of heme with vesicles, the fast phase is a second order process and correlates with the initial binding of heme to the outer layer of the membrane. The slow phase is a first order process, and the size of the absorbance change is inversely dependent on phosphate concentration (Figure 6.3). The fast phase observed when measuring the efflux of heme from various liposomes appears to be the result of simple dissociation from the outer portion of the bilayer (further discussion on this is presented in Chapter 7). Unlike the situation for the association reaction, a large slow phase was observed during the dissociation reaction even at high phospholipid concentrations (Figure 6.4). The relative magnitude of this slow phase was almost 45% of the total absorbance change. This would be expected if the heme was equally distributed across the membrane. The rate constants of the slow phases observed for both processes are comparable. A slow phase was also noted for the efflux of heme from long acyl-chain lecithin vesicles (Figure 6.6). The rate of the observed slow phase was a function of acyl chain length; the longer the chain, and the greater the distance the heme had to traverse, the slower the rate (Figure 6.5).

The temperature dependences of the slow phases seen in both the association and dissociation reactions closely matches that observed for the transmembrane movement of DMPC (Figure 6.8, De Kruijff & Van Zoelen, 1978). This result also supports the idea that these slow phases represent transmembrane movement of heme. Finally, two heme
fractions, membrane-retained and newly reconstituted Mb, can be separated by gel filtration from a solution of newly mixed apoMb and liposomes preincubated with CO-heme if the rate of the slow absorbance change is \( \leq 0.008 \text{ s}^{-1} \).

In view of these results it seemed appropriate to derive a kinetic model where heme flipping is relatively slow and to test the theoretical predictions with the results observed for the slow phase in our heme uptake experiments. For such a mechanism, three steps are needed to describe heme binding to a bilayer; heme partitioning into the outer layer, transmembrane movement, and partitioning into the inner aqueous phase encapsulated by the liposome:

\[
\begin{align*}
H_a^{o} & \xrightarrow{k_1} H_m^{o} \xleftarrow{k_2} H_m^{i} \xrightarrow{k_{-1}} H_a^{i} \\
\xrightarrow{k_{-1}} & \xrightarrow{k_{-2}} \xrightarrow{k_1}
\end{align*}
\]

The superscripts represent the outer, \( o \), and inner, \( i \), layers; \( k_1, k_{-1} \) represent the rate constants for partitioning between the aqueous, \( a \), and membrane, \( m \), phases; and \( k_2, k_{-2} \), the rate constants for transmembrane flipping. The volume fractions of the four phases in Equation 6.1 are defined by the concentration of lipid phosphate in the suspension, \( C_p \); the partial molar volume of the lipid, \( V_p \); and the outer and inner radii of the vesicle, \( R_o \) and \( R_i \).

\[
\begin{align*}
V_a^{o} &= 1 - V_p C_p \frac{R_o^3}{R_o^3 - R_i^3} \\
V_m^{o} &= V_p C_p \frac{R_o^2}{R_o^2 + R_i^2} \\
V_m^{i} &= V_p C_p \frac{R_i^2}{R_o^2 + R_i^2}
\end{align*}
\]
\[ V_m^i = V_o C_p \frac{R_i^2}{R_o^2 + R_i^2} \]

\[ V_a^i = V_o C_p \frac{R_i^3}{R_o^3 - R_i^3} \]

6.2

\( V_o \), \( V_m \), \( V_m^i \), and \( V_a^i \) are the volume fractions of the outer aqueous, outer membrane, inner membrane, and inner aqueous phases, respectively.

Equation 6.1 can be simplified for lipid vesicles because even for large liposomes \((R_o = 100 \text{ nm})\) the internal volume is small and, little or no heme will ever be present in the inner aqueous phase. This can be shown by considering the ratio of \( H_b^i / H_f^i \) which is given by:

\[ \frac{H_b^i}{H_f^i} = \frac{K_p V_m^i}{V_a^i} = \frac{K_p (R_o^3 - R_i^3)}{(R_o^2 + R_i^2) R_i} \]

Rearranging, the fraction of heme bound in the inner leaflet of the bilayer is:

\[ y_b^i = \frac{K_p V_m^i / V_a^i}{1 + K_p V_m^i / V_a^i} \]

6.3

In most of our experiments, the vesicle diameter is approximately 100 nm and the thickness of the lecithin membrane is roughly 4.5 nm \((i.e. \ R_o = 50 \text{ nm and } R_i = 45.5 \text{ nm})\). For these liposomes, \( V_m^i / V_a^i \) is 0.149 so that even for a lipid mixture which weakly binds heme with a \( K_p = 1000 \) (see Table 7.1), 99% of the heme on the inside of the vesicle will
be bound to the inner membrane monolayer. Most of the vesicles studied exhibit $K_p$ values around $10^5$ and for these samples heme would never be found in the inner aqueous phase. Thus, Equation 6.1 can be reduced to:

$$
\begin{align*}
H_a & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} H_m & \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} H_m^i \\
K_p &= \frac{k_1}{k_{-1}}; \quad K_{i,o} = \frac{k_2}{k_{-2}} = 1.0
\end{align*}
$$

Since there is no evidence to suggest that partitioning into the inner layer is different from that into the outer layer, $K_{i,o}$ is assumed to be 1.0.

