Articles

Encapsulation of Hemoglobin in Phospholipid Liposomes: Characterization and Stability†

J. Szebeni,‡ E. E. Di Iorio, H. Hauser,* and K. H. Winterhalter

Laboratorium für Biochemie, Eidgenössische Technische Hochschule Zürich, ETH-Zentrum, CH-8092 Zürich, Switzerland

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ABSTRACT: Hemoglobin is encapsulated in liposomes of different lipid composition. The resulting dispersion consists primarily of multilamellar liposomes (hemosomes) of a wide particle size distribution (diameter ranging mainly between 0.1 and 1 μm). The encapsulation efficiency is significantly larger with liposomes containing negatively charged lipids as compared to liposomes made of phosphatidylcholine. The integrity of the phospholipid bilayer is maintained in the presence of hemoglobin. The reaction rate of CO binding to encapsulated hemoglobin is reduced compared to that of free hemoglobin, but it is still greater than that observed in red blood cells. Hemoglobin encapsulated in liposomes made from negatively charged phospholipids is less stable than hemoglobin entrapped in isoelectric phosphatidylcholine. The instability of hemoglobin is due to the protein interacting with the negatively charged lipid bilayer. This interaction leads in turn to hemoglobin denaturation, possibly involving the dissociation of the heme group from the heme–globin complex. The nature of the negatively charged phospholipid is important in promoting the interaction with hemoglobin, the effect being in the order phosphatidic acid > phosphatidylinositol ≃ phosphatidylglycerol > phosphatidylserine. The presence of equimolar amounts of cholesterol in the phospholipid bilayer has a stabilizing effect on hemoglobin. This effect is pronounced with saturated phospholipids, but it is also observed, though to a lesser extent, with unsaturated ones, indicating that the bilayer fluidity has a modulating effect. The presence of cholesterol possibly interferes with secondary interactions following the binding of hemoglobin to the negatively charged lipid bilayer.

There is a growing interest in the encapsulation of hemoglobin (Hb)[1] in liposomes for two main reasons: (1) The Hb-containing liposomes, in this work referred to as hemosomes, have a potential use as a nontoxic and nonimmunogenic red blood cell substitute (Djordjevich & Miller, 1980; Miller, 1981; Gaber et al., 1983), and (2) they provide a useful erythrocyte model for studying the interaction between Hb and lipid bilayers (Szebeni et al., 1984a,b). A question of paramount importance is whether the Hb encapsulated in liposomes reversibly binds O_2 in a way comparable to the red blood cell. From a practical point of view, it is also important to show that the encapsulated Hb is sufficiently stable so that long-term storage is possible.

Here we report on the encapsulation of Hb in liposomes of different lipid composition and address ourselves to the question of Hb stability and the reaction kinetics of CO binding to Hb.

Materials and Methods

Egg yolk phosphatidylcholine (EPC), egg phosphatidic acid (EPA), the monosodium salt of ox brain phosphatidylserine, wheat germ phosphatidylinositol, and phosphatidylglycerol were purchased from Lipid Products (South Nutfield, U.K.). Dimyristoyl-, dipalmitoyl- (DPPC), and distearoylphosphatidylcholines and dipalmitoylphosphatidic acid (DPPA) were prepared by R. Berchtold (Biochemisches Labor, Bern, Switzerland). Cholesterol and cytochrome *c* from horse heart were used as supplied by Sigma Chemical Co. (St. Louis, MO). Phospholipids were pure by TLC standards.

Preparation of Hemolysate. Concentrated hemolysate was prepared according to a procedure slightly modified from that of Khachaturyan et al. (1979): fresh human erythrocytes were washed 3 times with 5 mM Tris-HCl, pH 7.4–0.15 M NaCl and lysed by adding an equal volume of tetrachloroethylene and stirring at 4 °C for 2 h. After centrifugation at 3000*g* for 20 min at 4 °C, the supernatant hemolysate was collected and the treatment with tetrachloroethylene repeated. The resulting hemolysate contained 30 ± 2 g/100 mL Hb and less than 2% met-Hb as determined according to Winterhalter (1978). The hemolysate was frozen in liquid nitrogen and stored at −20 °C.

