Biochimica et Biophysica Acta, 687 (1982) 63–70
Elsevier Biomedical Press

BBA 71088

THE INTERACTION OF HEMOGLOBIN WITH PHOSPHATIDYLSERINE VESICLES

YOCHEVED SHVIRO, ILANA ZILBER and NURITH SHAKLAI *

Department of Chemical Pathology, The Sackler School of Medicine, Tel Aviv University, Tel Aviv (Israel)

(Received July 17th, 1981)
(Revised manuscript received November 5th, 1981)

Key words *Hemoglobin, Phosphatidylserine, Protein-liposome interaction, Heme-globin dissociation*

The interaction of hemoglobin with phosphatidylserine vesicles at low ionic strength and pH conditions was studied. The fluorescence intensity of a lipid embedded probe was quenched by bound Hb but could not be reversed by an elevation of ionic strength and pH. The irreversibility of the fluorescence quenching is a time-dependent process associated with changes in the heme Soret and visible spectra. The rate of these changes was much faster for methemoglobin than for either cyanomethemoglobin or oxyhemoglobin. Elevation of ionic strength released out of the bound hemoglobin into the water phase most of the globin but only a small fraction of the heme. The data are interpreted as demonstrating the ability of phosphatidylserine vesicles to compete with globin for the heme group. When Hb binds to the liposome, heme is being transferred into the lipid phase and the rate-limiting step is the dissociation of the heme-globin complex. The fact that binding of heme to the lipid vesicles is very strong was demonstrated by the failure of hemin to interact with globin when the two were rapidly mixed in the presence of phosphatidylserine vesicles. A multi-step process is suggested to explain the results of Hb phosphatidylserine interaction.

Introduction

The red blood cell inner membrane surface exhibits a heterogeneous collection of binding sites for Hb [1,2]. Accumulated evidence [2,3] point to band 3 polypeptides, the integral membrane proteins which span the membrane, as being the specific high affinity sites for Hb. The lower affinity interaction was suggested to be the result of Hb attraction to the negatively charged inner surface lipids, namely phosphatidylserine (PS) [2].

As a consequence of these suggestions, it seemed natural to inquire as to the nature of PS-Hb interaction by studying the binding of Hb to PS liposomes. Such a study was undertaken using the technique developed for the red cell membrane, namely, the quenching of the fluorescence intensity of a membrane embedded probe by bound Hb [4]. It was found that although Hb interacts strongly with PS liposomes there is a basic difference between the PS and the biomembrane. While the quenching of the fluorescence intensity in the red cell membrane by Hb at low ionic strength could be reversed by ionic strength elevation, a large fraction of the fluorescence quenched in the liposome system could not be restored. These findings were interpreted as demonstrating irreversibility of Hb binding to PS. Later Szundi et al. [5] reported findings on Hb interaction with PS monolayers as revealed by measuring surface potential and pressure changes induced by bound Hb. They suggested that although the binding process is entirely irreversible, Hb preserves its

Abbreviations Hb, Hemoglobin; PS, phosphatidylserine, oxyHb, oxyhemoglobin, HbCO, carbomonoxyhemoglobin, metHb, methemoglobin, CNmetHb, cyanomethemoglobin, AS, 9-(1,2-anthroyl)stearic acid

0005-2736/82/0000–0000/$02 75

function, namely, oxygen binding.

Other studies pointing towards the possibility that various proteins could stimulate liposome permeability, showed that Hb is the most effective protein in increasing cation diffusion through PS-containing liposomes [1,2,6]. In order to exert the same permeability effect as Hb, 10-times the amount of myelin (the integral membrane protein) and 100-times of cytochrome *c* were needed [7]. Kimelberg [7] in reviewing protein liposome interactions calculated that the amount of Hb needed to cover only 2% of the total phospholipid area created the same permeability effect as cytochrome *c* in an amount sufficient to cover the entire surface. These findings, rather striking for a water-soluble protein such as Hb, were summarized by Kimelberg [7] who stated that 'the Hb case should prove to be an exception' for the general rule that only integral membrane proteins will interact strongly with phospholipid liposomes. Why should the Hb molecule represent such an exception? No reasonable explanation for that has been suggested to date. The present study was, therefore, undertaken to provide further information which might lead to an explanation of this unusual phenomenon.