The results in Tables 6.1 and 7.1 indicate that $k_2, k_{-2} \ll k_1$ or $k_{-1}$. Thus, the initial phase in uptake experiments can be analyzed by considering just the outer lipid phase. At the end of the initial uptake phase, the fractional amount of CO-Heme bound, $y_{fast}$, is given by:

$$
y_{fast} = \frac{K_p V_p C_p R_o^2 / (R_o^2 + R_i^2)}{1 + K_p V_p C_p R_o^2 / (R_o^2 + R_i^2)}
$$

where $R_o^2 / (R_o^2 + R_i^2)$ is just the fraction of total lipid present in the outer monolayer which for $R_o = 50$ nm is equal to 0.55. At the end of the slow phase when the system has achieved true equilibrium, the final fractional amount of heme bound, $y_e$, is given by equation 5.4. The amplitude of the slow phase is given by $y_e - y_{fast}$.
\[ y_e - y_{fast} = \frac{K_p V_p C_p}{1 + K_p V_p C_p} \cdot \frac{K_p V_p C_p}{(0.55)} - \frac{K_p V_p C_p}{1 + K_p V_p C_p} \cdot \frac{K_p V_p C_p}{(0.55)} \]

6.6

and the fractional amount of the slow phase, \( y_{slow} \), in the overall time course is given by:

\[ y_{slow} = \frac{y_e - y_{fast}}{y_e} = \frac{0.45}{1 + K_p V_p C_p (0.55)} \]

6.7

Thus, the relative magnitude of this phase is both expected and observed to decrease to zero as the lipid phosphate concentration, \( C_p \), is decreased (Figure 6.3a).

The rate of the fast phase in heme binding to phospholipid vesicles is determined by Equation 5.5 where the total lipid concentration is replaced by \( C_p R_o^2 / (R_o^2 + R_l^2) \) and is given by:

\[ k_{fast} = \frac{k_f V_p C_p (0.55)}{1 - V_p C_p (0.55)} + k_{-l} \]

6.8

for vesicles with an outer radius of 50 nm and a bilayer thickness of 4.5 nm. In effect, the bimolecular rate reported in Table 5.1 must be corrected for the fraction in the outer layer which is initially available for heme uptake (these corrections are further discussed in Chapter 7), but a linear dependence on lipid concentration, \( C_p \), is still expected (Figure 6.3b, filled circles).

The much slower rate of transmembrane movement can be derived from a consideration of the following differential equation:
\[
\frac{dH^i_m}{dt} = k_2 H^o_m - k_2 H^i_m
\]

6.9

Converting to suspension concentrations using the volume fractions defined in Equation 6.2, this becomes:

\[
\frac{dH^i_b}{dt} = \frac{k_2 V^i_m H^o_b}{V^o_m} - k_2 H^i_b
\]

6.10

Since what is followed experimentally is the change in free CO-heme, this first order differential equation must be rearranged to a linear form in \(dH^\rho / dt\) and \(H^\rho\). \(H^\rho\) and \(H^\rho\) are in equilibrium since \(k_1, k_1 \gg k_2, k_2\) (Figure 6.3b and equation 5.4) and the total CO-heme concentration is given by:

\[
H_t = H^\rho_f + H^\rho_b + H^i_b
\]

or

\[
H_t = H^\rho_f (1 + K_p V^\rho_m / V^\rho_a) + H^i_b
\]

6.11

Taking the derivatives of both sides of Equation 6.11 yields

\[
dH^i_b / dt = -(1 + K_p V^\rho_m / V^\rho_a) dH^\rho / dt \] since \(dH_t / dt = 0\). Substituting this expression for \(dH^i_b / dt\), \(H^\rho_b = K_p V^\rho_m / (V^\rho_a H^\rho),\) and \(H^i_b = H_t - H^\rho_f (1 + K_p V^\rho_m / V^\rho_a)\) into equation 6.10 yields:

\[
\frac{dH^\rho_f}{dt} = \frac{k_2 H_t}{(1 + K_p V^\rho_m / V^\rho_a)} - \left( \frac{k_2 K_p V^i_m / V^o_a}{1 + K_p V^\rho_m / V^\rho_a} + k_2 \right) H^\rho_f
\]

6.12

Thus, heme flipping observed in association experiments will exhibit simple exponential
behavior with an observed rate equal to:

\[ k_{\text{slow}}' = \frac{k' K_p V C_p (0.45)}{1 + K_p V C_p (0.55)} + k_{-2} \]  \hspace{1cm} (6.13)

when \( R_o = 50 \) nm and \( V_a o = 1 \). The dependence of \( k_{\text{slow}}' \) on lipid phosphate concentration is expected to be small since the rates of transmembrane flipping should be roughly independent of direction (i.e. \( K_{i,o} = 1.0 \) and \( k_2 = k_{-2} \)). At \( C_p = 0 \), the value of \( k_{\text{slow}}' \) will equal \( k_{-2} \); at \( C_p \rightarrow \infty \), \( k_{\text{slow}}' \) will equal 1.82 \( k_{-2} \). Thus, only an 80% increase in the slow rate would be expected over the entire range (> 20-fold) of lipid phosphate concentration used in our heme association experiments (Figure 6.3b).

A slow phase was also observed in heme dissociation time courses in which liposomes containing bound heme were mixed with apoMb (Figure 6.4). In these experiments, there was an initial rapid phase (2-50 s\(^{-1}\)) representing the release of heme from the more accessible outer half of the lipid bilayer (Cannon et al., 1984; and Rose et al., 1985). The second phase was much slower and exhibited a rate similar to that observed for the slow phase in CO-heme uptake experiments (Figure 6.2, Figure 6.4, and Table 6.1). Cannon et al. (1984) observed a similar slow phase when apoproteins were mixed with sonicated liposomes containing hemin. This slow phase represents myoglobin formation from CO-heme present initially in the inner monolayer and is limited by the rate of transmembrane flipping. To describe this process the total reaction scheme can be represented by:
The rate constants are the same as those defined earlier in this chapter; $k_{mb}$ is the bimolecular reaction rate described in Chapter 2; and $GB_a^o$ and $Mb_a^o$ are the apomyoglobin and myoglobin concentrations in the outer aqueous phase.