Stripped Hb was prepared by gel filtration of the hemolysate on a Sephadex G-25 column equilibrated with 5 mM Tris-HCl buffer, pH 7.6. The Hb-containing fractions were pooled and concentrated to 30 g of Hb/100 mL in an Amicon ultrafiltration apparatus using a Diaflo UM-10 membrane.

Preparation of Hemosomes (Liposomes Containing Entrapped Hb). A total of 40 mg of lipid or lipid mixture dissolved in $CHCl_3/CH_3OH$ (2:1 v/v) was rotary evaporated in a round-bottom flask and dried in vacuo for several hours. One milliliter of either freshly prepared hemolysate or hemolysate

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‡ Present address: Orszagos Elelmezes es Taplalkozastudomanyi Intezet, Budapest, Hungary 1476.

[1] Abbreviations: Hb, hemoglobin; EPC, egg phosphatidylcholine; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EPA, egg phosphatidic acid; DPPA, dipalmitoylphosphatidic acid; PG, phosphatidylglycerol; PS, bovine brain phosphatidylserine; PI, phosphatidylinositol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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stored at −20 °C was added to the dry lipid film and after addition of three to four glass beads, the lipid was dispersed by handshaking for 10 min at temperatures above the respective gel-to-liquid crystal transition temperature. Free Hb was removed from entrapped Hb by sedimenting the liposomes at 10000 rpm (~15000*g*) for 20 min at 4 °C. The pelleted liposomes were redispersed in buffer (5 mM Tris-HCl, pH 7.4–0.15 M NaCl), and the centrifugation was repeated. It was found that further washings in addition to the two washing cycles carried out routinely did not release any more Hb from the pellet into the supernatant, indicating that Hb does not leak out from the interior of the liposomes. Any Hb firmly bound to the outer surface of the liposomes and not removed by the two washing cycles would be included in the quantity of encapsulated Hb (see below and Table I). However, the existence of such firmly bound Hb is unlikely.

The final dispersion of hemosomes was used immediately or stored anaerobically at 4 °C. The lipid loss incurred by repeated washing cycles was negligible. The supernatants from the washing cycles were pooled and concentrated in an Amicon ultrafiltration apparatus using 0.05-μm pore-size polycarbonate membranes (from Nucleopore). Oxy-Hb was determined in the concentrated supernatant as described below. An aliquot of the concentrated supernatant was applied to a Sepharose 4B column (29 × 1.2 cm) equilibrated with the same buffer in order to determine the size of the particles present.

Measurement of Oxy-Hb Encapsulated in Liposomes. Encapsulated Hb was estimated in two ways. (1) After separating hemosomes by centrifugation as described above, the supernatant was filtered through a 0.05-μm polycarbonate filter (from Nucleopore) and its oxy-Hb content determined in the first 0.5 mL of the filtrate by using Drabkin's reagent. The amount of Hb encapsulated was obtained by subtracting the Hb in the supernatant from the total Hb added to the dry lipid film. (2) Alternatively, entrapped Hb was determined as follows: to washed hemosomes sodium cholate (~2%) was added, and the dispersion was sonicated with a Branson B12 sonifier (microtip) for 20 min (50% duty cycle) until it became translucent. The Hb content was then determined by using Drabkin's reagent. Within the experimental error, the two methods of determining encapsulated Hb gave consistent results.

Oxy-Hb encapsulated in phospholipid liposomes was unstable, and the decrease in encapsulated oxy-Hb content with time was determined spectrophotometrically as follows: spectra in the visible region were recorded on a Unicam SP 1700 spectrophotometer, and from the absorbance at 560, 577, and 630 nm, corrected by the absorbance at 700 nm, the oxy-Hb, met-Hb, and hemichrome concentrations (mM) were derived according to Winterbourne et al. (1976). The phospholipid concentration used in these experiments was 4–5 mg/mL, and the blank cuvette contained Hb-free liposomes. The use of liposomes as a blank was necessary to compensate for light scattering so that the base-line absorbance at 700 nm (used to correct the measured absorbance) was less than 0.1.

