Oxidative Reactions in Normal and Sickle Hemoglobin-Lipid Mixtures Under Various Conditions

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OXIDATIVE REACTIONS IN NORMAL AND SICKLE HEMOGLOBIN-LIPID MIXTURES UNDER VARIOUS CONDITIONS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

BY

MERITA NIRMALI DIAS

CHICAGO, IL
JANUARY 1997
To my
Parents
My Husband and Daughter Angelie
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\(O_2^-\) superoxide anion

(Hb)\(_0\) initial hemoglobin concentration

(Hb)\(_t\) hemoglobin concentration at a given time

(heme)\(_0\) initial heme concentration

(heme)\(_t\) heme concentration at a given time

A\(_{215}\) optical absorbance at 215 nm

A\(_{233}\) optical absorbance at 233 nm

A\(_{409}\) optical absorbance at 409 nm

apoMb apomyoglobin

BPS bovine phosphatidylserine

deoxyHb deoxygenated hemoglobin

E\(_a\) activation energy

EM electron microscopy

FerryHb HbFe\(^{4+}\)

FPLC fast protein liquid chromatography

GSH glutathione

Hb hemoglobin

HbA normal adult Hb

HbACO carbon monoxo HbA

HbAO\(_2\) oxygenated HbA

HbCO carbon monoxo Hb

HbS sickle hemoglobin

HbSCO carbon monoxo HbS

HbSO\(_2\) oxygenated HbS

HPTLC high performance thin layer chromatography

k\(_{HbS}/k_{HbA}\) ratios of the rate constants of HbS/rate constants of HbA

MDA malondialdehyde

metHb oxidized Hb

metHbA oxidized HbA

metHbS oxidized HbS

MLVs multi lamellar vesicles

oxyHb oxygenated Hb

PL/Hb Phospholipid to hemoglobin molar ratio

PL phospholipid

P0N 6.5 35 mM phosphate and 0 mM NaCl buffer at pH 6.5

P10N 7.4 35 mM phosphate and 10 mM NaCl buffer at pH 7.4

P10N 6.5 35 mM phosphate and 10 mM NaCl buffer at pH 6.5

P50N 6.5 35 mM phosphate and 50 mM NaCl buffer at pH 6.5

P110N 7.4 35 mM phosphate and 110 mM NaCl buffer at pH 7.4
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>QELS</td>
<td>quasi elastic light scattering</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobuturic acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobuturic acid reactive substances</td>
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<td>TCA</td>
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CHAPTER I

INTRODUCTION

Heme iron in oxygenated hemoglobin (Hb) can undergo autoxidation in erythrocytes since oxygen is a strong oxidant. Autoxidation converts oxygenated Hb (oxyHb) to oxidized (met) Hb (metHb). In a normal red blood cell (RBC), three percent of oxyHb is converted to metHb per day (Winterbourn, 1985a). Methemoglobin reductase converts metHb back to deoxy hemoglobin (deoxyHb) (Chiu et al., 1982). Superoxide anion \((O_2^-)\) (Misra and Fridovich, 1972) and hydrogen peroxide \((H_2O_2)\) are products of Hb oxidation (Winterbourn, 1983; Winterbourn, 1985b). Therefore in oxidized RBC these oxygen radicals initiate chain reactions as lipid peroxidation and further protein oxidation (Chiu and Lubin, 1989; Winterbourn, 1990a). However, the generation of radicals during oxidation of Hb and lipid peroxidation in erythrocytes (Halliwell, 1985) does not necessarily lead to cell death, since a normal RBC is equipped with an efficient cellular antioxidation system that remove radicals (Chiu et al., 1982). Extensive oxidation and heme release within the cytosol or adjacent to the membrane may occur in hemolytic disorders or when red blood cells are under oxidative stress (Hebbel and Eaton, 1989; Hebbel, 1986). The heme may cause an increase in membrane rigidity and membrane permeability on erythrocyte membranes. Our understanding of Hb toxicity and oxidative stress in erythrocytes under physiological and
pathophysiological conditions will depend on our knowledge on individual oxidative reactions and the exact role of their reaction products generated from Hb oxidation and lipid peroxidation in erythrocytes. A better understanding of Hb toxicity in the presence of lipid surface will also further efforts to prepare synthetic blood cell substitutes using Hb-based oxygen carriers and liposomes (Rudolph, 1988; Bunn, 1993; Tsuchida, 1994; Alayash and Cashon, 1995).

Despite the fact that the Hb toxicity has been studied for over 100 years and topics on Hb oxidation and lipid peroxidation in erythrocytes have been well documented in literature, our understanding of Hb toxicity is still not definitive. Hb toxicity, other than that related to antigenicity, is generally attributed to oxygen radicals and free iron generated during Hb oxidation. Although molecular oxygen itself is oxidative with respect to superoxide anion radical, hydrogen peroxide, hydroxyl radical and water, its oxidative potential is normally held in check by kinetic restrictions (Chiu and Lubin, 1989). Hydrogen peroxide and superoxide production in the presence of hydroxyl radicals occur very slowly in an aqueous solution. However, rapid productions of hydroxyl radicals may occur in membrane if ferrous salts are present in membrane, following a pathway like the Fenton reaction (Fenton, 1894; Haber & Weiss, 1934). Previous studies show that in the presence of oxygen radicals and H$_2$O$_2$, destruction of heme may occur releasing free iron (scheme I) (Brown and Jones, 1968; Cantoni et al., 1981). Thus heme iron release and the presence of iron in the aqueous and lipid phases are important steps in Hb oxidation in erythrocytes.

Hydroxyl radicals may interact with organic substrates such as lipid molecules
and efficiently oxidize lipids, especially those with polyunsaturated fatty acids (Stoock
and Domandy, 1971), and the implication of lipid peroxidation by hydroxyl radicals has
been extensive (Chiu and Lubinl., 1989; Sevanian and Hochstein, 1985; Chiu et al.,
1982). It is believed that extensive lipid peroxidation in the membrane could lead the
RBC to premature cell death. However, the intrinsic ability of the hydroxyl radical to
mediate membrane lipid peroxidation has been questioned. Since hydroxyl radicals are
extraordinarily reactive, they do not survive for more than a few collisions following
their formation, and thus their reactions are intrinsically site specific (Chiu et al., 1982).
It is highly unlikely that these highly reactive intracellular hydroxyl radicals are able to
migrate to the lipid acyl chain region to initiate lipid peroxidation. The hydroxyl radical
is more likely to react with substrates near the site of its generation. However, if heme
or heme iron act as a Fenton reagent in the lipid phase (Kanner and Harel, 1985; Puppo
and Halliwell, 1988; Gutteridge et al., 1994), and catalyze the production of hydroxyl
radicals in the lipid phase, this may provide a possible explanation for the ability of
highly reactive hydroxyl radicals to initiate lipid peroxidation (Chiu and Lubinl., 1989).
It has also been suggested that a Fe$^{3+}$-dioxygen-Fe$^{2+}$ complex, and not hydroxyl radical, was the oxidant to initiate lipid peroxidation (Minotti and Aust, 1987). In either model, the presence of iron in lipid phase is again a critical component for lipid peroxidation. However, little information is available on the kinetics of free iron released from Hb and its accumulation in lipid phase.

I used a model Hb-vesicle system with purified Hb in reduced form (oxyHb) and uniform size lipid vesicles with no initial lipid peroxidation products (LaBrake and Fung, 1992) to study the initial phase of Hb oxidation. This system allows us to specify the oxidation states of the reactants (Hb and lipid) and reactant concentrations as well as lipid hydrophobicity. The system also consists of no exogenous oxidants to complicate interpretation of experimental results, since different exogenous oxidants may exhibit different Hb oxidation kinetics and products (van den Berg et al., 1992). I also used different buffer conditions to study the pH and the ionic strength effects on heme transfer into the lipid phase. Thus this model system provides a well-characterized system for following various intermediate oxidation products in Hb oxidation in the presence of lipid surface. It is obviously critical to characterize the intermediates under the same experimental condition. Yet results of Hb oxidative studies under various conditions, including different salt concentrations (Marva and Hebbel, 1994), different pH (Sugawara et al., 1993) and different lipid compositions as well as lipid vesicles with different sizes (Yang and Huesstis, 1994), were often compared with each other. Sometimes, different \textit{in vivo} phenomenon has been interpreted using results from non-physiological conditions (Marva and Hebbel, 1994). Previous studies have shown that lipid surface
enhances the initial disappearance of oxyHb (LaBrake and Fung, 1992). I have now extended the study to include the kinetics of iron accumulation in lipid phase in the Hb-small unilamellar vesicle (SUV) system. I also characterized different intermediate reactions in Hb-lipid systems.

I also used sickle Hb (HbS) for a comparative study with HbA. It has been reported that HbS has abnormal oxidation properties (Hebbel, 1986). I found that oxidative reactions in Hb-SUV mixture depended on buffer conditions. The disappearance of oxyHb was slower in buffers with physiological ionic strength and pH than in buffers with low ionic strength and low pH, as expected. Under physiological buffer conditions, extensive iron accumulation in the lipid phase was observed. Surprisingly, iron concentrations in the aqueous phase, after initial increases, decreased as a function of time, whereas iron concentrations in the lipid phase continued to increase. Eventually all iron ions released from Hb were found in the lipid phase. Little difference was observed between HbA and HbS systems.
CHAPTER II
MATERIALS AND METHODS

2.1 Materials

An important aspect of these experiments was to have all glassware acid washed before use. All the buffers were prepared with HPLC grade water, treated with chelex 100 (BioRad, Richmond, CA) and filtered through an 0.2 micron filter (Gelman Science Ann Arbor, MI) to eliminate trace metal ions and any exogenous oxidants. Bovine phosphatidylserine (BPS) was purchased from Avanti Polar Lipids (Albaster, AL). All other chemicals were purchased from Sigma (St Louis, MO), Fisher (Pittsburgh, PA) or Aldrich (Milwaukee, WI).

2.2 Buffers

Phosphate (35 mM) or tris (10 mM) based buffers were used. Variation of ionic strength (osmolarity) was achieved by addition of either NaCl or KCl to buffers. Precise osmolarity values were measured using a Vapor Pressure 5500 Osmometer (Wercor, Logan, UT). The following abbreviations were used to represent different buffer systems. We designated phosphate buffers with P and tris buffers with T, followed by salt concentration (mM), then by N or K for NaCl and KCl salt used respectively and ended by its pH value. For example, P110N7.4 was a phosphate buffer containing 110
mM NaCl at pH 7.4, and T5K6.5 was a tris buffer containing 5 mM KCl at pH 6.5. P110N7.4 was used in our earlier studies (LaBrake and Fung, 1992) and was a buffer with ionic strength and pH similar to physiological conditions. T5K6.5 was the buffer used by others in a study of sickle Hb oxidation in the presence of lipid vesicles (Marva and Hebbel, 1994) and was a low salt and low pH buffer. Other buffers used include P10N7.4, P10N6.5, P50N6.5, P0N6.5, T85K6.5, T20K6.5, T5K6.5 and T5N6.5.