If the rate of heme transmembrane movement is slow then the fast phase represents only the efflux of heme initially bound in the outer bilayer and its subsequent rapid reaction with apoMb (i.e. heme flipping can be neglected). At high concentrations of apoMb, little or no free heme will be present in the aqueous phase and $dH_a^o / dt$ or $dH_p / dt$ will equal 0. The rate of myoglobin formation in the initial fast phase will be determined by:

$$\frac{dMb^o}{dt} = \frac{k_{-1} k_{Mb} Gb^o H_b^o}{k_j V_m^o + k_{Mb} Gb^o}$$  \hspace{1cm} 6.15$$

and

$$k_{fast} = \frac{k_{-1}}{\left( 1 + \frac{k_j V_p C_p (0.55)}{k_{Mb} Gb^o} \right)}$$  \hspace{1cm} 6.16$$

when $R_o = 50$ nm. When $k_{mb} Gb^o \gg k_j V_p C_p (0.55)$, this expression reduces to $k_{fast} = k_{-1}$.

The slow phase observed when apomyoglobin is mixed with heme containing liposomes appears to represent myoglobin formation from CO-heme present initially in the
inner monolayer and is limited by the rate of transmembrane flipping. Since the rates $k_{2,1}, k_{m}^{b}(G_{a}^{o}) >> k_2, k_{2,1}$, steady-state assumptions can be made for both $H_{m}^{o}$ and $H_{a}^{o}$.

Following a similar line of reasoning as that already presented for the derivation of the slow phase seen in the heme association experiments (equations 6.1-6.13), and using the steady-state assumptions, the rate of heme movement from the inner layer to the outer layer is determined by:

\[
\frac{dH_{b}^{i}}{dt} = - \frac{k_{2}H_{b}^{i}}{1 + \frac{k_{2}(k_{1}V_{m}^{o} + k_{M}^{b}G_{b}^{o})}{k_{1}k_{M}^{b}G_{b}^{o}}} \tag{6.17}
\]

where the observed rate constant will be:

\[
k_{slow} = \frac{k_{2}}{1 + \frac{k_{2}(k_{1}V_{m}^{o} + k_{M}^{b}(G_{b}^{o}))}{k_{1}k_{M}^{b}G_{b}^{o}}} \tag{6.18}
\]

At high globin concentrations, this expression reduces to $k_{slow} = \frac{k_{2}}{1 + \frac{k_{2}}{k_{1}}}$, and since $k_{1} >> k_{2}$ (Figures 6.2 & 6.4), $k_{slow}$ becomes simply $k_{2}$, the rate constant for transmembrane movement of CO-heme from the inner to the outer portion of the lipid bilayer. Cannon et al. (1984) derived a similar expression for $k_{slow}$ for this type of experiment using a theory with discrete heme binding sites in the lipid membrane. From these theoretical considerations, the rate of the slow phase observed during the dissociation step is expected to be almost half that measured for the slow phase during the association.
reaction at high lipid conditions. In Table 6.1, it can be seen that the rate measured during the association reaction was often greater than that measured during the dissociation reaction. It would appear, based on both theoretical as well as experimental observations, that the slow phases documented in this chapter represent the rate of heme transmembrane movement.

The unusual rate enhancement of heme flipping at the phase transition temperature indicates that the state of the liposome plays a significant role in transmembrane movement. At any given temperature near the $T_m$, the lipid population will be divided between those with sufficient energy to be in the liquid crystalline state and those in the gel state. Lipid packing is maintained in liquid-crystal phases because neighboring acyl-chains can fill created gaps, and in the gel phase packing is even closer because of reduced motion. At the interface of the two lipid packing states, however, gaps are caused by the motion of the liquid crystalline phase that are not compensated for by gel state molecules. While there is a decrease in the general fluidity of the acyl-chains for the whole vesicle as the temperature is lowered through the $T_m$ region (Figure 5.7), at the interface of the two phases there are large discontinuities that may act as gaps across the bilayer. The population of the two phases will be evenly divided at the $T_m$, and so the number of discontinuities would be maximal at the phase transition temperature. This phenomenon would influence heme already bound in the membrane but not necessarily aqueous heme in the process of binding to the liposomes. The fluidity of the bilayer is greatest in the center. Therefore, it is likely that the formation of gaps is more prevalent in the center, and may not occur at the surface where heme first inserts. Heme already in the membrane, however, would still be subjected to these transition gaps, no matter how transient. It is likely that the discontinuities formed at the interfaces between gel and liquid-crystalline phases cause the
large rate enhancement of transmembrane movement (Figure 6.7) at temperatures near the $T_m$.

The large affect of the phase state of the bilayer on the rate of heme flipping also explains some of the apparent deviations in Table 6.1. Although there is a general trend for the rate to decrease with acyl-chain length of the lecithin, there are a number of contradictions. Measurements made near the phase transition temperature of the lipid being studied may not be an accurate representation of the true rate for the liquid-crystal state. For example, the $T_m$ of DPDPC (34°C C) is close to 30°C. Unfortunately, 30°C is about the maximum temperature at which kinetic measurements can be reliably made with our stopped-flow apparatus.