Materials and Methods

Oxyhemoglobin was prepared according to the method developed by Drabkin [8], salt stripped on a Sephadex G-25 column and dialysed against the required solution. Carboxyhemoglobin (HbCO) was made by flushing oxyHb with CO. Methemoglobin (MetHb) was prepared using the method of ferricyanide oxidation according to the procedure set out by Taylor [9]. Methemoglobin was converted into cyanomethemoglobin by the addition of a twice molar excess of KCN to metHb. After a 0.5 h incubation excess KCN was removed by gel filtration column chromatography using Sephadex G-25. Hemoglobin solutions were used within one week.

Native globin, prepared by the method of Rossi-Fanelli et al. [10], was lyophilized and stored at −20°C. It was dissolved in the desired buffer solution and centrifuged to separate any insoluble material before use.

Phosphatidylserine vesicles were prepared by suspending the required amount of PS in the desired buffer solution using a vortex mixer and sonicating to clearness under nitrogen gas. The larger multilayer liposomes were sedimented by centrifugation at $40000 \times g$ for 10 min. The supernatant was then centrifuged for 90 min at $150000 \times g$ and the precipitate resuspended in the desired buffer. Fluorescent vesicles were prepared by sonication of the PS suspension in the presence of the fluorescent probe 9-(1,2-anthroyl)stearic acid (AS). The amount of 9-(1,2-anthroyl)stearic acid was about one-thousandth of the PS by weight. AS micelles formed were separated during the centrifugation. These vesicles will be referred to hereafter as PS-AS vesicles. Protein was determined quantitatively using the method of Lowry et al. [11]. Concentrations of the various hemoglobins were determined from their specific molar extinction coefficients. Heme was determined as heme pyridine using the procedure first described by De Duve [12].

All buffers were flushed with nitrogen and measurements were made with Thunberg cuvettes when slow reactions were followed. For preparation of heme solutions hemin was deoxygenated and then reduced by the injection of 1% solution of sodium dithionite.

Reagents were of analytical grade. Hematin was purchased from B.D.H., bovine brain phosphatidylserine from Sigma, and 9-(1,2-anthroyl)stearic acid was a product of Molecular Probe Inc.

Fluorescence measurements were performed using a Perkin-Elmer Model 44B spectrofluorimeter equipped with a thermostatically controlled cuvette. For spectrophotometric measurements a Cary 118 spectrophotometer was used.

Results

The reaction of hemoglobin with PS vesicles as demonstrated by fluorescence measurements

A typical sample of sonicated PS vesicles, as demonstrated in Fig. 1C, contained mainly vesicles in the diameter range of 250–800 Å but some larger multilayer liposomes could be seen in these preparations. When PS vesicles containing the fluorescence probe AS (PS-AS) were mixed with oxyHb, a reduction in fluorescence intensity due to binding was observed. The fraction of the fluo-

rescence intensity quenched was increased at pH values lower than 7.0 reaching a maximal value at pH = 5.6. Restoration of fluorescence intensity by ionic strength elevation was found to be time dependent.

Heme alone interacted readily with the liposomes, as shown by the quenching of the fluorescence intensity of the PS-AS by the addition of heme (Fig. 1B). In contrast to the hemoglobin interaction, when the ionic strength was raised after the interaction with heme, no change in fluorescence intensity was observed. The effect of cytochrome *c*, another heme contaning protein known to react with liposomes [13], is shown in Fig. 1A. Elevation of ionic strength reversed part of the fluorescence intensity, but in contrast to the hemoglobin case no time dependency of the reversibility was demonstrated.