2.3 Hemolysate Preparation

Packed RBCs (normal RBC or sickle RBC) were washed using 4 volumes of 35 mM phosphate and 150 mM NaCl pH 7.4 buffer (PBS). The washing was performed by suspending the RBC in the buffer and then centrifuging at 1500 g for 10 min. The supernatant and the buffy coat was removed by aspiration. This washing was repeated 4 times until the supernatant were clear. The washed RBCs were then lysed by addition of 2 volumes of cold DI water. A 77% ammonium sulphate solution at pH 7.0 was used for the precipitation of membrane proteins in the lysed RBCs. This solution was then centrifuged and the supernatant was obtained. The solution was CO gassed and dialyzed overnight against 5 mM phosphate pH 7.4 buffer to remove 2,3 -diphosphoglycerate and other small molecules. The resulting solution was then CO gassed, concentrated and stored at -70 °C (LaBrake and Fung, 1992).

2.4 Hemoglobin Preparation

Hb (HbA or HbS) was isolated from stripped hemolysate that was previously
prepared and was stored at -70 °C. The hemolysate was separated on a DEAE-sephadex A-50 (Pharmacia, Piscataway, NJ) column under a pH gradient made from 5 mM tris and 150 mM EDTA at pH 8.3 and pH 7.0. HbA was eluted at pH 7.75 and HbS was eluted at pH 7.9 (Winterbourn, 1985c; LaBrake and Fung, 1992). The purified hemoglobin fractions were CO gassed and dialyzed against 35 mM phosphate and 110 mM NaCl pH 7.4 buffer. Samples were tested for SOD and catalase activity (Watkins et al., 1985). No enzyme activity was found in the hemoglobin samples. The dialyzed Hb was frozen drop-wise in liquid N2 and stored at -70°C.

2.5 Oxyhemoglobin Preparation

Carbon monoxy Hb (HbACO and HbSCO) was oxygenated for 45 min on ice while illuminated by a flood light. The concentration was adjusted to the required concentrations using the absorbance at 577 nm and the extinction coefficient of 15.0 (mM/ heme)\(^{-1}\) cm\(^{-1}\) (Winterbourn, 1985c; LaBrake and Fung, 1992).

2.6 MetHemoglobin Preparation

HbA or HbS (160 µM) was treated with potassium ferrithiocyanate in a molar ratio of 1 mole heme : 3 moles of potassium ferrithiocyanate. The solution was stirred for 10 min and the excess potassium ferrithiocyanate was separated on a Sephadex G25 (Pharmacia, Piscataway, NJ) column equilibrated with 35 mM phosphate, 110 mM NaCl pH 7.4 buffer (Perutz, 1972). This column separation allowed us to remove the excess potassium ferrothiocyanate from methHb. The methHb fractions were pooled and the
concentration was determined by using the absorbance at 405 nm and its extinction coefficient of 149 mM$^{-1}$ (Perutz, 1972).

2.7 SUV Preparation

The BPS in CHCl$_3$ was evaporated under N$_2$ on a rotary evaporator to remove the CHCl$_3$ and to obtain a thin lipid film. The film was solubilized in N$_2$ gassed phosphate buffer or tris buffers to prepare multi lamellar vesicles (MLVs). SUVs were prepared by sonication (LaBrake and Fung, 1992) using a VC 50T Vibra Cell™ sonicator (Sonics & Materials Inc. Danbury, CT) and a 2 mm probe. A volume of 1 ml, of MLVs in phosphate buffers was sonicated for 4 min at power 40 and/or 1 ml of MLVs in tris buffers was sonicated for 2 min at power 20. The clear solution obtained after sonication was centrifuged at 38,000 g for 20 min to pellet large lipid vesicles and particulates (LaBrake and Fung, 1992).

2.8 Conjugated Diene Assay

The conjugated diene assays ($A_{233}/A_{215}$ index) were performed on the lipid samples to determine lipid peroxidation (Kim and LaBella, 1987). A sample of 10 µl of the lipid vesicles were mixed with 1 ml of ethanol and the absorbance at 233 nm and 215 nm were measured. The wave lengths were calibrated by using 10µl phosphatidylcholine in 1 ml of absolute ethanol. The blank phosphatidylcholine solution was made the same concentration as the sample to be assayed in absolute ethanol. (Kim and LaBella, 1987). Samples with an index of 0.08 are considered to be low lipid peroxidation and with 0.66
are considered to be high index of lipid peroxidation (Szebeni and Toth, 1986). The samples having an index above 0.2 were rejected (Szebeni and Toth, 1986; LaBrake and Fung, 1992).

2.9 Phospholipid Assay

The lipid concentrations of SUV samples were determined by the method of Stewart (1980). A sample of 25 µl of the BPS SUV was extracted with a mixture of 1.5 ml of water, 2 ml of CHCl₃ and 2 ml of MeOH. The CHCl₃ layer was evaporated to dryness under vacuum or N₂. It was then redissolved in 2 ml of CHCl₃ and 2 ml of 10 N ammonium ferrothiocyanate solution was added to obtain a red complex. The mixture was vortexed for 2 min. The CHCl₃ layer was separated, and the absorbance at 452 nm was obtained. The concentration of the lipid was calculated using the ε of 1.9 for a 0.5 mg / ml PS solution (Stewart, 1980).

The average effective diameter of SUVs determined by quasi elastic light scattering (QELS) particle size analyzer (Brookhaven Instruments, New Haven, CT) was 87.5 ± 5 nm (n = 3) for SUVs made in P110N 7.4 buffer and 88 ± 6.8 nm (n = 3) for SUVs made in T5K 6.5 buffer. The effective diameters of SUVs obtained from the QELS are generally larger than what were obtained by electron microscopy (EM) (Mayer et al., 1986). An effective diameter of ~ 90 nm corresponds to a 10-30 nm diameter measured by EM (LaBrake and Fung, 1992). Thus the SUVs used in this study were similar to those used in our earlier studies of Hb oxidation (LaBrake and Fung, 1992).
2.10 Hemoglobin-SUV Mixtures

Hb and SUVs solutions were incubated at 37°C in phosphate buffers or at 28°C in tris buffers of different ionic strength and were mixed to give a sample with a specific phospholipid/Hb molar ratio. For samples with phospholipid to Hb molar ratio of 800, 240 µL of Hb (17.7 µM) and 1.0 ml of SUVs (3.4 mM BPS) were mixed to give a final concentration of 3.4 µM for Hb and 2.7 mM for BPS. For other phospholipid/Hb ratios (360, 200 or 45), sample volume and Hb concentration were held constant, BPS concentrations were varied. The phospholipid to Hb ratio of 45 was the same as that used by Marva and Hebbel (1994).

2.11 Disappearance of OxyHemoglobin

UV-Vis absorption intensities from 500 nm to 700 nm were monitored for 1 h. The oxidation of Hb was determined by using the four component (oxyHb, metHb, hemichrome and choleglobin) analysis method (Labrake and Fung, 1992). The oxyHb, metHb, hemichrome and choleglobin concentrations were determined by matrix equations assay the absorbance at 577, 630, 560, and 700 nm, (Labrake and Fung, 1992; Winterbourn, 1985c). The pseudo first order rate constant (k) for the disappearance of oxyHb was obtained by fitting the logarithmic values of oxyHb concentration ([oxyHb], (%)) versus time (t) to an linear equation of ln [oxyHb], = kt.
2.12 Disappearance of Heme/Porphyrin

Fluorescence signals of porphyrin were used to detect heme concentration (Schwartz et al., 1983). An oxalic acid/FeSO₄ solution was used to dissociate Fe³⁺/Fe²⁺ from porphyrin in free heme or heme moiety in Hb. Five microliter of Hb-SUV mixtures were mixed well with 3 µl of Triton-X-100 and added to 1 ml of FeSO₄/oxalic acid solution (2.5 g of FeSO₄ and 30.0 g of oxalic acid in 100 ml of DI water) (Schwartz et al., 1983). The sample was heated at 100°C for 0.5 h and then cooled for 15 min at room temperature. The fluorescence emission at 601 nm, with excitation at 405 nm, was measured. Porphyrin concentration in the mixture was determined by using a calibration curve made with Hb (concentration range of 0.1 µM to 30 µM) as the standard. The porphyrin concentration was monitored as a function of time (t) in the Hb-SUV mixtures. The decrease in the fluorescence signals was used as an indication of porphyrin disappearance. A semi-logarithmic plot of [porphyrin] (%) versus time (t) was obtained, and the slope from a linear regression analysis provided the first order rate constant (k) for porphyrin disappearance in the presence of lipid vesicles.

2.13 Heme Partitioning into the Aqueous Phase

MetHb was used as the heme donor and apomyoglobin (apoMb) as the acceptor for free heme in solution (Paul et al., 1991). MetHb concentration was adjusted to 3.4 µM per heme and the apoMb concentration to 17 µM to give a 5 molar excess of apomyoglobin in the reaction mixture. Changes in absorbance at 409 nm were monitored as a function of time at 25°C instead of 37°C, since absorbance changes were too fast
to follow at 37°C. The difference in absorbance values at 409 nm between metHb (3.4 µM) and myoglobin (heme-containing myoglobin) (3.4 µM) in specific buffer was used to represent complete heme extracted in that buffer.

2.14 Heme Transfer from Hemoglobin to SUVs

SUVs were separated from Hb in Hb-SUV mixtures by size exclusion column chromatography. Samples were loaded immediately after mixing (< 5 min) to a FPLC Superose 12 (Pharmacia) column, and the elution time was about 15-20 min for SUV fractions and about 60 min for Hb. Porphyrin and lipid concentrations were determined on pooled SUV fractions. A hundred microliter sample from the pooled SUV fractions in 3 µL Triton X-100 was used for porphyrin assays. Fractions of heme transferred from Hb to SUVs were calculated as [porphyrin per mg lipid in SUV pooled fractions]/[porphyrin per mg lipid in Hb-SUV mixture] (%). Since the heme transfer process was faster than our separation process (we estimated it to be about 10 min or less on our Superose column) for systems in T5K6.5 buffer, we were not able to determine kinetic properties of heme transfer to SUVs. Therefore only the extent of heme transfer to lipid phase was obtained.

2.15 Free Iron Concentration

Free iron concentration in samples was determined with ferrozine/neocuprion reagent (75 mg ferrozine, 75 mg of neocuproine hydrate and one drop of conc. HCl in 25.00 ml water) (Carter, 1971). Equal volumes (125µL) of sample and ascorbic acid
(0.02 % in 0.2 N HCl) were mixed and left at room temperature for 5 min. Trichloroacetic acid (10 %) solution (125 µL) was then added, mixed and centrifuged at 16,000 g for 10 min to obtain a clear supernatant. Ferrozine/neocuprion reagent (25 µL) and 10 % sodium acetate (100 µL) were added to the supernatant (250 µL). The concentration of free iron was determined by monitoring absorbance at 562 nm for the formation of magenta colored complex of Fe$^{3+}$ with ferrozine. A calibration curve was obtained using Fe(NH$_4$)$_2$SO$_4$·6H$_2$O (10$^{-4}$ µM to 30 µM).