Equations 6.2, 6.5, and 6.7 help to explain why Rose et al. (1985) were unable to observe transmembrane movement of CO-heme in either stopped-flow or chromatographic experiments. The geometric factor, assuming random heme distribution, for the fraction of heme in the inner portion of the bilayer is given by $R_i^2 / (R_o^2 + R_i^2)$. For the liposomes prepared in this study 45% of the total heme content can be expected to be in the inner portion of the bilayer ($R_o = 50nm, R_i = 45.5nm$), but only 23% would be in the inner portion of the bilayer for the liposomes used by Rose et al. (1985) ($R_o = 10nm, R_i = 5.5nm$). Even at equilibrium, most of the heme was present in the outer portion of the bilayer in the smaller liposomes and readily available for binding to ApoMb. Thus, under optimum conditions, only a small portion of the heme would be retained by the small liposomes utilized by Rose et al. (1985) after the first few seconds of mixing with apoMb. For chromatographic analysis, this problem was compounded by the incubation time allowed between mixing and loading on the column (at least 2-4 minutes). The measurable separation of the two heme pools shown in Figure 6.9 was the result of the greater fraction
of heme in the inner portion of the bilayer in our larger liposomes and the optimization of
the loading procedure for speed. In addition to the small amplitude problem, the conditions
used by Rose et al. (1985) were such that heme transmembrane movement in small egg
lecithin liposomes was fast enough not to be obvious by column chromatography but slow
enough to be very difficult to measure with stopped-flow techniques (i.e. $t_{1/2} = 5$ minutes).
In our best experiments, DMPC vesicles were used, and these liposomes have a relatively
fast rate for heme transmembrane movement (Figures 6.6 & 6.4) which is easily measured
in the stopped-flow apparatus (i.e. $t_{1/2} = 5$ seconds).
Chapter VII

Partition Rate and Equilibrium Constants for Heme Binding to Liposomes

Introduction

The kinetic parameters for the binding of CO-heme to liposomes were presented in Chapter 5 as bimolecular association rate constants on a per phosphate basis. Given that the physical process is actually a unimolecular partitioning phenomenon, it is more appropriate when comparing various lipid mixtures to report the rates as partition rate and equilibrium constants as indicated in equations 5.4 and 5.5. It is also necessary to make two corrections to the partitioning rates. First, the total lipid content of the membrane, not just the phosphate content, must be considered (i.e. account for cholesterol when used). Second, since the rate of heme transbilayer movement is slow, only the lipid content of the outer portion of the membrane should be considered for the initial association process (Equations 6.5 and 6.8).

As described in Chapter 6, heme efflux from lipid vesicles was measured by the formation of COMb from apoMb, and this method was also used for probing for the rate of heme transmembrane movement. The rate equation for heme flipping (Equation 6.18) was derived from theoretical considerations which included the initial efflux of heme from the outer portion of the bilayer (Equation 6.15). At high apoMb concentrations, two observed phases were observed when membrane-bound heme was mixed with apoMb. The rate of the fast phase is directly related to the dissociation rate constant, $k_{1}$, and the rate of the slow phase represents the speed of transmembrane movement of heme from the inner to the outer layer of the membrane, $k_{2}$ (Equation 6.4). Both rates were measured, but only the flipping rate constants were reported in Chapter 6. In principle, the initial heme dissociation rates should equal those indirectly determined from the $y$-intercept of plots of $k_{obs}$ versus $C_{p}$ which were obtained from association experiments (Table 5.1).
However, in most cases, the $k_j$ values measured directly by extraction with apoMb were lower by a factor of = 2 than those obtained from the dependence of the observed binding rate on lipid concentration. This difference was examined carefully to determine which rate should be used in the calculation of the equilibrium partition constant. After these corrections were made, it was possible to examine more quantitatively the effects of acyl-chain composition, lipid charge, and cholesterol content on the binding of heme to lipid bilayers.

Results

New Considerations in the Partitioning of Heme into Lipid Bilayers:

The unimolecular rate constant describing heme partitioning into the membrane phase, $k_I$, is related to the apparent bimolecular rate constant, $k'$, by the partial molar volume of the lipid, $k_I = k'/V_p$. The partial molar volume of lipid mixtures based on phosphate content is an inconvenient parameter, and it is easier to consider the partial specific volume of the membrane, $V_L$, which is = 1.0 ml/gm for all the lipid mixtures examined (White et al., 1987). Since $k'$ was defined as the slope of a plot of $k_{obs}$ versus $C_p$, what needs to be derived is an expression for $k_{obs}$ in terms of $V_L$ and $C_p$. In general, the observed rate for CO-heme binding to the outer layer of liposomes is given by (see Equation 6.8):

$$k_{obs} = \frac{k_I V_L C_L (R_o^2 / (R_o^2 + R_i^2))}{1 - V_L C_L (R_o^2 / (R_o^2 + R_i^2))} + k_{-1} \quad 7.1$$

$C_L$ is the total weight concentration of lipid in gm/ml. For the vesicles used in our study, $R_o = 50$ nm, $R_i = 45.5$ nm, and $V_L C_L(0.55) << 1$. Equation 7.1 then reduces to:

$$k_{obs} = k_I V_L C_L (0.55) + k_{-1} \quad 7.2$$
$C_L$ can be derived from the molar concentration of lipid, $C_m$ (moles/l): $C_L = C_mMW_{avg} / 1000$. $MW_{avg}$ is the average molecular weight of the lipid mixture and is determined by the sum of the mole fractions of the individual molecules multiplied by their molecular weights, $MW_{avg} = f_1MW_1 + f_2MW_2 + \ldots f_nMW_n$. The total molar concentration of lipid is related to the lipid phosphate concentration by $C_m = C_p / f_p$, where $f_p$ is the mole fraction of phosphate containing lipids. The final expression for the observed association rate is:

$$k_{obs} = k_1V_L\frac{MW_{avg}}{1000} \times \frac{C_p(0.55)}{f_p} + k_{-1}$$  \hspace{1cm} 7.3

and thus, the slope of $k_{obs}$ versus $C_p$ is given by $k_1V_LMW_{avg}(0.55)/(1000f_p)$ or

$$k_1 = \frac{k'f_p(1000)}{V_LMW_{avg} \times 0.55}$$  \hspace{1cm} 7.4

Unimolecular rate constants for heme movement from the aqueous to the membrane phase were calculated using Equation 7.4 and are listed in Table 7.1 for a variety of lipid compositions.