Spectral changes of oxyHb interacting with PS

Typical to the interaction of Hb with the liposomes were slow spectral changes in the Soret and visible region. Since at pH 5.6 the spectral changes were observed after a few minutes, the data are shown at the latter pH value. At higher pH values the same changes could be followed but at a slower rate. Fig. 2A demonstrates spectral changes in the Soret band of oxyHb after 30 min: a reduction in the absorption and a blue shift in the absorption maximum wave length. The sample underwent most of its change after 3 h at pH 5.6. Incubation up to 48 h did not cause any further changes in the absorption pattern of Hb. Reduction of the absorbance in the Soret, rules out metHb as the product, since the molar extinction coefficient of metHb is larger than that of oxyHb [14]. Spectral changes in the visible region are demonstrated in panel B of Fig. 2. Samples were reduced by the addition of dithionite and the spectrum of the reduced sample is demonstrated in panel B of Fig. 2. The spectral features of heme after interaction with PS are shown in panel C of Fig. 2. The spectrum of the dithionite-reduced hemoglobin does not resemble that of the reversible hemochromes. The changes may result from a mixture of oxyHb being reduced and free heme disolved in the lipid phase (Fig. 2C). Another possibility is a denatured unreversed hemochro-

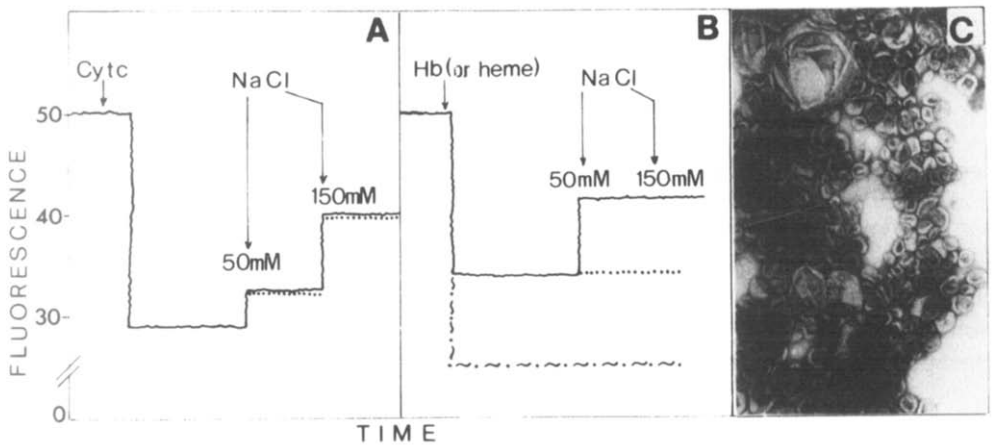

Fig 1 The binding of heme and heme-containing proteins to PS as reflected by quenching of the fluorescence intensity (in arbitrary units) of AS. (A) Reduction of the fluorescence intensity of a membrane probe induced by cytochrome *c* 1 μM cytochrome *c*, 0.5 mg/ml PS 5 mM phosphate at pH 5 6, Temperature, 20°C The ionic strength is elevated by addition of NaCl at concentrations indicated in the figure Ionic strength elevation ———, within 1 min, · · , after 60 min of incubation (B) binding of heme (~ · ~ ·) 0.5 mg/ml PS and 1 μM Hb, 0.2 μM heme. The intensity after 1 or 60 min of incubation and ionic strength elevation did not change the intensity Binding of oxyHb, 0 5 mg/ml PS and 1 μM of Hb heme Effect of ionic strength elevation by addition of the indicated NaCl concentrations ———, within 1 min; · · · , after 60 min of incubation at low ionic strength. Fluorescence quenched by the addition of heme after 1 or 60 min (~ · ~ ~). (C) Electron micrograph of PS-sonicated liposomes, negatively stained with uranyl acetate The multilayer liposome in the field are much larger than the usual diameter range of the vesicles, but their quantity is small