Triton X-100 was added to the sample prior to the addition of assay reagents to determine total (lipid phase plus aqueous phase) Fe ion concentrations. Aqueous phase iron concentrations were assayed in the absence of Triton X-100. Iron concentrations in the lipid phase were obtained by subtraction. Iron concentrations were monitored as a function of time in Hb-SUV mixtures.

2.16 Cross-Linked Hemoglobin in the Hb-SUV mixture

Hb-SUV mixture was incubated at 37 °C. Samples were obtained every hour and loaded to a 16 % SDS PAGE stained with Coomassie Blue. The bands on the gel were scanned using the Logitech Scan-Man II (Logitech Inc., Fremont, CA), and the band intensities as a function of time in each lane were analyzed with Sigma Gel software (Jandel, San Rafael, CA). The experiments was performed in P110N 7.4 buffer at 37 °C, with a phospholipid/Hb molar ratio of 800, and in T5K 6.5 buffer at 28 °C, with a phospholipid/Hb molar ratio of 360.
2.17 Appearance of Malondialdehyde (MDA)

Thiobarbituric acid (TBA) solution was prepared by mixing TBA (0.375 mg) and trichloroacetic acid (TCA) (10 g) in 100 ml of 0.2 N HCl solution. A sample of 50 µl of the Hb-SUV mixture was added into 500 µl of TBA solution. The sample was boiled for 30 min, cooled at room temperature for 15 min and centrifuged at 16,000 g for 10 min. The TBA forms a complex with the MDA giving rise to a pink colored compound. The absorbance of the TBA-MDA complex (thiobarbituric acid reactive substances, TBARS) formed was determined at 532 nm. The concentration of TBARS was determined by using a calibration curve of MDA within a concentration range of 0.10 µM to 10.00 µM (Buege and Aust, 1978). The experiments were performed in P110N 7.4 buffer at 37 °C and at a phospholipid/Hb molar ratio of 800.

2.18 Oxidative Products in the Hemoglobin-SUV Reaction Mixture

Hb-SUV mixture was incubated for 0, 2, and 6 h, followed by separation on a Sepharose 4B-CL (Pharmacia, Piskataway, NJ) column (42 x 1.5 cm) equilibrated with the experimental buffer (13.5 ml/min). The molecular weights of the separated components were determined using a calibration curve prepared with ribonuclease A (13.7 kDa), carbonic anhydrase (23 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa) and ferritin (440 kDa) as the molecular weight standards. The experiments were performed in P110N 7.4 buffer at 37 °C and at a phospholipid/Hb molar ratio of 800 and in T5K 6.5 buffer at 28 °C and at a phospholipid/Hb molar ratio of 360.
The separated peaks from HbA-SUV mixture at PI/Hb molar ratio of 800 were tested for carbonyl functions using UV/Vis spectra.

2.19 Lipid Bound to the Hemoglobin

The pooled fractions separated on a Sepharose 4B-CL column as described above were extracted with CHCl₃/MeOH (1:1 v/v) mixture. The CHCl₃ phase was separated and evaporated to dryness. The dried sample was redissolved in 2.0 ml CHCl₃ and the lipid content was determined by the lipid assay (Stewart, 1980). Alternatively the dried sample was redissolved in 200 µl CHCl₃ and loaded on to a high performance thin layer chromatogram (HPTLC). The HPTLC plate was first activated with a CHCl₃/MeOH (1:1 v/v) mixture and the plate was dried for 5 h at 120 °C. The plate was then cooled for 1 h in a CaCO₃ desiccator. A 20 µl sample was loaded to the plate and eluted with CHCl₃/MeOH/NaOH (60 : 20 : 4 v/v) to half the length of the plate for high polar components. After air drying the plate was eluted with hexane/diethyl ether/HOAc (80 : 20 : 0.5 v/v) for the full length of the plate. The plate was visualized by spraying the plate with 10% CuSO₄ in 10% phosphoric acid and then charring the plate at 160 °C for 10 min. The charred spots of the sample were compared with those from BPS (Avanti Polar Lipids) and stearic acid (Sigma Chemicals). The experiments were performed in P110N 7.4 buffer at 37 °C and at a phospholipid/Hb molar ratio of 800 and in T5K 6.5 buffer at 28 °C and at a phospholipid/Hb molar ratio of 360.
CHAPTER III

RESULTS

Previous studies have shown that the oxidation kinetics of oxyHb in Hb-SUV mixtures depends linearly on phospholipid to Hb molar ratio (LaBrake and Fung, 1992). The higher the molar ratio, the faster the reactions. In this study, I found that oxidation kinetics depended on buffer conditions. I used smaller phospholipid to Hb molar ratio values in samples with buffer conditions that provided faster kinetics and larger molar ratios in samples with buffer conditions that provided slower kinetics. A molar ratio of 800 was used for systems in P110N7.4 buffer and molar ratios of 360, 200 and 45 for systems in T5K6.5 buffer. These adjustments in molar ratios positioned oxidative reactions in Hb-SUV mixtures under different buffer conditions to similar time frame (minutes to hours) and thus provided results under different conditions with similar reliability. If the molar ratio was kept the same for systems with different buffer conditions, the oxidative reactions would span vastly different time frames (e.g. msec or days).

3.1 Oxidative Reactions Under Physiological pH and Ionic Strength Conditions (P110N 7.4)

3.1.1 Disappearance of OxyHb

Disappearance of Hb was determined by analyzing the UV-Vis absorption spectra.
Figure 1. Spectral changes from 500 - 700 nm of HbO$_2$ + BPS SUVs in P110N 7.4 buffer as a function of time at lipid to Hb molar ratio of 800 and 37 °C. The time interval between each spectrum was 6 min, with the bottom spectrum taken immediately after mixing HbO$_2$ and SUVs and the top spectrum taken at 45 min after mixing. Characteristic absorption peaks for oxyHb at 577 nm and 565 nm was observed at $t = 0$, and a prominent peak at 630 nm were observed at $t = 45$ min. A. HbAO$_2$ + BPS SUVS and B. HbSO$_2$ + BPS SUVs
Spectral Changes in HbA and HbS
PN 7.4 Buffer, at PL/Hb = 800 & 37°C
Figure 2. The semi-logarithmic plot of oxyHb concentration (%) versus time (h) for the disappearance of Hb in the presence of BPS SUVs in P110N 7.4 buffer at lipid to Hb molar ratio of 800 and 37 °C. The value at each time point was the mean value of 2 experimental runs. ○ HbAO₂ + BPS SUVs and ■ HbSO₂ + BPS SUVs
Disappearance of OxyHb in the Presence of BPS SUVs

PN 7.4 Buffer, 37°C, PL/Hb = 800
A typical set of spectra obtained on HbA-SUV sample and HbS-SUV sample in P110N 7.4 buffer as a function of time (Fig. 1A & B) shows a decrease in the two characteristic peaks for oxyHb at 577 and 565 nm, due to the disappearance of oxyHb, and an increase in absorbance at 630 nm, due to the formation of metHb as well as a slight increase in absorbance at 700 nm which was attributed to the formation of choleglobin and fusion of lipid vesicles (La.Brake and Fung, 1992). A four component (oxyHb, metHb, hemichrome and choleglobin) analysis (LaBrake and Fung, 1992) of these spectra allowed us to determine oxyHb concentrations as a function of time in our Hb-SUV mixture. The semi-logarithmic plot of oxyHb concentration (%) versus time revealed a linear relationship during the initial phase (up to about 0.5 h) for both HbA and HbS SUV oxidation reaction mixtures (Fig. 2). Linear fitting of the first 11 data points (0 - 0.5 h) provided a pseudo first order rate constant for the initial disappearance of oxyHb in the presence of SUVs. The mean rate constant of $2.77 \pm 0.08$ h$^{-1}$ ($n = 3$) was obtained for HbA-SUV mixture. The time for the disappearance of 50% of the maximum value ($t_{1/2}$) of oxyHb concentration was 0.25 h. The reported rate constant of the HbA oxidation under similar conditions was $3.20 \pm 0.52$ h$^{-1}$ (LaBrake and Fung, 1992). The mean rate constant of HbS oxidation was $2.70 \pm 0.09$ h$^{-1}$ ($n = 3$) (Fig. 2) and the $t_{1/2}$ for the disappearance of oxyHbS was 0.25 h. These values were very similar to those of HbA system in P110N 7.4 buffer.

3.1.2 Oxidative Products in Hb-SUV Mixtures in P110N7.4

Upon mixing (t ~ 5 min) of oxyHbA and SUVs in P110N7.4 with a phospholipid
ratio of 800, two fractions were separated by size exclusion column (Sepharose 4B-CL) (Fig. 3). The first fraction eluted at void volume of 23.6 ml (elution time of 1.75 h) and was identified as SUVs. This peak consisted of 2.4 - 2.6 mg of phospholipid, as determined by phospholipid assays, which corresponded to about 80 - 85 % of the total amount of BPS loaded to the column. The second fraction eluted at 65.7 ml (elution time of 4.8 h), corresponding to a molecular mass of 64 ± 0.2 kDa (n = 2), and was assumed to contain mostly HbA (Fig. 3 a). Small amounts of phospholipid (0.11 ± 0.02 mg or about 4 % of total BPS) was detected by phospholipid assays. As shown earlier, about 36 % of HbA molecules already exhibited inter-chain cross-linking, but remained as tetramers under these conditions.

After 2 h incubation, the lipid vesicle peak (at the void volume of 23.6 ml) became more intense, probably due to vesicle fusion induced by Hb molecules to give extensive light scattering at 280 nm (LaBrake and Fung, 1992). This fraction consisted about 80 - 85 % of the total amount of BPS loaded, as determined by phospholipid assays. No fraction was collected at the 65.7 ml position. However, a fraction at 63.7 ml (elution time of 4.7 h), corresponding to a molecular mass of 94 kDa, and a fraction at 81.6 ml (elution time of 6.0 h), corresponding to a molecular mass of 2 kDa, were collected (Fig. 3 b). Globin cross-linking studies discussed earlier showed about 62 % of Hb as monomers, 26 % as dimers and 12 % as trimers, and these values were very similar to those observed at t ~ 5 min. The shift in molecular mass from 64 kDa to 94 kDa was thus assumed due to an increased amount of SUV/BPS associated with Hb molecules. The fraction with low molecular weights (2 kDa) was not analyzed but was
The elution profiles of the separation of the HbA-SUV mixture on the sepharose 4B-Cl gel filtration column. The HbAO₂⁺ BPS SUVs in P110N 7.4 buffer at a lipid to Hb molar ratio of 800 and at 37 °C were incubated for 0, 2 and 6 h and was separated on a sepharose 4B-Cl gel filtration column.
Elution Profiles of HbA-SUV Mixture Separated on a Sepharose 4B-Cl Column, in P110N 7.4 Buffer at 37°C
assumed to be similar to the 79.7 ml fraction at $t = 6$ h, as described in detail below.