Measurement of CO Binding to Hb Encapsulated in Liposomes. CO rather than O_2 was used to investigate the kinetics of the ligand binding to encapsulated Hb for reasons of its lower combination rates and higher affinity for the protein (Antonini & Brunori, 1971). Rapid mixing experiments were performed with the stopped-flow apparatus described elsewhere (Oertle et al., 1983). Dispersions of hemosomes or, alternatively, solutions of free hemolysate were diluted anaerobically with Ar-saturated buffer (5 mM Tris-HCl, pH 7.4–0.15 M NaCl) so that the Hb concentration was about 20 μM before mixing. CO solutions (~1 mM) were made by saturating water with the gaseous ligand at 20 °C and atmospheric pressure and diluted to the desired concentration with Ar-saturated buffer (Di Iorio, 1981). To ensure complete removal of oxygen, a few grains of dithionite were added to both solutions. The two solutions were mixed in a 1:1 volume ratio, and the reaction at 20 °C was followed by monitoring the absorbance at 419 nm. In order to check if hemosomes underwent lysis during rapid mixing, they were spun down at 15000*g* for 20 min after completion of the kinetic measurements, and Hb was determined spectrophotometrically in the supernatant. Lysis was negligible by this criterion.

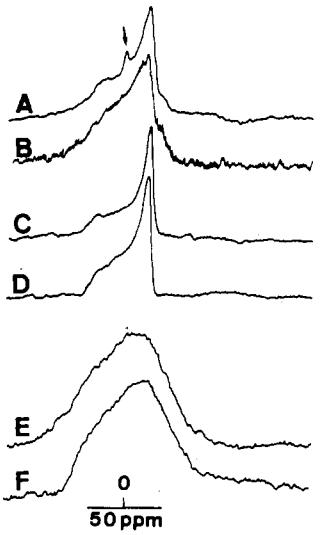

FIGURE 1: Proton-decoupled ^{31}P NMR spectra recorded at 121.47 MHz on a Bruker CXP 300 Fourier-transform spectrometer at 2 °C. Hemosomes were prepared with fresh hemolysate as described under Materials and Methods. DPPC/DPPA/cholesterol (3:1:4 mole ratio) with Hb (A); DPPC/DPPA/cholesterol (3:1:4) without Hb (B); EPC/cholesterol (1:1) with Hb (C); EPC/cholesterol (1:1) without Hb (D); DPPC/DPPA (3:1) with Hb (E); DPPC/DPPA (3:1) without Hb (F).

^{31}P NMR. Proton-decoupled ^{31}P NMR spectra were recorded on a Bruker CXP 300 spectrometer operating at 121.47 MHz in the Fourier-transform mode. Spectra were accumulated by using the Hahn spin-echo pulse sequence $(90_x-\tau-180x-\tau-AQ-PD)_n$ with a 90° pulse of 3 μs, a refocusing time τ = 90 μs, an acquisition time AQ = 150 ms, and a pulse delay PD = 8–10 s.

RESULTS

Characterization of Hemosomes. The fact that hemosomes were almost quantitatively pelleted by centrifugation at 10000 rpm (15000*g*) for 20 min suggests that they consist of large, possibly multilamellar structures. Consistent with this suggestion was the finding that hemosomes (DPPC/DPPA/cholesterol, 3:1:4 mole ratio) pelleted as described above, resuspended in buffer, and applied to Sepharose 4B are too big to enter the Sepharose gel. When the supernatant of the hemosome pellet was concentrated and applied to the Sepharose 4B column, all phospholipid was eluted in the void volume of the column. This indicates that our hemosome preparation is devoid of small unilamellar vesicles. Electron micrographs of freeze-fractured preparations of hemosomes (DPPC/DPPA/cholesterol, 2:1:3 mole ratio) showed that such a preparation consists predominantly of large, multilamellar particles as are characteristic of unsonicated EPC dispersions (data not shown). Most of the liposomes have a diameter in the range 0.1–1.0 μm.