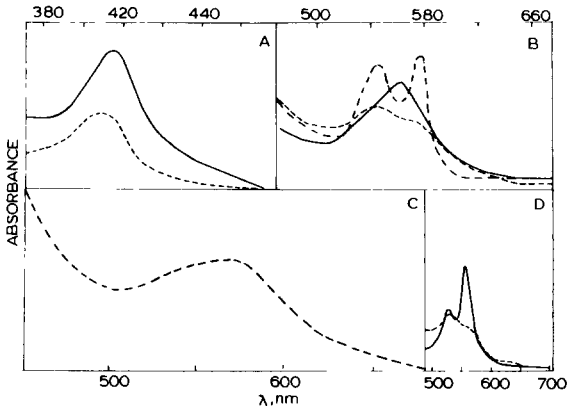

Fig 2 Absorption changes of oxyHb resulting from interaction with liposomes (A) Soret band Liposome concentration, 0 5 mg/ml, Hb concentration, 2 μM of heme, in 5 mM phosphate at pH 5 6 Temperature, 20°C The solutions were deaerated and kept under nitrogen ————, 1 min after mixing of Hb and PS, , 60 min after mixing (B) Visible spectrum – – –, oxyHb, 1 min after mixing with PS, - - - - - -, 60 min after mixing, ————, the 60 min incubation sample reduced with aliquots of dithionite (C) Heme visible band in the presence of PS 0 2 μM hemin and 0 5 mg/ml PS (aliquots of dithionite were added to the nitrogen flashed solution in order to reduce hemin to heme) Heme visible band is the same as that of the reduced sample in panel B (D) Spectral features of reversible hemichrome (- - - - -) and hemochrome (————) taken from Refs 15 and 16

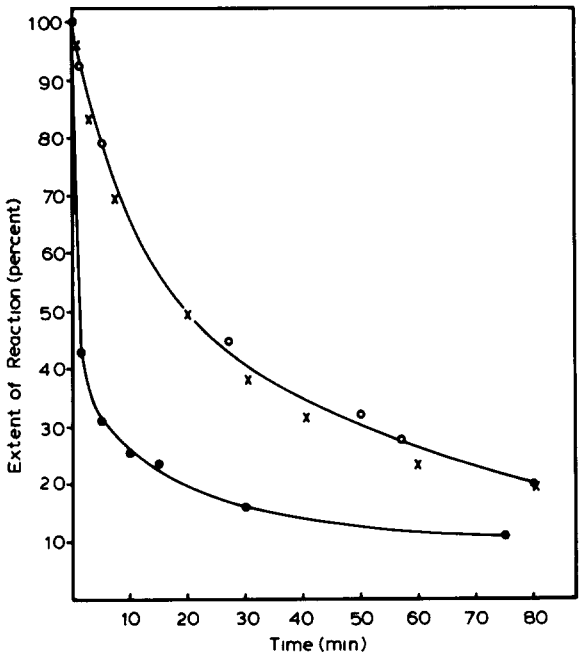

Fig 3 Rates of Hb-PS interactions as observed by Soret spectral changes PS concentrations. 0 5 mg/ml, Hb concentration 3 μM, phosphate 5 mM at pH 5 6, temperature 20°C Changes in absorbance were followed at the wavelength of 419 nm for HbCO and 405 for metHb and CNmetHb The extent of reaction is demonstrated as $(1-\Delta A/\Delta A)\times 100$ or percent of reaction A_∞ is the absorbance changes after 24 h ○, CNmetHb, ×, HbCO, ●, metHb

mogen [15,16]. The alternative of a mixture of all three, namely of oxyhemoglobin, irreversible hemochromogen and heme dissociated from globin must also be considered.

Rates of reaction followed by absorption changes

The rate of Hb-PS interaction was observed by studying the extinction coefficient of Hb in the Soret band. It was observed that species other than oxyHb also undergo time dependent reductions in their absorbance in the Soret band. The results are shown in Fig. 3. MetHb was found to react much faster than liganded oxy- or carbomonoxyhemoglobin both showing the same reaction rate pattern. At the experimental conditions of Fig. 3 the half-life time of the metHb reaction was 2 min and that of either oxy or CO hemoglobins 20 min. When metHb was converted to the CNmetHb form the rate of the reaction became slower and had the same pattern of the HbCO form (Fig. 3).