**Evaluation of CO-Heme Dissociation Rate Constants, $k_{-1}$:**

The determination of $k_{-1}$ from the y-intercept of a plot of the observed association rate as a function of lipid phosphate concentration assumes that such a plot is linear. Rose *et al.* (1985) suggested that the extent of heme partitioning into a bilayer is limited by electrostatic repulsion between the propionate groups of the heme. Evidence for this
Table 7.1  
Partition Constants for the Association and Dissociation of  
CO-Heme with Various Phosphatidylcholine Liposome Mixtures at 30° C

<table>
<thead>
<tr>
<th>Liposome Composition</th>
<th>$k_I \times 10^{-6} \text{ s}^{-1}$</th>
<th>$k_{I_{1}} \text{ s}^{-1}$</th>
<th>$K_p \times 10^{-6} \text{ s}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC</td>
<td>2.7</td>
<td>3.3</td>
<td>0.81</td>
</tr>
<tr>
<td>DTPC</td>
<td>3.1</td>
<td>5.0</td>
<td>0.62</td>
</tr>
<tr>
<td>DMPC</td>
<td>1.7</td>
<td>10$^a$</td>
<td>0.17$^a$</td>
</tr>
<tr>
<td>DMPC/DCP (95:5)</td>
<td>1.8</td>
<td>12$^a$</td>
<td>0.15$^a$</td>
</tr>
<tr>
<td>(90:10)</td>
<td>1.7</td>
<td>13$^a$</td>
<td>0.13$^a$</td>
</tr>
<tr>
<td>(85:15)</td>
<td>1.1</td>
<td>16$^a$</td>
<td>0.07$^a$</td>
</tr>
<tr>
<td>DMPC/Chol/DCP (8:1:1)</td>
<td>0.77</td>
<td>15.3</td>
<td>0.05</td>
</tr>
<tr>
<td>(7:2:1)</td>
<td>0.60</td>
<td>25.4</td>
<td>0.024</td>
</tr>
<tr>
<td>(5:4:1)</td>
<td>no binding observed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis $\Delta^9$ DMPC</td>
<td>3.2</td>
<td>6.8</td>
<td>0.48</td>
</tr>
<tr>
<td>cis $\Delta^9$ DMPC/DCP (9:1)</td>
<td>2.7</td>
<td>19.6</td>
<td>0.14</td>
</tr>
<tr>
<td>DPDPC</td>
<td>0.62</td>
<td>8.1</td>
<td>0.076</td>
</tr>
<tr>
<td>DPPC/DCP (9:1)</td>
<td>0.21</td>
<td>14</td>
<td>0.015</td>
</tr>
<tr>
<td>cis $\Delta^9$ DPPC</td>
<td>5.0</td>
<td>9.0</td>
<td>0.55</td>
</tr>
<tr>
<td>cis $\Delta^9$ DPPC/Chol (8:2)</td>
<td>5.7</td>
<td>5.7</td>
<td>1.0</td>
</tr>
<tr>
<td>cis $\Delta^9$ DPPC/Chol (6:4)</td>
<td>3.5</td>
<td>5.2</td>
<td>0.67</td>
</tr>
<tr>
<td>cis $\Delta^9$ DPPC/DCP (95:5)</td>
<td>3.3</td>
<td>16</td>
<td>0.20</td>
</tr>
<tr>
<td>cis $\Delta^9$ DPPC/DCP (9:1)</td>
<td>3.3</td>
<td>13$^a$</td>
<td>0.25$^a$</td>
</tr>
<tr>
<td>cis $\Delta^9$ DPPC/DCP (85:15)</td>
<td>2.4</td>
<td>22</td>
<td>0.11</td>
</tr>
<tr>
<td>POPC</td>
<td>2.9</td>
<td>6.0</td>
<td>0.50</td>
</tr>
<tr>
<td>POPC/Chol (8:2)</td>
<td>3.0</td>
<td>6.1</td>
<td>0.49</td>
</tr>
<tr>
<td>POPC/Chol (6:4)</td>
<td>3.6</td>
<td>8.4</td>
<td>0.43</td>
</tr>
<tr>
<td>POPC/DCP(95:5)</td>
<td>2.7</td>
<td>9.7</td>
<td>0.28</td>
</tr>
<tr>
<td>POPC/DCP (9:1)</td>
<td>2.7</td>
<td>23</td>
<td>0.12</td>
</tr>
<tr>
<td>POPC/DCP(85:15)</td>
<td>2.2</td>
<td>12</td>
<td>0.19</td>
</tr>
<tr>
<td>POPC/DMPG (95:5)</td>
<td>2.9</td>
<td>7.4</td>
<td>0.39</td>
</tr>
<tr>
<td>POPC/DMPG(85:15)</td>
<td>2.0</td>
<td>15.6</td>
<td>0.13</td>
</tr>
<tr>
<td>EL</td>
<td>4.2</td>
<td>7.0</td>
<td>0.60</td>
</tr>
<tr>
<td>EL/Chol (6:4)</td>
<td>3.5</td>
<td>9.1</td>
<td>0.38</td>
</tr>
<tr>
<td>OSIPC/DCP (9:1)</td>
<td>2.1</td>
<td>14</td>
<td>0.15</td>
</tr>
</tbody>
</table>

a) The $k_{I_{1}}$ values were not measured directly but estimated from Table 5.1 and are approximate values.
phenomenon was observed at low phospholipid to heme ratios. In simple binding experiments, the observed rate of heme association with egg lecithin membranes became independent of lipid concentration at low phosphate levels. The limiting rate as $C_p$ approached zero was 20-40 s$^{-1}$ which was 5-10 times greater than the dissociation rate constant measured directly by mixing apoHb with liposomes containing bound CO-heme. Similar curvature was observed in Figure 7.1a where the observed rate for the association of 1 μM CO-heme with egg lecithin liposomes prepared by our extrusion method is plotted versus phosphate concentration. This curvature was even more pronounced when 3 μM CO-heme was mixed with the membranes. In the absence of interactions between the heme groups, the observed association rates should be independent of the heme concentration. Thus, membrane-bound heme interactions also occur when heme is bound to large vesicles. Even at high phospholipid to heme ratios where a plot of the observed association rate versus phosphate concentration appears to be linear, the y-intercept may not reflect the true dissociation rate constant. Rose et al. (1985) accounted for interactions between bound heme molecules by attenuating the observed partition constant with an electrostatic potential factor that assumes uniform distribution of the anionic change along the surface of the membrane.