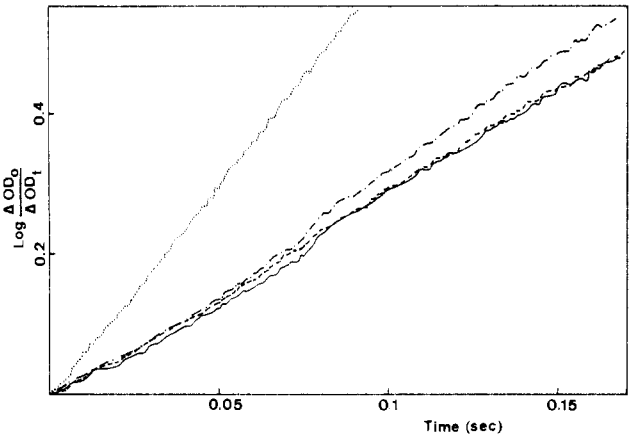

FIGURE 2: Reaction kinetics of the CO binding to Hb encapsulated in liposomes of different lipid composition. Equal volumes of CO solutions in Ar-saturated 5 mM Tris-HCl buffer, pH 7.4–0.15 M NaCl and hemosomes in the same buffer, both at appropriate concentrations, were mixed as described under Materials and Methods, and the CO binding at 20 °C was followed by monitoring the absorbance at 419 nm. The experimental data are plotted as a pseudo-first-order plot, where ΔOD_0 and ΔOD_t are the changes in optical density at time zero and t, respectively. Fresh, free hemolysate (not encapsulated) (···); DPPC/DPPA/cholesterol (3:1:4) (−−); DPPC/cholesterol (1:1) (−·−); EPC/cholesterol (1:1) (—).

Table I: Hb Encapsulation

composition of liposomes (mole ratio)	encapsulated Hb (g of Hb/g of lipid)[a]	encapsulation efficiency (%)[b]
EPC/EPA/cholesterol (3:1:4)	4.1	50
EPC/EPA (3:1)	3.0	37
EPC/PS/cholesterol (3:1:4)	5.1 ± 0.6	66
EPC/PS (3:1)	4.1 ± 0.2	52
EPC/PI/cholesterol (3:1:4)	5.1	68
EPC/PI (3:1)	4.8	63
EPC/PG/cholesterol (3:1:4)	4.5	60
EPC/PG (3:1)	4.8	63
DPPC/DPPA/cholesterol (3:1:4)	1.8 ± 0.9	24
DPPC/DPPA (3:1)	1.8 ± 0.7	25
DPPC (at 45 °C)	0.8	10
DPPC	1.2	12

[a] Results are expressed as the mean ± SD whenever three to six measurements were made. Other measurements were done in duplicate. Unless otherwise stated, the Hb encapsulation was measured at room temperature. Hb encapsulated was determined by measuring Hb in the supernatant and subtracting this amount from the total Hb. [b] Encapsulation efficiency is expressed as percent of total Hb that is entrapped in liposomes.

Table II: Apparent Initial Rate Constants k_0 and Half-Times $t_{1/2}$ for the Reaction of CO with Hemosomes and Free Hemolysate[a]

sample	[CO] (mM)	k_0 (s^{-1})	$t_{1/2}$ (s)[b]
hemolysate	0.05	13.0	0.05 (13.8)
DPPC/cholesterol (1:1)	0.05	5.2	0.09 (7.7)
DPPC/cholesterol (1:1)	0.025	3.8	0.15 (4.6)
DPPC/DPPA/cholesterol (3:1:4)	0.05	5.6	0.10 (6.9)
EPC/cholesterol (1:1)	0.05	5.0	0.10 (6.9)

[a] Lipid mixtures are expressed as mole ratios. [b] The values in parentheses represent rate constants (s^{-1}) calculated from $t_{1/2}$.