Determination of heme to globin ratio in hemoglobin dissociated from the vesicles

A sample of PS vesicles (1 mM) was reacted with oxyHb (8 μM heme) for 1 h in 5 mM phosphate (pH 5.6) and then sedimented. One portion of the washed vesicles was incubated with 0.1 M NaCl at pH 7.0 for 60 min, while another was incubated with 5 mM phosphate buffer at pH 5.6. The vesicles were sedimented and the water phase analysed for protein and heme using the procedure of Lowry et al. [11] and the heme-pyridine procedure [12], respectively. Under these conditions in the sample incubated in low ionic strength neither heme nor protein were released into the water phase. On the other hand, the sample incubated in 0.1 M NaCl released most of the globin leaving its heme in the lipid phase. In another set of experiments the ratio of PS to hemoglobin was reduced so that not all the hemoglobin in the solution could be absorbed on to the vesicles' surface. The mixtures were incubated for various periods of

TABLE I

PS (μM)	Hb (μM)	Heme/globin (% from hemoglobin)	
		2 h incubation	24 h incubation
36	8	39	25
166	8	28	10

time in low ionic strength and pH conditions and then centrifuged to separate the liposomes from the water phase now containing excess of hemoglobin which was in dynamic equilibrium with the liposomes. It was found that this hemoglobin also lost some of its heme. The reduction in the heme content was found to depend on time of incubation and the ratio of PS to hemoglobin. In Table I the average percent of heme remaining in the hemoglobin samples in some typical experiments is summarized.

Fluorescence characteristics of the released protein

The fluorescence intensity of the tryptophan in Hb is quenched by resonance energy transfer to the neighboring heme groups [17]. Variations in tryptophan emission pattern are therefore expected to follow changes in the Hb molecule. In Fig. 4 the results of the following experiment are demonstrated. Hemoglobin solution (1 μM) at pH 5.6 in 5 mM phosphate buffer was mixed with PS vesicles (0.5 mg/ml) and incubated for 60 min. The ionic strength of the solution was then elevated to 0.1 M and the mixture centrifuged. The fluorescence emission was recorded and compared to that of the original Hb solution having exactly the same absorbance at the excitation wavelength (280 nm) as that of the protein being disconnected from the membrane. The data of Fig. 4 show the same emission pattern but about 6-fold increase in the

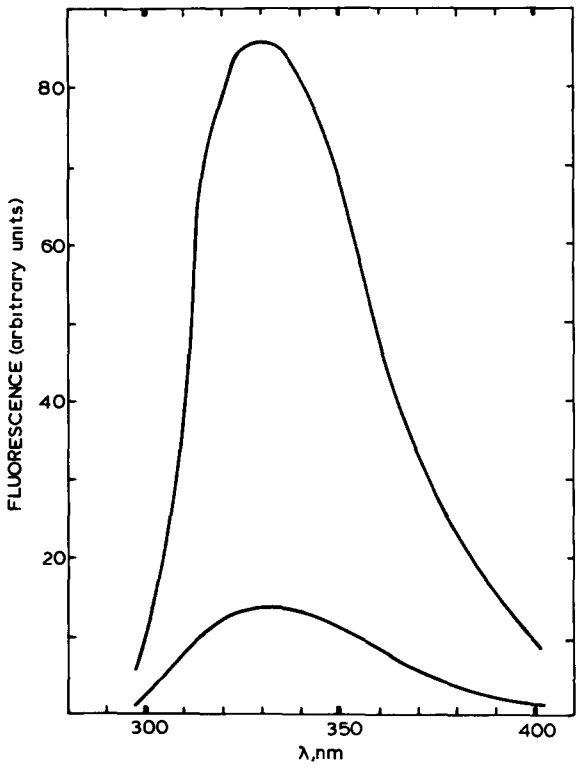

Fig 4 Tryptophan fluorescence characteristics of Hb· relative quantum yields. Excitation at 280 nm Slits widths 6 mm The lower curve shows the fluorescence of oxyHb solution (A_{280} = 0 126) The upper curve shows the fluorescence intensity of the oxyhemoglobin solution detached from the membrane after 60 min incubation at low ionic strength (see text for details) The Hb solution was matched to have exactly the same A_{280} as that of the lower curve solution The fluorimeter was set at the same conditions exactly as those of the lower curve