\[
K_p = K_p^0 \exp(-AH_b/V_p C_p)
\]

7.5

\(K_p^0\) is the ideal equilibrium constant at low levels of bound heme and \(A\) is a constant reflecting unfavorable electrostatic interactions which decrease the amount of heme which can partition into the bilayer. This was incorporated into the differential equation for heme binding by assuming that this unfavorable electrostatic potential increases the dissociation rate constant.
\[
\frac{dH_b}{dt} = k_I V_p C_p (0.55) \left( \frac{H_b}{1 - V_p C_p (0.55)} \right) (H_I - H_b) - (k_{0,I}^0 \exp (AH_b / V_p C_p (0.55))) H_b
\]

This equation was numerically integrated to generate the theoretical dependences of the observed rate for the binding of CO-heme to egg lecithin vesicles on the phospholipid and heme concentration which are shown in Figure 7.2. \( k_I \) was taken from the slope of the curves at high \( C_p \) values, and \( k_{0,I} \) from dissociation experiments in the presence of apomyoglobin (Table 7.1). A value of 8 M\(^{-1}\) for the \( A \) constant gave the best fit to the data. The value of \( k_{0,I} \) is shown in Figure 7.1 as the open diamond on the y-axis, and is significantly lower than the apparent intercept generated by either the theoretical curve or a least-squares fit to the data. Even a least-squares fit to the region of the theoretical curve that appears to be linear ([Phosphate] \( \geq \) 100 \( \mu \)M and [heme] = 1\( \mu \)M) results in a y-intercept that is higher than the rate measured by the apoMb uptake method (Figure 2.7b). The slope, \( k' \), from this type of plot is also influenced but to a much smaller degree. The apparent bimolecular rate from a least-squares fit to the full hypothetical curve in Figure 7.2b is only 10% less than that obtained when only the linear portion ([Phosphate] \( \geq \) 100 \( \mu \)M) is fitted. In practice, only phosphate concentrations \( \geq \) 25 \( \mu \)M were used in the determination of the association rate constants and curvature is only readily apparent at concentrations < 25 \( \mu \)M. On the other hand, the dissociation rate constant can not be accurately determined from plots of observed association rate versus lipid concentration because of the electrostatic factors which increase the apparent \( k_{0,I} \) value.

Accurate determinations of \( k_{0,I} \) must be obtained by the apoMb uptake method which can be carried out at high lipid to heme ratio. When apomyoglobin is mixed with liposomes containing bound CO-heme, a rapid phase is observed and limited by the rate of heme dissociation from the outer lipid layer. The observed rate is given by: \( k_{obs} = k_{I,I} / (1 + k_I V_p C_p / k_{MbMb}) \) (Equation 6.15). However, as shown in Figure 7.2, at high protein
Figure 7.1. Observed association rates of CO-heme with egg lecithin liposomes as a function of lipid phosphate concentration. The reactions of CO-heme with egg lecithin liposomes were followed at both 408 and 420 nm, 30° C with the stopped-flow apparatus. The average of the fitted observed rates were plotted as a function of total lipid phosphate. The curved lines are the theoretical rates determined as described in the text. The open diamonds on the y-axis are values for $k_{1}$ measured by the apoMb uptake method. A, the data points are from reactions of 1 μM and 3 μM CO-heme (closed circles and open circles, respectively). B, the data points are from the reaction of 1 μM CO-heme with the egg lecithin vesicles (closed circles) and the straight line represents a least-squares fit to the linear portion of the theoretical curve ([Phosphate] ≥ 100 μM).
Figure 7.2. The heme dissociation rate from DTPC vesicles as a function of apomyoglobin concentration. 100 μM DTPC vesicles equilibrated with 1 μM CO-heme was mixed with apoMb in the stopped-flow apparatus and the average of the rates observed at 414 and 424 nm were plotted as a function of apoMb concentration at 30°C (all reported concentrations are those after mixing).
concentrations this rate is independent of protein levels and directly equal to $k_f$. Corrected values of $k_I$ and $k_f$ values from direct dissociation experiments are listed in Table 7.1. $K_p^o$ was calculated from $k_I / k_o - 1$ and represents the ideal partition constant in the absence of interactions between bound heme molecules.

Discussion

At $30^\circ$ C, the single largest effect of lipid composition was a decrease in CO-heme binding with saturation of the phosphatidylcholine acyl-chains. As shown in Figure 7.3, the overall partition constant, $K_p$, decreased by almost a factor of 3 when comparing cis$\Delta^9$DMPC to DMPC and more than a factor of 15 when comparing cis$\Delta^9$DPPC/DCP (9:1) and DPPC/DCP (9:1). From Table 7.1, it can be seen that this change in affinity is primarily the result of a decrease in the association rate constant, $k_I$. In Chapter 5, a dramatic decrease in the apparent association rate constant for heme binding to membranes was noted when the temperature was lowered through the phase transition region. The measurements presented in Figure 7.3 were at $30^\circ$C, which is $6^\circ$ C higher than the phase transition temperature of DMPC and at least $4^\circ$C lower than the $T_m$ of DPPC/DCP (9:1). The results obtained with cis$\Delta^9$DPPC/DCP (9:1) and DPPC/DCP (9:1) confirm that heme binds extremely poorly to a lecithin below or near its phase transition temperature. The generalization that heme binds better under all conditions to unsaturated than fully saturated lecithins can not be made because even though the measurements were made above the $T_m$ of DMPC the measurement temperature is still close enough that there may be effects of residual gel phases. Unfortunately, accurate measurements at temperatures much greater than $30^\circ$ C were not possible with our current stopped-flow apparatus. Measurements were made with C$_{12}$ and C$_{13}$ acyl-chain lecithins with a lower $T_m$, but the corresponding unsaturated lecithins were not available. The unimolecular partition rate constants obtained for these lecithins, DLPC and DTPC, were comparable to those of the longer acyl-chain
Figure 7.3. The effect of acyl-chain saturation on heme partitioning with lipid vesicles at 30° C. The overall partition constant, $K_p$, was determined as described in the text for CO-heme binding to $\text{cis}\Delta^9\text{DMPC}$, DMPC, $\text{cis}\Delta^9\text{DPPC/DCP}$ (9:1), and DPPC/DCP (9:1) vesicles. The light gray bars represent the unsaturated lecithins and the dark gray bars their fully saturated counterparts.
unsaturated lecithins (Table 7.1; Figure 7.5). Thus, the major effect of adding double bonds to C14 and C16 saturated lecithin appears to be lowering the $T_m$ so that heme binding occurs to membranes in the liquid crystal state.