After the Hb-SUV mixture was incubated for 6 h, other than the lipid peak, which still consisted about 80% phospholipid, a fraction at 61.2 ml (4.5 h), corresponding to a molecular mass of 184 ± 11 kDa, and a fraction at 79.7 ml (4.7 h), corresponding to a molecular mass of 3 ± 0.5 kDa ($n = 2$), were detected (Fig. 3c). The fraction at 61.2 ml (184 kDa) exhibited no absorption peaks characteristic of the heme moiety and was assumed to be mostly globin aggregates or choleglobin (gel electrophoresis data showed ≥75% globin cross-linking). Pacifici and coworkers, in their studies of Hb oxidation by hydrogen peroxide, observed a peak on a Sepharose 4B column that corresponded to 120 - 180 kDa (Pacifici et al., 1993). This fraction also consisted of small amounts of phospholipid (0.26 ± 0.02 mg, $n = 2$ phospholipid or about 8% total, as detected by phospholipid assay). HPTLC data also indicated the presence of BPS as well as fatty acids and aldehydes (Fig. 6, Lanes 2). The fraction at 79.7 ml (3 kDa) exhibited a sharp absorbance at 265 nm that corresponded to conjugated carbonyl groups (Fig. 5). This fraction also exhibited a trace level of BPS and fatty acids and other aldehydes, as detected by HPTLC (Fig. 6, lane 5). Only about 1.5% of total BPS (0.05 ± 0.01 mg; $n = 2$) was detected in this fraction. Thus this fraction probably consisted of low molecular weight lipid peroxidation products.

I observed similar changes when HbS-SUV mixture that was separated on the sepharose 4B-Cl column (Fig. 4). Initially when the Hb-SUV mixture was separated the Hb fraction with a molecular weight of 64 kDa and the lipid fraction eluted off with the void volume (Fig. 4 a). When the HbS-SUV mixture was incubated for 6 h the
Figure 4. The elution profiles of the separation of the HbS-SUV mixture on the sepharose 4B-Cl gel filtration column. The HbSO$_2$+ BPS SUVs in P110N 7.4 buffer at 37 °C with a lipid to Hb molar ratio of 800 and were incubated for 0, 2 and 6 h and was separated on a sepharose 4B-Cl gel filtration column.
Elution Profiles of HbS-SUV Mixture Separated on a Sepharose 4B-Cl Column, in P110N 7.4 Buffer at 37°C
Figure 5. The UV/Vis spectrum of 3 kDa fraction obtained from the column separation of HbA-SUV mixture at 37 °C with phospholipid to Hb molar ratio of 800.
UV/Vis Spectrum of 3 kD Fraction

Separated from HbA-SUV mixture
The high performance thin layer chromatogram of the CHCl$_3$ extract of the Hb fractions and the byproduct fractions separated from the size exclusion chromatography at $t = 6$ h (see Fig. 2). The TLC was eluted with CHCl$_3$/MeOH/NH$_3$OH (60:20:4) up to 50 % of the plate length and then the plate was eluted with hexane/diethyl ether/HOAc (80:20:0.5) up to 100 % of the plate length. The plate was visualized using 10 % CuSO$_4$ in 10 % phosphoric acid and charring at 160 °C for 10 min.

Lane 1- BPS (Avanti Polar Lipids)

Lane 2- CHCl$_3$ extract of the 61.2 ml (184 kDa) fraction of HbA-SUVs mixture

Lane 3- CHCl$_3$ extract of the 61.8 ml (190 kDa) fraction of HbS-SUVs mixture

Lane 4- Stearic acid

Lane 5- CHCl$_3$ extract of the 79.7 ml (3 kDa) fraction of HbA-SUVs mixture

Lane 6- CHCl$_3$ extract of the 79.7 ml (3 kD) fraction of HbS-SUVs mixture
eluted fractions we observed two new peaks appearing with molecular weights of 190 kDa and 3 kDa (Fig. 4c) similar to HbA-SUV mixture.

3.1.3 Globin Cross-Linking

The reaction mixture was also characterized using SDS PAGE. The Hb-SUV (t = 5 min) exhibited bands at 15 kDa (64 % of total), 30 kDa (24 %) and a faint band at 45 kDa (12 %) of Hb (Fig. 7 A & B) for both HbA and HbS SUV mixture. Since a native Hb gave rise to only a band at 15 kDa, under similar gel conditions the 15 kDa band in Hb-SUV samples corresponded to those Hb molecules that were not cross-linked and the higher molecular weight bands were due to covalently cross-linked α and β chains (cross-linked αβ at 30 kDa, dimers and αβα or αββ at 45 kDa, trimers) (Fig. 8). Thus at t = 5 min about 36 percent of globin were already cross-linked. At 2 h of incubation SDS PAGE data showed 62 % of Hb as monomers 26 % as dimers and 12 % as trimers chains. These values were similar to those obtained immediately after mixing (t = 5 min).

I observed an increase in globin cross-linking in the Hb-SUV oxidation sample with prolonged incubation with 43 ± 12 % at 3 h, 64 ± 6 % at 4 h and 75 ± 5 % at 5 h (n = 2) (Fig. 8). Similarly I observed the cross-linking of HbS in the presence of SUV and observed 30.0 %, 27 %, 41 %, 48 %, 66 % and 70 % at ~ 5 min, 1 h, 2 h, 3 h, 4 h, and 5 h respectively (Fig. 8). The samples showed the appearance of bands with a higher than 45 kDa. These results agreed with previous findings of Hb oxidation in the presence of H₂O₂ (Pacifici et al., 1993). They observed the appearance of the
Figure 7. The 16% SDS PAGE performed on the Hb-SUV mixture in P110N 7.4 buffer at lipid to Hb molar of 800 and 37°C, at different incubation times. The 50 µl of the sample was loaded to the gel. A. HbA-SUV mixture, B. HbS-SUV mixture
SDS PAGE of Hb-SUV Mixture in P110N 7.4 Buffer
at PL/Hb = 800 & 37 °C
Figure 8. The % of cross-linked Hb in the Hb-SUV mixture in P110N 7.4 buffer at lipid of Hb molar of 800 and 37 °C as a function of incubation time (h).

The cross-linking was monitored by performing a 16 % SDS PAGE. The % intensity of each band was obtained by using Logitech Scan Man for scanning and Sigma Gel analyzing software for the gel % analysis.

■ HbAO₂ + BPS SUVs and □ HbSO₂ + BPS SUVs
Cross-Linking of Hemoglobin In the Presence of BPS SUVs

In P110N 7.4 Buffer at PL/Hb = 800 & 37 °C
kDa, and 48 kDa bands on a SDS PAGE. The $t_{1/2}$ values for the cross-linking of Hb was estimated to be 3.5 h for both HbA and HbS in SUVs. The cross-linked globin was also identified as choleglobin, a species that is not well identified in the literature, but is generally considered as denatured globin.

3.1.4 Disappearance of Heme/Porphyrin

I observed the fluorescence intensity of the porphyrin in Hb-SUV samples decreasing with time (Fig. 9). The first order rate constant for the initial porphyrin (heme) disappearance was $0.76 \pm 0.12 \text{ h}^{-1}$ (n = 3) for HbA-SUV mixture and $0.79 \pm 0.11 \text{ h}^{-1}$ (n = 3) for HbS-SUV mixture followed by a second first order rate constant of $0.43 \pm 0.23 \text{ h}^{-1}$ (n = 3) for HbA and $0.50 \pm 0.35 \text{ h}^{-1}$ for HbS. At 0.5 h 63 % of heme was left in the oxidation reaction mixture, and at 1.0 h it decreased to 45 % for both HbA and HbS - SUV mixtures. The $t_{1/2}$ value for the destruction of heme was 0.75 h for both HbA and HbS. The results obtained for HbS-SUV mixtures were very similar to those of HbA-SUV mixture.

3.1.5 Heme Transfer into the Lipid Phase (SUVs)

I observed only about 1 % heme transferred to the lipid phase upon mixing oxyHb with SUVs in P110N 7.4 buffer for both HbA ($1.10 \pm 0.02 \%$, n =2) and HbS ($1.15 \pm 0.07 \%$, n =2).
Figure 9. The semi-logarithmic plot of heme concentration (%) versus time (h) in Hb-BPS SUV mixture in P110N 7.4 buffer at lipid to Hb molar ratio of 800 and 37 °C. The heme concentration was determined by fluorescence spectroscopy as described in the Methods section. The fluorescence emission of the solution was obtained at 601 nm with the excitation at 405 nm. The values presented were mean values of 3 experimental runs.

HbAO₂ + BPS SUVs and O HbSO₂ + BPS SUVs
Disappearance of Heme/Porphyrin in the Presence of BPS SUVs

In P110N 7.4 Buffer at PL/Hb = 800 and 37 °C

Heme (%) in the Hb-SUV Mixture

Time (h)
3.1.6 Appearance of Free Iron

The free iron concentration in the Hb-SUV mixture increased as a function of incubation time. However the semi-logarithmic plot of free iron concentrations in both aqueous phase and lipid phase as a function of time could not be fitted with a linear equation (Fig. 10). Therefore I was not able to describe the iron released from Hb with a first order rate constant. The mean value of the free iron concentration was 27.4 ± 4.9 % (n = 3) for HbA-SUV mixture and 27.1 ± 8.0 for HbS-SUV mixture at 0.5 h, and 100 % at 10 h (Fig. 10) for both HbA and HbS-SUV mixtures. All the iron in Hb molecules was released after 10 h incubation with SUVs. The free iron concentrations in HbS-SUV mixture were similar to those in HbA-SUV mixture. The $t_{1/2}$ values for the total iron released from HbA and HbS in the presence of SUVs were about 1.3 h.

The concentrations of free iron in the aqueous phase increased initially and reached a maximum value of 33.8 ± 0.0 % (n = 2) at 2.5 h for HbA-SUV mixture (Fig. 4), and 37.1 ± 0.0 % (n = 2) for HbS-SUV mixture. The concentrations in the aqueous phase decreased after 2.5 h in HbA-SUV mixture and HbS-SUV mixture. At 10 h the mean concentration decreased to 23.1 ± 4.8 % (n = 2) for HbA-SUV mixture and 16.4 ± 1.0 % (n = 2) for HbS-SUV mixtures. The $t_{1/2}$ value was about 0.8 h for both HbA and HbS.