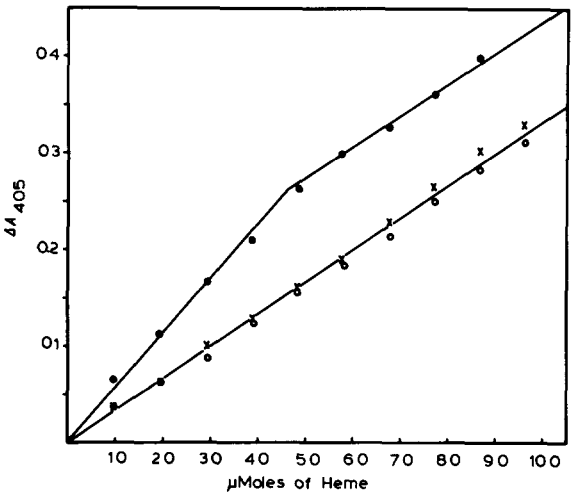

Fig 5 Titration of globin with hemin In 5 mM phosphate at pH 5 6, 4 5 nmol globin are titrated Absorbance is measured at a wavelength of 405 nm. O, Hemin added to globin solution in buffer only; ×, hemin added to the same amount of globin in the presence of 2 mg of PS, O, hemin added to buffer solution only. *Note.* The first two points in the lower curve show superimposed cross and an open circle and should not be confused with full circles belonging to the upper curve

fluorescence yield of the hemoglobin detached from the PS.

Binding of hemin to globin in the presence of PS vesicles

The reaction between globin and hemin was studied using the changes in absorption at 405 nm (the absorption maximum of metHb). Such a titration is shown in Fig. 5. The inflection point indicates the titration end-point. In a parallel experiment hemin was added to a solution containing globin and PS vesicles. As shown in Fig. 5, the slope of the absorption dependency on the amount of hemin added is the same as that of hemin added to buffer solution only. This result indicates that in the presence of PS no reaction between globin and hemin occurred.

Discussion

The absorption of Hb to the surface of PS vesicles, being electrostatic, is expected to be very fast. Slow spectral changes observed in this study are, therefore, attributed to secondary slower changes in the Hb molecule. Inspite of the fact that the Hb-PS electrostatic interaction is not expected to change in the pH range where neither Hb nor PS alter their charge [18,19], the reaction is faster at pH 5.6 than that at pH 6. This is also consistent with the assumption that slower changes in the Hb molecule occur and result in spectral changes.

The spectral changes summarized in Fig. 2 rule out reversible hemochrome production [15,16]. Two possibilities are therefore left. Irreversible hemochrome production or dissociation of heme from the globin. The increase in tryptophan fluorescence quantum yield could also be related to either denaturated hemochrome or heme dissociation from the globin. Since no shift in the tryptophan emission pattern which is known to follow protein denaturation [20], was observed, the most logical explanation of the reaction mechanism is dissociation of heme from globin as the end product. The fact that inspite of the release of most of the protein from the PS membrane by ionic strength elevation, the fluorescence intensity of the AS probe did not reverse also proves that the heme group which is responsible for the radiationless energy transfer [17] was left in the lipid core.

The observation of a very slow reaction rate is not surprising considering the heme-globin dissociation as the reaction rate limiting step [21]. It therefore appears that the fast association of hemoglobin with the membrane surface is consequented by the following events:

$$\text{Globin-heme} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \text{globin} + \text{heme}$$

$$\text{Heme} + \text{PS} \overset{k_2}{\rightarrow} \text{heme-PS}$$

$$\text{Globin-heme} + \text{PS} \overset{k_{app}}{\rightarrow} \text{heme-PS} + \text{globin}$$