The effect of adding negative charges to liposomes is easier to interpret. The addition of dicetyl phosphate (DCP) during the preparation of four separate lecithin vesicles decreased the equilibrium partition constant for CO-heme binding (Figure 7.4a). A 3-fold decrease in the $K_p$ was generally observed at a level of 10% (per mole) DCP. Similar results were obtained when dimyristoylphosphatidylglycerol (DMPG) was added to POPC vesicles; $K_p$ decreased with increasing mole percent of the anionic lipid. Thus, the influence of DCP does not appear to depend on its chemical structure but rather its anionic character. In Figure 7.4b, it can be seen that the major effect of negative charge is an increase in the dissociation constant, $k_{-1}$; only a minor decrease in the association constant was observed. This result supports the assumption made by Rose et al. (1985) that electrostatic repulsion limits heme uptake by increasing the dissociation rate (see Equation 7.2).

In Figure 7.5, partition rate and equilibrium constants are presented as a function of $n$-acyl-chain length for simple lecithins as well as mixed acyl-chain phospholipids. Several conclusions can be made. First, the overall partition constant, $K_p$, decreased by less than 2-fold in going from C12 to C16 acyl-chains. Second, association rate constants are also relatively invariant; cis$\Delta^9$DPPC and egg lecithin exhibit the highest $k_I$ values, $4\times10^6$ s$^{-1}$ and DLPC the lowest, $2.7\times10^6$ s$^{-1}$. Third, the dissociation rate constant increases roughly 3-fold with increasing acyl-chain length in simple lecithins (C12 to C16) but the trend is less clear with the mixed acyl-chain lipids. This small increase in $k_{-1}$ with increasing acyl-chain length is the opposite of what was observed for the rate of transmembrane movement. As shown in Figure 6.5, $k_{-2}$ decreased roughly 100-fold in going from DLPC to POPC vesicles. This demonstrates that dissociation into the aqueous phase and flipping are
Figure 7.4. The effect of DCP on heme partitioning with lipid vesicles at 30°

C. DCP levels were varied from 0 to 15% (per mole) during the preparation of liposomes from four separate phosphatidylcholines, DMPC, cisΔ⁹DMPC, cisΔ⁹DPPC, and POPC. The kinetic parameters were determined for the binding of CO-heme to the 14 lipid compositions studied as described in the text. The lipid compositions are listed along the x-axis, the major lecithin is listed below the relative percentage of DCP used (i.e. DMPC, 10% is the same as DMPC/DCP (90:10)). A, the overall partition constant, determined by k / k', as a function of lecithin and %DCP. The white, light gray, dark gray, and black bars represent 0, 5, 10, and 15% DCP levels in the various phosphatidylcholines. B, the individual partition constants for heme going into, k (black bars), and out of, k' (gray bars), the same series of liposomes as in panel A.
Figure 7.5. The effect of the acyl-chains on CO-heme partitioning with phosphatidylcholine vesicles. The partition constants were determined for heme binding to a series of n-acyl lecithins, 12 to 16 carbons in length, as well as to the mixed acyl-chain lecithins POPC and egg lecithin. The light gray, black, and dark gray bars represent the measured values for heme partitioning into, out of, and the overall partition constant with the membrane phase, respectively, for each vesicle composition.
The image contains a bar chart labeled "KINETIC PARAMETERS". The x-axis represents various lipid molecules: DLPC, DIPC, cis Δ^0 DMPC, cis Δ^0 DPPC, POPC, and EL. The chart shows different sets of data represented by bars: $k_f \times 10^{-6}$ s$^{-1}$, $k_l$ s$^{-1}$, and $K_p \times 10^{-5}$. The chart also includes a legend indicating the types of acyl chains: $n$-acyl chains and mixed chains.
governed by quite different physical and chemical constraints.

The small but systematic increase in dissociation rate constants in going from DLPC to cisΔ⁹DPPC liposomes may be the result of acyl-chain packing constraints. When heme inserts into the bilayer, it is not freely miscible; the charged propionate groups must stay in close contact with the polar region surrounding the outside of the bilayer. Thus, the porphyrin ring is anchored to the surface of the bilayer. The width of the porphyrin ring is only 8-10 Å, and as a result, the portion of the heme that can penetrate the aliphatic region of the bilayer is shorter than the surrounding acyl-chains (see Figure 1.2). The acyl-chains must compensate by wrapping around the lower region of the heme and filling the gap. But as the fatty acyl-chains become longer the gap grows larger (=2.5 Å per 2 carbon unit). The formation of a gap behind the heme must destabilize the bound complex to some extent and this could account for the small increase in $k_J$ and decrease in $K_p$ with increasing chain length. In mixed acyl-chain lecithins the core region of the acyl-chains would already be heterogeneous and may be able to compensate for packing defects more readily. This might account for the lower $k_J$ values for POPC and EL vesicles. However, in spite of the trends just described, the major conclusion from the results in Figure 7.5 is that the partitioning parameters do not vary greatly with changing lecithin compositions as long as measurements are made at temperatures well above that of the phase transition.