The free iron concentrations in the lipid phase continued to increase with time for both HbA-SUV and HbS-SUV mixtures. At $t = 2.5$ h, 23.8 ± 5.8 % of iron was present in the lipid phase in HbA-SUV sample and 28.0 ± 0.0 % in HbS-SUV sample.
Figure 10. A. The semi-logarithmic plot of free iron concentration (%) in HbA-SUV mixture as a function of time (h). The iron concentrations were determined by the foreseen assay. The iron concentration in the lipid phase was determined by subtracting the free iron concentration in the aqueous phase from the total free iron concentration. A. ■ total free iron concentration in HbA-SUV mixture △ Free iron in the lipid phase in HbA-SUV mixture, and ○ free iron in the aqueous phase in HbA-SUV mixture. B. ▽ total free iron concentration in HbS-SUV mixture ● Free iron in the lipid phase in HbS-SUV mixture, and ■ free iron in the aqueous phase in HbS-SUV mixture
Free Iron Release From Hb in the Presence of BPS SUVs

in P110N 7.4 Buffer at P/LHb = 800 and 37°C

A

Free Iron (%) in Hb-SUV Mixture

Time (h)

B

Free Iron (%) in Hb-SUV Mixture

Time (h)
Within a period of 10 h, 76.9 ± 4.8 % of free iron partitioned into the lipid phase for HbA-SUV mixture, and 83.6 ± 1.0 % for HbS-SUV mixture. The $t_{1/2}$ values for the iron partition into the lipid phase for both HbA and HbS was about 4.6 h. Thus after 10 h most of the iron released from Hb was partitioned into the lipid phase, with slightly higher values for HbS-SUV mixture than for HbA-SUV mixture in P110N 7.4 buffer. The results obtained for the oxidative reactions in P110N 7.4 buffer conditions are summarized in Table 1.

3.1.7 MDA Assay

The MDA assay showed a gradual increase in MDA levels in Hb-SUV system at phospholipid to Hb molar ratio of 800 in P110N7.4 buffer (Fig. 11). Both HbA and HbS systems exhibited similar rate of MDA formation. Initially about 0.4 µM MDA was detected and this value increased to 4 µM at 2.5 h. The MDA concentration leveled to a maximum value of 6.22 ± 0.01 µM ($n = 2$) at 10 h for both HbA- and HbS-SUV mixtures.

3.2 Oxidative Products Under Low pH and Low Ionic Strength Conditions (T5K 6.5 Buffer)

3.2.1 Disappearance of OxyHb

Similarly the oxidation of Hb in T5K 6.5 buffer at phospholipid to Hb molar ratio of 360 and 45 was determined by analyzing the absorption spectra. A typical set of spectra obtained on HbA-SUV sample and HbS-SUV sample in T5K 6.5 buffer at
Figure 11. Formation of MDA (µM) in a Hb + SUV mixture as a function of time (h). The Hb was incubated with BPS SUVs at phospholipid to Hb molar ratio of 800 and 37 °C in P110N 7.4 buffer. The production of MDA was determined by TBARS assay. ■ HbA + BPS SUV and □ HbS + BPS SUV.
MDA Formation in a Hb-SUV mixture

In P110N 7.4 Buffer at PL/Hb = 800 & 37°C

- ■ MDA in HbA-SUV mixture
- □ MDA in HbS-SUV mixture

MDA (μM)

Time (h)
Table 1. Summary of the First Order Rate Constants and $t_{1/2}$ Values for the Disappearance of OxyHb, Disappearance of Heme and Appearance of Free Iron, Appearance of MDA and Cross-Linking of Hb in P110N 7.4 Buffer at PL/Hb Molar Ratio of 800.

<table>
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<tr>
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<th>First Order Rate Constants</th>
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<td>HbA</td>
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<tr>
<td>Disappearance of oxyHb</td>
<td>2.77±0.08</td>
<td>2.70±0.09</td>
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<tr>
<td>Disappearance of Heme</td>
<td>0.76±0.12</td>
<td>0.79±0.11</td>
</tr>
<tr>
<td>Appearance of Free Iron</td>
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<td>0.79±0.11</td>
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**a** - Mean value of 2-3 measurements ± the standard deviation.

**b** - Reaction was not first order. Thus the value was not obtained.
phospholipid to Hb molar ratio of 360 as a function of time (Fig. 12 A & B) shows a decrease in the two characteristic peaks for oxyHb at 577 and 565 nm and an increase in absorbance between 600 - 700 nm. The disappearance of oxyHb in HbA- or HbS-SUV mixture in T5K6.5 buffer was found to be much faster than corresponding ones in P110N7.4 buffer at molar ratio of 800. In order to obtain reliable data, I decreased the rate by reducing the phospholipid to Hb molar ratio to 360 and determined the first order rate constants for the initial (up to 0.3 h) disappearance of oxyHb. The semilogarithmic plot of oxyHb concentration (%) versus time revealed a linear relationship during the initial phase (up to 0.3 h) of the reaction (Fig. 13). Linear fitting of the first 9 data point provided a pseudo first order rate constant for the initial disappearance of oxyHb in the presence of SUVs. The mean rate constants were $0.84 \pm 0.1 \text{ h}^{-1}$ ($n = 4$) for HbA, and $4.34 \pm 1.05 \text{ h}^{-1}$ ($n = 4$) for HbS. The $t_{1/2}$ for the initial disappearance of oxyHbS was $0.62$ h for HbA and $0.2$ h for HbS. I also monitored the oxidation of HbA and HbS at phospholipid to Hb molar ratio of 45 and obtained the rate constant of $0.1 \pm 0.01 \text{ h}^{-1}$ ($n = 4$) for HbA, and $0.16 \pm 0.02 \text{ h}^{-1}$ ($n = 4$) for HbS (Fig. 13).

Previous studies also show that HbS has a higher oxidation rate compared to HbA oxidation in T5K 6.5 buffer at phospholipid to Hb molar ratio of 45 in a Hb-LUV mixture. The observed rates of $0.25 \pm 0.02 \text{ h}^{-1}$ for HbA and $0.85 \pm 0.18 \text{ h}^{-1}$ for HbS (Marva and Hebbel 1994). I obtained higher rate constants when buffers were not treated with chelex-100 to remove the trace metal ions or when glassware used was not acid washed.
Figure 12. Spectral changes from 500 - 700 nm of HbO₂ + BPS SUVs in T5K 6.5 buffer as a function of time at lipid to Hb molar ratio of 360 and 28 °C. The time intervals between each spectrum was 6 min, with the bottom spectrum taken immediately after mixing HbO₂ and SUVs and the top spectrum taken at 45 min after mixing. Characteristic absorption peaks for oxyHb at 577 nm and 565 nm were observed at t = 0, and a prominent peak at 630 nm were observed at t = 45 min. A. HbAO₂ + BPS SUVs in T5K 6.5 buffer, and B. HbSO₂ + BPS SUVs in T5K 6.5 buffer.
Spectral Changes in HbA and HbS
TK 6.5 Buffer, at PL/Hb = 360 and 28°C

[Graph A]

Absorbance
500 550 600 650 700
Wavelength (nm)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

[Graph B]

Absorbance
500 550 600 650 700
Wavelength (nm)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

Wavelength (nm)
Figure 13. The semi-logarithmic plot of oxyHb concentration (%) versus time (h) for the disappearance of Hb in the presence of BPS SUVs in T5K 6.5 buffer at lipid to Hb molar ratio of 360 and 28 °C. The value at each time point was the mean value of 4 experimental runs. ■ HbAO₂ + BPS SUVs in T5K 6.5 buffer, and ◦ HbSO₂ + BPS SUVs in T5K 6.5 buffer at phospholipid to Hb molar ratio of 45 and ◇ HbAO₂ + BPS SUVs in T5K 6.5 buffer and □ HbSO₂ + BPS SUVs in T5K 6.5 buffer at phospholipid to Hb molar ratio of 360.
Disappearance of OxyHb in the Presence of BPS SUVs

In T5K 6.5 Buffer at PL/Hb = 360 & 28 °C
3.2.2 Disappearance of Heme /Porphyrrins

The fluorescence intensity of the porphyrin in Hb-SUV mixture, at molar ratio of 360, in TSK6.5 buffer decreased with time (Fig. 14) in a manner similar to systems at molar ratio of 800 in P110N7.4 buffer. Pseudo first order rate constants for disappearance of heme at PL/Hb molar ratio of 360 were obtained by fitting the logarithmic values of heme concentration (%) versus time (t) to a linear equation. The rate constants that calculated were 0.63 ± 0.30 h⁻¹ (n = 6) for HbA-SUV mixture and 0.93 ± 0.30 h⁻¹ (n = 6) for HbS-SUV mixture. At 0.5 h 43 % of heme was remaining in HbS reaction mixture and 73 % of heme was remaining in HbA reaction mixture (Fig. 14). The t₁/₂ value was 1.1 h for HbA and 0.7 h for HbS. I saw a very little difference in rate constants between HbA and HbS at phospholipid to Hb ratio of 360, in TSK 6.5 buffer.

At phospholipid to Hb molar ratio of 45, the initial rate lasted for 0.7 h for both HbA and HbS (Fig. 14). I fitted the data in this region to linear equation and only marginal fit was obtained, as for the data with phospholipid to Hb ratio of 360. The first order rate constants for the destruction of heme were 0.37 ± 0.16 h⁻¹ (n = 4) for HbA and 0.34 ± 0.09 h⁻¹ (n = 4) for HbS. However due to the poor fit and the fact that the error bars were large, it was difficult to determine whether results of HbA differed from those of HbS.

3.2.3 Heme Transfer into the Lipid Phase (SUVs)

I observed a dramatic increase in the heme transfer to the lipid phase in T5K 6.5
Figure 14. The semi-logarithmic plot of heme concentration (%) versus time (h) in Hb- SUV mixture in T5K 6.5 buffer at lipid to Hb molar ratio of 360 and 45 and at 28 °C. The heme concentration was determined by fluorescence spectroscopy. The fluorescence emission of the solution was obtained at 601 nm with the excitation at 405 nm. The values presented were mean values of 4 experimental runs. ● HbAO₂ + BPS SUVs mixture in T5K 6.5 buffer, and ■ HbSO₂ + BPS SUVs mixture in T5K 6.5 buffer at phospholipid to Hb molar ratio of 45 and ○ HbAO₂ + BPS SUVs in T5K 6.5 buffer and □ HbSO₂ + BPS SUVs in T5K 6.5 buffer at phospholipid to Hb molar ratio of 360.
Destruction of Heme in the Presence of BPS SUVs

in TK 6.5 Buffer, 28°C, PL/Hb = 45 & 360
Figure 15. The semi-logarithmic plot of free iron concentration (%) in Hb-SUV mixture as a function of time (h) in T5K 6.5 buffer at phospholipid to Hb molar ratio of 360 and incubated at 28 °C. The iron concentrations were determined by the foreseen assay. □ HbA-SUV mixture, ○ HbS-SUV mixture
Free Iron Release From Hemoglobin in the Presence of BPS SUVs

In T5K 6.5 buffer at PI/Hb = 360 and 28°C

Free iron (% Released From Hb) vs Time (h)

- □ - HbA
- ○ - HbS
buffer. Instead of ~ 1 % observed in P110N 7.4 buffer, 22.5 ± 0.7 % of heme transferred rapidly (t > 10 min) to the lipid phase upon mixing Hb and SUVs for HbA and 94.3 ± 4.9 % for HbS.