The data of Fig. 5 demonstrate that heme fails to interact with globin when competing with PS. In other words, for the set of reactions described above $k_2 > k_1$. It is known that $k_{-1} \gg k_1$ and therefore both heme binding reactions are much faster than heme dissociation from Hb or $k_2 \gg k_{-1}$. The fact that the rate limiting step of the reaction observed is the Hb dissociation, is demonstrated by the comparison of the reaction rate of oxyHb, metHb and CNmetHb. It is known that the heme moiety of metHb is more loosely bound to the globin in metHb than in oxyHb [22], while in CNmetHb, the low spin derivative of the metHb form, heme is again strongly attached to the globin. The data indeed show (Fig. 3) that the reaction rates of HbCO and CNmetHb are the same, while metHb reacts much faster. Since it is known that Hb species which more readily lose heme, undergo denaturation easily; it is possible that the reaction includes reversible hemochromes as intermediates. It is interesting to note that a mechanism including hemichrome intermediates was suggested for the transfer of heme from globin to albumin [23]. The analysis of the results discussed above refers to that fraction of Hb which was detached from the membrane. It is possible that the rest of the protein carried within the phospholipid is in a denatured hemochrome form.

Surface bound Hb transfers the dissociated heme readily but we have shown in this study (Figs. 1 and 5) that free heme as well, when available, binds to the PS liposomes. One can imagine

that liposomes serve as traps for free heme in aqueous solution. Szundi et al. [5], stated that Hb binding over a pH range of 4.0–6.0 could be observed for the charged PS monolayers. On the other hand, for the uncharged outer surface lipids of the red cell membrane, changes due to Hb binding were observed at pH 4 only. Since at pH 4 enhanced dissociation of heme from globin in the water phase is expected [21], the latter findings could be attributed to trapping of the free heme by the lipid layer rather than to absorption of the whole Hb molecule.

The conclusions of this work are schematically summarized in Fig. 6. The first step is the fast electrostatic absorption of Hb to the liposome surface which is, of course, pH dependent (Fig. 6A). This first step of interaction was previously postulated for other proteins interacting with phospholipid vesicles [13]. In a second step, a fraction of the surface-absorbed Hb molecules will penetrate the membrane partially (Fig. 6B) leaving its dissociated heme in the lipid core (Fig. 6C). Since surface and lipid embedded protein molecules attain a dynamic interchange, after a time most of the surface protein as well as water-soluble protein will appear as globin. Whenever neutral salt is added, it will cause the detachment of any protein electrostatically bound to the surface. The fraction of globin in the detached protein will increase and the heme containing protein will decrease (Fig. 6D). This process occurs with any form of Hb but the rate of heme 'loss' will depend on the type of Hb bound.

In view of the findings of this work, the enhanced liposome permeability induced by Hb discussed by Calissano et al. [24] and Papahadjopoulos and Kimelberg [13] may be explained as heme rather than Hb effect. Cytochrome *c* which also binds readily to liposomes is unable to cause such permeability effects [25]. Since heme is covalently bound in cytochrome *c*, it is reasonable to assume that it is impossible for the liposome to compete for heme in this system.

The data of this study indicate that the interaction of hemoglobin with PS vesicles causes inactivation of hemoglobin. In contrast hemoglobin associated with the inner surface of the red cell membrane was shown to preserve its oxygen binding properties [26]. It therefore seems that membrane proteins normally protect the hemoglobin from undergoing the irreversible interaction with the hydrophobic core of the phospholipid. Hydrophobic proteins such as sickle cell hemoglobin are possibly capable of undergoing hydrophobic interactions with the phospholipids [27]. It is of interest to note here that residual hemoglobin in the red cell membranes in considerable amounts in the sickle cell membranes [28], was found to have the same spectral characteristic as those demonstrated in the study presented here.