As shown in Figure 7.6, cholesterol appears to have little effect on the CO-heme partition constant for POPC, egg lecithin liposomes, or at high levels in cisΔ⁹DPPC vesicles. The $K_p$ value for cisΔ⁹DPPC/Chol (8:2) appears to be ~two-fold greater than that for simple cisΔ⁹DPPC liposomes. As shown in Table 7.1, this increase in the partition constant is primarily due to a decrease in the cisΔ⁹DPPC dissociation rate constant in the presence of cholesterol. In the other lipid compositions cholesterol had little effect on the off-rate. However, again when looked at as a whole, these results indicate that cholesterol does not inhibit heme binding to membranes, at least at temperatures well above the $T_m$ for
the lipid being tested. Comparing Figures 7.6 and 5.4, dramatic effects of cholesterol are only observed in DMPC vesicles at temperatures below or near the phase transition.
Figure 7.6. The effect of cholesterol on heme partitioning with lipid vesicles. Cholesterol levels were varied from 0 to 40% (per mole) during the preparation of liposomes from three separate phosphatidylcholines, cis\(\Delta^9\)DPPC, POPC, and egg lecithin. The kinetic parameters were determined for the binding of CO-heme to the 8 lipid compositions studied as described in the text. The lipid compositions are listed along the x-axis. For each lecithin the light gray, dark gray, and black bars represent 0, 20, and 40% cholesterol.
Chapter VIII
Conclusion

Heme binding to either apomyoglobin or a lipid bilayer is extremely rapid, $= 9 \times 10^7$ M$^{-1}$ s$^{-1}$ and $= 3 \times 10^6$ s$^{-1}$, respectively, at 30° C. In apomyoglobin, this is seen as a lack of kinetic discrimination between two heme orientations in the heme binding pocket. Thus, the driving force for the initial complex between the protein and the prosthetic group is both strong and nonspecific. The major factor is presumably a "hydrophobic effect" involving preferential partitioning of the aromatic porphyrin ring into the lipophilic region between the F and E helices of apomyoglobin. The amphipathic nature of the heme group causes the propionic acids to be exposed to solvent and the methyl and vinyl substituents to be buried in the interior.

The rapid rate and high affinity constant for heme incorporation into apomyoglobin allow tight coupling between the synthesis of the protein and the prosthetic group. Excess production of either heme or globin is pathological, particularly in the case of hemoglobin biosynthesis in maturing erythrocytes. The speed of heme incorporation appears to preclude precise kinetic discrimination between the methyl and vinyl positions in the protein interior. However, the rate and equilibrium constants for O$_2$ binding are the same for the various orientational conformers. As a result the physiological properties of myoglobin are unaffected by the slow heme reorientation process and, therefore, independent of time after synthesis of holoprotein.

Heme binding to membranes can be described as a partitioning process; there was no evidence for discrete binding sites. The equilibrium partition constant is so high ($= 5 \times 10^5$ at 30° C) for simple lecithins with low $T_m$ values that under physiological conditions
there would be no significant levels of free aqueous heme. However, heme transfer can
occur between the outer layers of separate phosphatidylcholine membranes through the
aqueous medium and is limited only by the rate of heme efflux from the bilayer (≈7 s\(^{-1}\) at
30° C). Heme is also readily available for binding by apoproteins but the large partition
constant for association with a lecithin bilayer (≈ 5 \times 10^5 s\(^{-1}\) at 30° C) requires that the
affinity of the apoprotein for free heme must be high.

In this study the only factor seen to influence dramatically the equilibrium partition
constant for heme binding to liposomes was the physical state of the membrane. A sharp
decrease in the association rate of CO-heme with a lipid vesicle was seen near the transition
temperature as the lipid state changed from a liquid-crystalline to a gel phase and the bilayer
became much more viscous. This may not be directly relevant to physiological conditions
since most mammalian membranes are in the liquid-crystalline state, but it did confirm that
the heme group directly interacts with the acyl-chains. The addition of cholesterol to
DMPC liposomes decreased the affinity of these vesicles for heme in a concentration
dependent manner at temperatures at or below the \(T_m\). There was no effect of cholesterol
on heme binding with unsaturated lecithins. Thus, heme binding to natural membranes is
not inhibited by their cholesterol content.

The transmembrane movement of heme in liposomes containing long, unsaturated
acyl-chains is very slow (\(k_2 = 0.001 \text{ s}^{-1}\)). If this value is an indication of the \textit{in vivo}
flipping rate, then the time required for heme to travel from the inner matrix of the
mitochondria where it is synthesized through the inner and outer mitochondrial membranes
(Figure 1.1) could limit the rate of heme protein formation in the cytoplasm. It is possible
that the rate constants measured in these model membrane system are lower than those \textit{in}
vivo. Significant rate enhancement was observed \textit{in vitro} at temperatures near the phase
transition temperature, and there may be proteins or other factors that perturb membranes in a similar manner by creating discontinuities in the aliphatic region. In addition, the half-time of 10 minutes measured for heme flipping in egg lecithin liposomes at 30° C may not be all that large with respect to the rate of protein synthesis. It has been estimated that the rate of complete formation of a hemoglobin α-chain in reticulocytes is = 3 minutes per complete peptide at 37° C (Stryer, 1981). Finally, the slow rate of heme flipping could be advantageous under certain conditions. If there were no kinetic barriers to movement through the membrane, heme would rapidly disperse within a cell and be lost to the surroundings.
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