3.2.4 Appearance of Free Iron

As for systems in P110N7.4 buffer, I was not able to fit the semi-logarithmic plot of free iron concentrations in T5K6.5 buffer as a function of time with a linear equation. Total free iron concentration increased as a function of time (Fig. 15) in a manner similar to systems in P110N7.4 buffer. After 4 h incubation, 67.7 ± 1.0 % iron were found as free iron in HbA system and 71.6 ± 2.0 % in HbS system. The $t_{1/2}$ values for the iron released from Hb in the presence of SUVs were 2.3 h for HbS and 1.5 h for HbA. The iron concentration in the lipid or aqueous phase were not determined for these systems in T5K 6.5 buffer.

The results obtained for the oxidative reactions in T5K 6.5 buffer conditions are summarized in Table 2.

3.2.5 Globin Cross-Linking

I also characterized the components in the oxidation reaction mixture by gel electrophoresis to detect Hb chains and cross-linked products in Hb-SUV mixture in T5K 6.5 buffer at a phospholipid to Hb molar ratio of 360. The HbA- and HbS-SUV samples both showed bands at 15 kDa, 30 kDa and a faint band at 45 kDa soon after mixing (t ~ 5 min) (Fig. 16 A & B). At t ~ 5 min I observed about 36 % of covalently cross-
Table 2. Summary of the First Order Rate Constants and $t_{1/2}$ Values for the Disappearance of OxyHb, Disappearance of Heme and Appearance of Free Iron and Cross-Linking of Hb in TSK 6.5 Buffer at PL/Hb Molar Ratio of 360.

<table>
<thead>
<tr>
<th></th>
<th>First Order Rate Constants (h$^{-1}$)</th>
<th>$t_{1/2}$ (50 % Concentration) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HbA</td>
<td>HbS</td>
</tr>
<tr>
<td>Disappearance of oxyHb</td>
<td>0.84±0.10$^a$</td>
<td>4.34±1.05</td>
</tr>
<tr>
<td>Disappearance of Heme</td>
<td>0.63±0.30</td>
<td>0.93±0.30</td>
</tr>
<tr>
<td>Total Free Iron Released</td>
<td>N/D$^b$</td>
<td>N/D</td>
</tr>
<tr>
<td>Hb Cross-Linking</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

$^a$ Mean value of 2-3 measurements ± the standard deviation.

$^b$ Reaction was not first order. Thus the value was not obtained.
Figure 16. The 16 % SDS PAGE performed on the Hb-SUV mixture in TSK 6.5 buffer at lipid to Hb molar of 360 and 28 °C, at different incubation times. The 50 µl of the sample was loaded to the gel. A. HbA-SUV mixture, B. HbS-SUV mixture
SDS PAGE of Hb-SUV Mixture in T5K 6.5 Buffer at PL/Hb = 360 and 28 °C
Figure 17. The % of cross-linked Hb in the Hb-SUV mixture in TSK 6.5 buffer at lipid to Hb molar of 360 and 28 °C as a function of incubation time (h). The cross-linking was monitored by performing a 16 % SDS PAGE. The % intensity of each band was obtained by using Logitech Scan Man for scanning and Sigma Gel analyzing software for the gel % analysis. ■ HbA-SUV mixture and ○ HbS-SUV mixture as a function of incubation time (h).
Cross-Linking of Hemoglobin in the Presence of BPS SUVs

in T5K 6.5 Buffer at PL/Hb = 360 and 28°C

Cross-linked Hemoglobin (%)

Time (h)

- ■ HbA + SUV
- ○ HbS + SUV
linked Hb in the HbA-SUV mixture and 47 % in HbS-SUV mixture (Fig. 17). After prolonged incubation of the HbA-SUV mixture, cross-linking increased to 41.61 % at 1 h, 63 % at 2 h, 70 % at 3 h, 71 % at 4 h and the cross-linking reached a plateau value of ~ 75 % at 5 h. The cross-linking of HbS appeared to be slightly faster with about 60 % at 1 h, 73 % at 2 h, and 81 % for t = 3 -5 h. The t_{1/2} value for the cross-linking of Hb 0.25 h for the HbS-SUV mixture and 1.4 h for the HbA-SUV mixture, showing a difference between HbA and HbS in covalently cross-linking between their subunits.

3.3 Globin-Heme Affinity

I used apomyoglobin (apoMb) to extract heme from metHb to measure the heme and globin affinity in both HbA and HbS molecules under both P110N7.4 and T5K6.5 buffer conditions. The heme extraction profiles as a function of time in both P110N7.4 and T5K6.5 buffers at 25°C were similar (Fig. 18 and Fig.19 respectively). A fast phase kinetic followed by a slow phase kinetic was observed. These results were qualitatively similar to those published with an initial fast phase kinetics, which was attributed to heme extraction from β-chains, followed by a slower phase which attributed to heme extraction from α-chains (Benesch and Kwong, 1990). I used the time for 25 percent of the heme to be transferred to apoMb (Benesch and Kwong, 1990) to represent rates of the heme transfer. The time taken for 25 % of the heme to be transferred from metHbA to apoMb in P110N 7.4 buffer was 1.7 ± 0.2 h (n = 3) and that for metHbS was 1.6 ± 0.1 h (n = 3) (Fig. 18). The heme release from Hb in the T5K 6.5 buffer
Figure 18. The heme partitioning into apomyoglobin from metHb as a function of time in P110N 7.4 buffer. MetHb and apoMb was mixed in a 1:5 molar ratio and was incubated at 25 °C. An increase in absorbance at 409 nm was monitored as an indication of heme being coordinated to apoMb. Heme containing Mb at the same concentration as metHb was used to give absorbance reading for 100% heme transfer. The ratio of absorbance difference for apoMb and Mb (%) were used to represent heme transferred from Hb to apoMb. ■ HbA + apoMb and ○ HbS + apoMb
Heme Release From MetHb into apoMb

in P110N 7.4 buffer at 25°C

Heme (%), Extracted by ApoMb

Time (h)
Figure 19. The heme partitioning into apomyoglobin from metHb as a function of time in T5K 6.5 buffer. MetHb and apoMb was mixed in a 1:5 molar ratio and was incubated at 25 °C. An increase in absorbance at 409 nm was monitored as an indication of heme being coordinated to apoMb. Heme containing Mb at the same concentration as metHb was used to give absorbance reading for 100 % heme transfer. The ratio of absorbance difference for apoMb and Mb (%) were used to represent heme transferred from Hb to apoMb. ■ HbA + apoMb and ○ HbS + apoMb.
Heme Release From MetHb into apoMb
in T5K 6.5 buffer at 25 °C

Heme Release From MetHb into apoMb
in T5K 6.5 buffer at 25 °C

Heme (%) Extracted by ApoMb

Time (h)

- □ methbA + apoMb
- ○ methbS + apoMb
Figure 20. Heme partitioning into the lipid phase as a function of ionic strength and osmolarity. The Hb in Hb + BPS SUV mixture in the experimental buffer was mixed with SUV to Hb molar ratio of 360, and 200 was immediately separated on a superose 12 FPLC column. The lipid fraction was collected, and the heme concentration in the lipid phase was determined. Values were presented as a percentage of heme partitioned into the lipid phase. • HbA + BPS SUV and □ HbS + BPS SUV in tris buffer at 70, 90 and 130 osmolarity (mmol/kg) at phospholipid to Hb molar ratio of 200, ◊ HbA + BPS SUV and ◇ HbS + BPS SUV in T5N 6.5 buffer at phospholipid SUV in T5K 6.5 buffer at phospholipid to Hb molar ratio of 360.
Heme Transfer into the SUVs from Hb Under Tris Buffer Conditions

[Graph showing the partitioning of heme into the lipid phase vs. osmolarity (mmol/kg) for different Hb samples.]
was 3.2 ± 0.2 h (n = 2) for metHbS and 3.8 ± 0.0 h (n = 2) for metHbA (Fig. 19). I observed that at low pH and low ionic strength conditions less heme transferred to apoMb compared to that of in high pH and higher ionic strength. A small difference between HbA and HbS was observed in T5K 6.5 buffer. These results are similar to the previously reported values of heme transfer under low pH which are due to the decrease in the affinity between free heme and apoMb at low pH (Benesch and Kwong, 1990).

3.4 Ionic Strength and pH Effects on Heme Transfer in Hb-SUV Systems

Since the heme transfer from Hb to lipid phase in P110N7.4 buffer was found to be very different from that in T5K6.5 buffer for both HbA and HbS -SUV systems, I also studied the heme transfer as a function of ionic strength and pH in both phosphate and tris buffer systems. Since phosphate buffers were prepared with 35 mM phosphate, whereas tris buffers were prepared with 10 mM tris, osmolarities rather than salt concentrations were used to show ionic strength effects on heme concentrations in the lipid phase. As shown in Fig. 20 and Fig. 21, a strong osmolarity dependent was observed for the HbS system in tris buffers. In addition, a large difference in heme concentrations in lipid phase was observed between HbA and HbS systems at low osmolarities. Replacement of KCl with NaCl did not affect the heme concentrations in the lipid phase. For HbS systems, 97 ± 7 % (n = 2) of heme were found in the lipid phase in T5K6.5 buffer and 100 ± 0 % (n = 2) in T5N6.5 buffer (Fig. 20). For HbA systems, 26 ± 5 % (n = 4) heme was released from Hb in T5K6.5 buffer and 29 ± 0 % (n = 2) in T5N6.5 buffer. I observed little difference between the results obtained
for T5K 6.5 buffer systems and in T5N 6.5 buffer systems. This indicated that the use of KCl or NaCl did not affect the amount of the heme release. In T20K 6.5 buffer, I observed $50 \pm 5\%$ (n = 3) heme was transferred to the lipid phase for HbS-SUV mixture and $20 \pm 1\%$ (n = 2) for the HbA-SUV system. In T85K 6.5 buffer, $21 \pm 1\%$ (n = 2) of heme was transferred into the lipid phase for HbS system and $11 \pm 2\%$ (n = 2) for HbA-SUV system. I observed, with the increase of the ionic strength, the extent of heme transfer decreased and the differences between heme transferred in HbA and HbS system decreased.