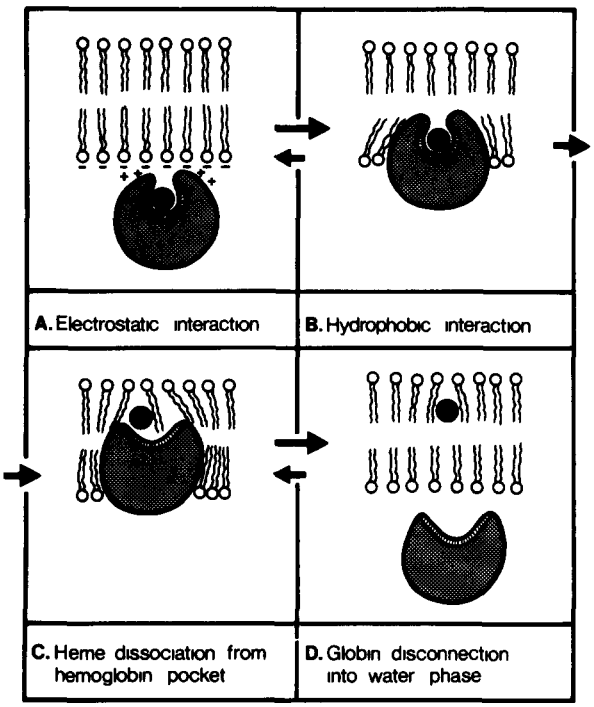

Fig 6 A schematic representation of heme-PS interaction See text for explanation

Acknowledgement

This work was supported by Research Funds from the Binational Science Foundation No. 1879/79.

References

1 Shaklai, N, Yguerabidae, I and Ranney, H M (1977) Biochemistry 16, 5585–5592
2 Shaklai, N., Yguerabidae, I and Ranney, H M (1977) Biochemistry 16, 5593–5597

3 Salhany, J M , Cordes, K A and Gaines, E D (1980) Biochemistry 19, 1447–1454
4 Shaklai, N and Ranney, H M (1978) Isr J Med Sci 14, 1152–1155
5 Szundi, I , Szenlenyi, J G , Breuer, J H and Berczi, A (1980) Biochim Biophys Acta 595, 41–46
6 Papahadjopoulos, D., Cowden, N and Kimelberg, H (1973) Biochim Biophys Acta 330, 8–26
7 Kimelberg, H K (1976) Mol Cell Biochem 10, 171–190
8 Drabkin, D (1946) J Biol Chem 146, 703–708
9 Taylor, J F (1957) in Conference on Hb, National Academy of Science, National Research Council, Washington, DC, Publications, 557, pp 155
10 Rossi-Fanelli, A , Antonini, E and Caputo, A (1958) Biochim Biophys Acta 30, 608
11 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265–269
12 De Duve, C (1948) Acta Chem Scand 2, 264–268
13 Papahadjopoulos, D and Kimelberg, H K (1971) Biochim Biophys Acta 233, 805–809
14 Antonini, E and Brunori, M (1971) in Hemoglobin and Myoglobin in their Reactions with Ligands (Neuberger, A and Tatum, E L , eds), North-Holland Publishing Company, Amsterdam
15 Rachmilewitz, E A , Peisach, J and Blumberg, J (1971) J Biol Chem 246, 3356–3362
16 Rachmilewitz, E A. (1974) Semin Hematol 11, 441–461
17 Teale, F W J (1959) Biochim Biophys Acta 35, 289–292
18 Brunori, M , Giacomelli, G M and Antonini, E (1972) J Mol Biol 63, 139–145
19 Papahadjopoulos, D and Kimelberg, H K (1972) Progr Surface Sci 4, 190–205
20 Korman, M J (1976) in Biochemical fluorescence, Vol 2 (Chen, R F and Edelhoch, H , eds), chap 10, Marcel Dekker, New York
21 Brunori, M , Wyman, J , Antonini, E and Rossi-Fanelli, A (1965) J Biol Chem 240, 3317–3324
22 Bunn, F H and Jandle, J H (1966) Proc Natl Acad Sci USA 56, 974–978
23 Adachi, K and Asakura, T (1976) Biochim Biophys Acta 427, 536–548
24 Calissano, P , Alema, S and Rusca, G (1972) Biochim Biophys Acta 255, 1009–1013
25 Kimelberg, H K and Papahadjopoulos, D (1971) J Biol Chem 246, 1142–1148
26 Salhany, J M and Shaklai, N (1979) Biochemistry 18, 893–899
27 Shaklai, N , Sharma, V S and Ranney, H W (1981) Proc Natl Acad Sci USA 78, 65–68
28 Asakura, T , Minakata, K , Adachi, K , Russell, M D and Schwartz, E (1977) J Clin Invest 59, 633–640