The concentrations of heme in the lipid phase decreased as pH increased. For HbS systems, at pH 6.5, $28 \pm 6\%$ (n = 3) of the heme were found in the lipid phase in P10N6.5 buffer, but at pH 7.4 only $3 \pm 0\%$ (n = 3) were found in P10N7.4 buffer (Fig. 21). For HbA system, at pH 6.5, $16 \pm 3\%$ (n = 3) heme were found in the lipid phase in P10N6.5 buffer, but at pH 7.4 only $3 \pm 1\%$ (n = 2) in P10N7.4 buffer. Thus in phosphate buffer with 10 mM NaCl, a difference in heme concentrations in lipid phase was observed at pH 6.5 between HbA and HbS systems, but not at pH 7.4. Under physiological ionic strength and pH, very little heme was found in the lipid phase in both the HbA and HbS systems. In P0N6.5 buffer $24 \pm 2\%$ (n = 3) heme was transferred into the lipid phase for HbS system and $10 \pm 3\%$ (n = 3) for the HbA system. In P50K 6.5 buffer $9.02 \pm 1\%$ (n = 3) heme was transferred into the lipid phase for HbS and $11 \pm 1\%$ for HbA system. Similarly, in phosphate buffers I observed that the extent of heme transfer decreases with the increase of ionic strength. At low ionic strength phosphate buffers (P0N6.5 and P10N6.5) I observed differences between the
Figure 21. Heme partitioning into the lipid phase as a function of ionic strength and osmolarity. The Hb in Hb + BPS SUV mixture in the experimental buffer was mixed with SUV to Hb molar ratio of 800, and 200 and was immediately separated on a Superose 12 FPLC column. The lipid fraction was collected, and the heme concentration in the lipid phase was determined. The values were presented as a percentage of heme from the initial heme in the reaction mixture. ⋄ HbA + BPS SUV and ■ HbS + BPS SUV in phosphate buffer at 70, 90 and 130 osmolarity (mmol/kg) at phospholipid to Hb molar ratio of 200. ○ HbA + BPS SUV and □ HbS + BPS SUV in P10N 7.4 buffer at phospholipid to Hb molar ratio of 800 and ◊ HbA + BPS SUV and ▽ HbS + BPS SUV in P10N 7.4 buffer.
Heme Transfer into SUV from Hb Under Phosphate Buffer Conditions

![Graph showing heme transfer into SUV from Hb under phosphate buffer conditions.](image)

- Hb\(_{A_{PN9.5200}}\)
- Hb\(_{S_{PN9.5200}}\)
- Hb\(_{A_{P110N7.4800}}\)
- Hb\(_{S_{P110N7.4800}}\)
- Hb\(_{A_{P10N7.4200}}\)
- Hb\(_{S_{P110N7.4200}}\)
HbS and HbA systems. As the ionic strength and/or pH was increased (P50N6.5 and P10N7.4) the HbA and HbS systems exhibited similar values for the heme transfer. The results are summarized in Table 3.
Table 3. Heme (%) Transfer into BPS SUVs Under Different Buffer Conditions

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Osmolarity (mnoles/kg)</th>
<th>PL/Hb</th>
<th>Hb</th>
<th>n</th>
<th>% Heme</th>
<th>%Heme(\text{HbS})/ %Heme(\text{HbA})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON 6.5</td>
<td>70</td>
<td>200</td>
<td>HbA</td>
<td>3</td>
<td>10.2±3.2*</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>3</td>
<td>24.3±2.1</td>
<td></td>
</tr>
<tr>
<td>P10N 6.5</td>
<td>90</td>
<td>200</td>
<td>HbA</td>
<td>3</td>
<td>15.6±3.8</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>3</td>
<td>26.7±4.6</td>
<td></td>
</tr>
<tr>
<td>P50N 6.5</td>
<td>130</td>
<td>200</td>
<td>HbA</td>
<td>3</td>
<td>10.0±1.0</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>3</td>
<td>10.7±0.6</td>
<td></td>
</tr>
<tr>
<td>P10N 7.4</td>
<td>90</td>
<td>200</td>
<td>HbA</td>
<td>2</td>
<td>6.0±0.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>3</td>
<td>6.0±1.0</td>
<td></td>
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<tr>
<td>P110N 7.4</td>
<td>240</td>
<td>800</td>
<td>HbA</td>
<td>2</td>
<td>1.0±0.0</td>
<td>1.0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>2</td>
<td>1.0±0.0</td>
<td></td>
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<tr>
<td>T5K 6.5</td>
<td>70</td>
<td>360</td>
<td>HbA</td>
<td>3</td>
<td>22.5±0.7</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>3</td>
<td>94.3±4.9</td>
<td></td>
</tr>
<tr>
<td>T5K 6.5</td>
<td>70</td>
<td>200</td>
<td>HbA</td>
<td>2</td>
<td>28.3±2.2</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>4</td>
<td>97.4±4.6</td>
<td></td>
</tr>
<tr>
<td>T5N 6.5</td>
<td>70</td>
<td>200</td>
<td>HbA</td>
<td>2</td>
<td>28.5±0.1</td>
<td>3.5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>2</td>
<td>100±0.0</td>
<td></td>
</tr>
<tr>
<td>T20K 6.5</td>
<td>90</td>
<td>200</td>
<td>HbA</td>
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<td>20.0±1.4</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>3</td>
<td>50.3±5.1</td>
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<tr>
<td>T85K 6.5</td>
<td>130</td>
<td>200</td>
<td>HbA</td>
<td>2</td>
<td>10.5±2.1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbS</td>
<td>2</td>
<td>20.5±0.7</td>
<td></td>
</tr>
</tbody>
</table>

a- Mean value of 2-3 measurements ± the standard deviation

b- Number of individual runs
CHAPTER IV
DISCUSSION

Oxidation of oxyHb in aqueous solution is complex and involves many intermediate species, including metHb, hemichrome, choleglobin, superoxide anion, hydrogen peroxide, hydroxyl radical, etc. (Winterbourn, 1990b; Winterbourn 1985b). And some of these intermediate species include a subset of compounds. For example, hemichrome is a general classification of many different compounds (Peisach and Blumberg, 1975) and choleglobin is often not well defined (Winterbourn, 1990b; Pacifici et al., 1993). Oxidation of oxyHb in the presence of lipid surface is further complicated not only by additional intermediate species from lipid molecules but also by reactions in the aqueous phase and in the lipid phase. The presence of a lipid phase also promotes the disappearance of oxyHb (LaBrake and Fung, 1993) and the accumulation of hydrophobic species such as heme and porphyrin in the lipid phase. In this study, we systematically monitored the disappearance of oxyHb and the formation of three intermediate species in oxyHb and SUV mixtures to further our understanding of the oxidative reactions of oxyHb in the presence of lipid surface under different buffer conditions. These three intermediates were heme/porphyrin, free iron ion and MDA. The iron concentration in both the lipid and aqueous phase was monitored.

My results support the earlier finding that lipid surface enhances oxyHb
disappearance and metHb formation. In a system consisting of simply oxyHb and lipid molecules (with no or little initial Hb oxidation and lipid peroxidation products) the lipid surface enhances the oxidation of oxyHb. When the oxyHb-SUV mixture was incubated in physiological buffer (pH 7.4) the disappearance of oxyHbA was observed immediately after mixing, with a pseudo first order rate constant of 2.8 h⁻¹. I also observed the Hb undergoing cross-linking immediately after mixing (30% cross-linked Hb products at ~5 min). Subsequent to the disappearance of oxyHbA, I observed the disappearance of heme, with a first order rate constant of 0.8 h⁻¹, and the appearance of free iron. The data on the appearance of iron could not be fitted to first order reaction kinetics. At 0.3 h about half of the oxyHb was disappeared and, at 0.8 h about half of the heme was disappeared and at about 1.3 h 50% of the heme iron was detected as free iron (iron free from Hb).

The oxygen radicals (O₂⁻) formed during the process of the Hb oxidation were more likely to react at its generation site. Therefore the decrease in the heme concentration that I detected was probably due to the presence of these oxygen radical, since reactions between heme and oxygen radicals lead to the destruction of the porphyrin (Cantoni et al., 1981). The first step of the destruction of porphyrin would be the cleavage of the methylene bridge of the porphyrin ring carbon which leads to the formation of biliverdin. Since biliverdin undergoes further oxidation, resulting in the formation of smaller fragments (Brown and Jones, 1968), I did not detect any biliverdin in our reaction mixture. The destruction of heme caused the release of free iron from
One of the most interesting findings was the distribution of the iron released from Hb molecules. As expected iron concentrations in Hb-SUV mixtures increased as a function of time. However, iron concentrations in the aqueous phase reached a maximum value of 30 - 40 % at about 2.5 h before decreasing to only about 24 % at 10 h. Initially the iron concentrations in the lipid phase increased with slightly slower rates than that in the aqueous phase, but the concentration continued to increase after 2.5 h and reached almost 100 % at 10 h. The $t_{1/2}$ value for total Fe release was found to be 1.3 h.

As a consequence of the accumulation of iron ions in the lipid phase, lipid peroxidation became significant. MDA concentration increased rapidly and exhibited a 10 fold increase (4µM) at 2.5 h. At the end of 10 h, about 6 µM of MDA was detected. It was interesting to see that the $t_{1/2}$ of the MDA formation was 1.4 h, a value very similar to that of iron release.

At $t = 2$ h when about 30 % of iron were found in the lipid phase, substantial amount of lipid was degraded. A fraction with molecular masses much smaller than Hb (2-3 kDa) was separated from other oxidative components. This fraction exhibited a sharp absorption at 265 nm suggesting the presence of conjugated carbonyl groups.

The initial cross-linking of globin chains was much faster than the appearance of iron. Immediately after mixing of oxyHb with SUVs, about 36 % of globin already appeared to be crossed-linked (mostly between chains within the Hb tetramer such that the molecular mass remained as 64 kDa). During this time interval, disappearance of
oxyHb was detected. Thus the cross-linking of globin chains was probably due to the generation of oxygen radicals but not necessarily in the presence of iron. Previous studies show that the reaction of Hb with H$_2$O$_2$ lead to the formation of ferryl-Hb (Fe$^{4+}$). The Fe$^{4+}$ in ferryl-Hb can undergo electron transfer with neighboring amino acid tyrosine 42 forming a radical on the tyrosine 42 residue (McArthur and Davies, 1993). This radical may initiate the formation of radicals on other tyrosine residues in globin subunits leading to the formation of a bi-tyrosine cross-link and the association of subunits in the Hb molecule (Giulivi et al., 1994; Pacifici et al., 1993).

It is interesting to compare the results from HbA with those from HbS since HbS has been considered to exhibit faster oxidation rates that lead to membrane abnormalities (Hebble, 1986; Marva and Hebbel, 1994). However I found little difference between HbA and HbS in buffers under physiological conditions (P110N7.4) in the disappearance of oxyHb, Hb cross-linking, heme destruction, Fe release MDA formation and heme release into the lipid phase. All the $t_{1/2}$ values and the rate constant obtained for HbS were found to be very similar to those of HbA.

Under low salt and low pH conditions (T5K6.5), the disappearance of oxyHb was considerably faster than in high salt, high pH buffer condition. Since the oxidation reaction under low salt low pH buffer condition was fast, we reduced the phospholipid to Hb molar ratio from 800 to 360 to slow down the reaction to monitor and compare the kinetics between HbS and HbA reliably. The oxidation rate constants were 4.34 h$^{-1}$ for HbS and 0.84 h$^{-1}$ for HbA, showing a 5.2 times increase in HbS system. We also observed a difference in the cross-linking of Hb subunits in HbA and HbS systems.
The destruction of heme in the presence of SUVs did not show much difference between the two Hbs. Since the destruction of Hb led to the release of free iron, we also did not see differences in the release of total free iron. We believe that the destruction of heme could proceed when heme is in either the aqueous phase or the lipid phase.

The heme transfer into SUV from HbS and HbA showed no difference in P110N 7.4 buffer but showed a drastic difference in T5K 6.5 buffer. The heme transfer from HbS was 10 times greater in HbS-SUV system than from HbA in HbA-SUV system in T5K 6.5 buffer. The difference could not be explained by the globin-heme affinity in HbS in T5K 6.5, since we found that both HbA and HbS exhibited similar heme-globin affinity in both P110N7.4 and T5K 6.5 buffers, as determined by apomyoglobin-heme extraction. At lower pH (T5K 6.5), we observed that less heme (30 %) was extracted by apoMb as compared to 50 % in high salt high pH buffer conditions (P110N 7.4). This is probably due to reduced affinity of heme towards apoMb at low pH (Benesch and Kwong, 1990).

Heme partitioning into the lipid phase may be represented by two steps. First, heme release from the globin to the aqueous phase, and then the free heme is transferred to a heme acceptor (Smith et al., 1984). The heme transfer from Hb is biphasic. The fast phase is the release of heme from \( \beta \) chains and slow phase represents the release from \( \alpha \) chains (Paul et al., 1991). The \( \text{Fe}^{2+} \)- proximal histidine covalent bond in oxyHb prevents the heme loss from globin (Whitaker et al., 1995) and also histidine 64, valine 68 and porphyrin interactions inhibit the release of heme (Hargrove et al., 1994). My results showed that heme from Hb partitioning into apoMb was a rather a slow process.
under both physiological and non-physiological buffer conditions. In 150 mM phosphate buffer at pH 7.0 a complete release of heme occurs within 3 -4 days (Hargrove et al., 1994). Due to the protonation of the proximal histidine under low pH conditions the release of heme is much faster than at physiological pH (Hargrove et al., 1994). I did not see much difference in the rate of heme release between HbA and HbS in P110N 7.4 buffer or in T5K 6.5 buffer (low salt and low pH); although we observed a large difference in the heme transfer into the lipid phase between HbA and HbS in T5K 6.5 buffer.

It has been found that CO-heme partitions between the external solvent phase and the outer lipid layer of the membrane at a rapid rate (rate constant of about $7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). The rate limiting steps of this reaction are the separation of heme from the globin and the solvation of the heme in the aqueous phase (Smith et al., 1991). Since I did not see much difference in the heme release into the aqueous phase under both buffer conditions we believe that the rate for the heme-proximal histidine bond breaking and the heme solvation in the aqueous phase to be the same for both HbA and HbS. I observed a difference in the acceptance of the heme by the SUV at low salt and low pH buffer conditions. It appears that the ionic strength affects the heme transfer into the lipid phase. I observed that the release of heme into the lipid phase drastically decreased as the KCl concentration (osmolarity) was increased in tris buffers. Similarly when the osmolarity of the phosphate buffers were increased I observed a decreased in the heme transfer as well as a decrease in the differences between HbA and HbS.

It is also interesting to discuss the effects of tris and the phosphate buffers. I
clearly showed that tris buffers have a greater effect on heme transfer as compared to phosphate buffers. Previous investigations showed that tris buffers could cause many adverse effects on the proteins (Bubb et al., 1995; Gillespie and McKnight, 1976). They showed that tris(2-amino-2-hydroxymethylpropane-1-3-diol), can react with proteins and small molecules as aldehydes. Pinder and co workers (1995) showed the use of tris solutions as a denaturating agent similar to guanidinium chloride, for depolymerization of proteins. The capability of tris to form complexes with a wide range of metal ions (group IIA metals and transition metals) (Scheller et al., 1980) may produce adverse effects on Hb oxidation. Since the presence of metal ions have shown to enhance the oxidation of Hb (Winterbourn and Carrel, 1977)

Differences between HbA and HbS in oxidative reactions (disappearance of oxyHb, Hb cross-linking and heme transfer into SUVs) were mostly at low salt and low pH conditions. The heme transfer experiments performed at different osmolarities showed that an increase in the Cl⁻ concentration decreased the heme transfer into the lipid phase. This is probably due to a stabilizing effect of the Cl⁻ ions on the globin structure. Previous studies have shown that the denaturation of myoglobin occur much faster under low salt (Cl⁻) and low pH conditions (Loh et al., 1995). Since Mb and Hb have similar structural characteristics one can assume that the Cl⁻ effects on Hb structure are similar to those on Mb structure. The Cl⁻ stabilizes the helical structure of the proteins and at low Cl⁻ concentration B, C, E and H helices are unfolded (Loh et al., 1995). Unfolding the E helix changes the conformation of the distal histidine (F8 -histidine) and causes the exposure of the heme to the aqueous environment, leading to an increase Fe²⁺
oxidation and the release of heme. Oxidation of Hb leads to conformational changes in F and E helices (Dong et al. 1995). Whitaker et al. (1995) showed that in the absence of the D helix in Mb the mobility of C- and E-helices increased and the rate of hemin loss increased by 50-fold. These helices (E and F) carry the proximal histidine (F8) and the distal histidine (E7). The orientation of these histidine residues are very important in the retention of heme at the heme pocket (Hargrove et al., 1994). Oxidation Fe²⁺ to Fe³⁺ in Hb weakens the proximal histidine-Fe bond and releases the heme from the oxidized Hb. Protonation of the histidine residues under low pH also makes the release of heme easier. HbS molecule exhibits a higher surface activity and more surface hydrophobicity than HbA (Hirsch et al., 1980). It is also shown that HbS has a greater affinity towards the membrane compared to HbA (Fung et al., 1975). These studies showed that under low pH and low salt conditions (5 mM phosphate at pH 6.0) HbS had a greater affinity towards the membrane and upon addition of Cl⁻, under high salt high pH (physiological) conditions HbS had a lower affinity towards membrane. Since HbS interacts with SUV to a greater extent compared to HbA especially at low salt and low pH buffer conditions it is possible for the HbS as compared to HbA, to have a higher rate of oxidation and a higher reactivity towards SUVs.

Results also show that SUVs act as a better heme acceptor than apoMb. It has been suggested that the carboxylic side chains and the Fe³⁺ atom in the porphyrin (heme) occupies site close to phospholipid head groups when partitioned into the lipid bilayer (Kuzelova and Brault, 1994; Tipping et al., 1979) and the dimethyl-divinyl "end" of the heme is immersed into the hydrocarbon region (Tipping et al., 1979).
I saw very little differences between HbA and HbS-systems that the disappearance of heme and in the release of total free iron. Since I argue that the iron is the major little differences in the lipid peroxidation in these two systems are expected even at low ionic strengths.

HbS has a mutation at the β6 position. β6 glutamic acid is substituted by valine. The most prominent feature of the HbS is the polymerization of deoxyHbS. Crystal structure did not show differences between oxyHbA and oxyHbS. NMR studies showed some irregulations on some surface residues in HbS in both oxy and deoxy states, but no structural differences at the heme pocket was observed (Fung et al., 1975). Fronticelli (1978) showed differences between the CD spectra of HbA and HbS at the soret region which were believed to be due to the changes in the β globin chain. It is also found that the β globin chains of HbA has a higher affinity to the α globin chains than the β globin chains of HbS, giving HbA a higher tetramer stability than HbS (Shaeffer, 1980). It is believed that the abnormalities observed in the sickle cell membrane is due to the faster rate of oxidation in HbS (Hebbel, 1986). My experiments show that under physiological conditions HbS does not undergo higher rate of oxidation to give membrane abnormalities. However faster disappearance of oxyHb in HbS was observed at pH and low ionic strength. Differences were also observed on Hb cross-linking and heme release from Hb into SUVs. When the sickle RBCs undergo some cellular change to cause the pH and the ionic strength to be lowered, faster oxidation in HbS are expected. This increase in the oxidation of HbS could lead to the higher level of Hb cross-linking and heme release from Hb. However little difference in the heme destruction and in the free
iron release from Hb even under low pH and low ionic strength was observed between HbA and HbS. Thus it is possible that the abnormalities in sickle erythrocyte membranes are due to the Hb denaturation (Hb cross-linking) leading to the buildup of hemichrome in the RBC membrane and not due to abnormal lipid peroxidation.

Results obtained from these studies also provide insight towards the development of blood substitute using liposome encapsulated Hb. Research efforts on blood substitute have mainly focused on oxygen binding capacity and stability of Hb at low temperatures (Tsuchida, 1994). An important factor to consider is the release of free iron from Hb which may cause many toxic effects on the endothelial cell. I believe it is also important to avoid non-physiological buffers such as tris since these buffers may initiate destructive reactions in the Hb-liposome systems.
REFERENCES


Winterbourn, C. C. (1985a) Hemoglobin Oxidation and inter-relationship with lipid


VITA

The author, Merita Nirmalie Dias, was born in Sri Lanka on May 17, 1965. After finishing her high school (GCE / AL) she entered to the Institute of Chemistry Ceylon, Sri Lanka to pursue higher studies. At the same time she worked part-time at the University of Peradeniya as a Research Technician on a project funded by the World Health Organization (WHO), and later did her practical training as a part of her graduateship program at the same place. Her work involved isolation, purification and characterization of anti-fertility compounds and anti-cancer compounds from higher plants. From her research conducted at the University of Peradeniya under the guidance of Professor Leslie Gunatilake she co-authored two publication in refereed journals. She graduated in 1990 from the Institute of Chemistry Ceylon with the Graduateship in Chemistry which is equivalent to a special degree in honors in chemistry. After graduating in 1990 she joined Chemical Industries Colombo Ltd., as a member of the management staff and was involved in quality control and research and development of adhesives, detergents and agro-chemicals. She resigned Chemical Industries Colombo Ltd in 1991 to join her husband in Chicago, IL, USA. To pursue her higher studies she joined University of Illinois at Chicago, Chicago as Visiting Research Specialist at the Department of Medicinal Chemistry and Pharmacognosy. Her work involved cytotoxicity assay and receptor binding assay to identify anticancer compounds. In 1992 she joined Loyola University Chicago as a full time graduate student at the Department of
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This Dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

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