^{31}P powder-type NMR spectra of hemosomes of different lipid composition and of the Hb-free liposomes as the blank are shown in Figure 1. Three conclusions are obvious. (1) ^{31}P NMR is consistent with other results discussed above in that small unilamellar vesicles are essentially absent. Only hemosomes made from DPPC/DPPA/cholesterol (3:1:4) show a small isotropic component (see arrow in Figure 1A), which could arise from a minor proportion of small unilamellar vesicles. (2) Both in the presence and absence of Hb the spectra are typical for a bilayer structure with the phosphate group undergoing rapid motional averaging about the bilayer normal. As a result, the spectral line shape is characteristic of an axially symmetric chemical shielding tensor. (3) Hb encapsulated in liposomes has no dramatic effect on the structure and motion of the phospholipid polar group although in some cases small differences in line width and chemical shielding anisotropy are observed between hemosomes and the blank. The spectra of DPPC/DPPA (3:1 mole ratio) were run at 2 °C, well below the gel to liquid crystal phase-transition temperature of both DPPC and DPPA, which are 41.5 and 67 °C, respectively. Under these conditions the motional averaging is greatly reduced as evident from a significant increase in line width and in the chemical shielding anisotropy $\Delta\sigma$ (Figure 1E,F). Upon storage of the DPPC/DPPA hemosomes at 4 °C, the Hb was denatured (see below); however, no change in the ^{31}P NMR spectrum was observed (data not shown).

Consistent with ^{31}P NMR, no ^{1}H high-resolution NMR spectrum was obtained with hemosomes, indicating that these dispersions are devoid of small unilamellar vesicles. Sonication produced a ^{1}H NMR high-resolution spectrum characteristic of sonicated phospholipid dispersions.

Hb Encapsulation. The amount of Hb encapsulated in liposomes of different lipid composition was determined at room temperature as described under Materials and Methods, and the results are summarized in Table I. Wide variancies were observed in the encapsulation efficiency, which ranged from about 10% to 70%. The lowest values were observed with pure PC. Addition of a negatively charged phospholipid to EPC or DPPC increased the encapsulation efficiency by a factor of about 2–5. The presence of equimolar amounts of cholesterol in negatively charged phospholipid bilayers either increased the encapsulation efficiency, e.g., by 35% with EPC/EPA or by 27% with EPC/PS, or had a negligible effect, as was the case with EPC/PI, EPC/PG, and DPPC/DPPA.

Binding of CO to Hb Encapsulated in Phospholipid Liposomes. Progress curves for CO binding to Hb encapsulated in liposomes of different chemical composition are shown in Figure 2. The initial apparent pseudo-first-order rate constants and half-times of CO binding derived from Figure 2 are given in Table II. CO binding is fastest with pure hemolysate. The reaction rate is reduced when the Hb is encapsulated in liposomes, but it is still greater than that reported for red blood cells (Coin & Olson, 1979). Table II also reveals that the reaction is autocatalytic, i.e., its velocity increases as the reaction proceeds, as rate constants calculated from $t_{1/2}$ are larger than the initial ones. The differences in k observed between hemosomes differing in lipid composition are within the experimental error of the measurement.

Stability of Encapsulated Hb. The oxy-Hb content of hemosomes made from isoelectric PC was fairly stable at least within about 10% when hemosomes were stored at 4 °C for 2–3 weeks (Figure 3A,B). Cholesterol had no effect on the stability of oxy-Hb encapsulated in EPC or DPPC. The loss of oxy-Hb in liposomes made from unsaturated EPC was slightly higher as compared to DPPC liposomes (cf. Figure 3A,B). However, cholesterol had a marked stabilizing effect on oxy-Hb encapsulated in charged liposomes. This is also demonstrated in Figure 3A,B. The oxy-Hb content of hemosomes made from saturated DPPC/DPPA/cholesterol

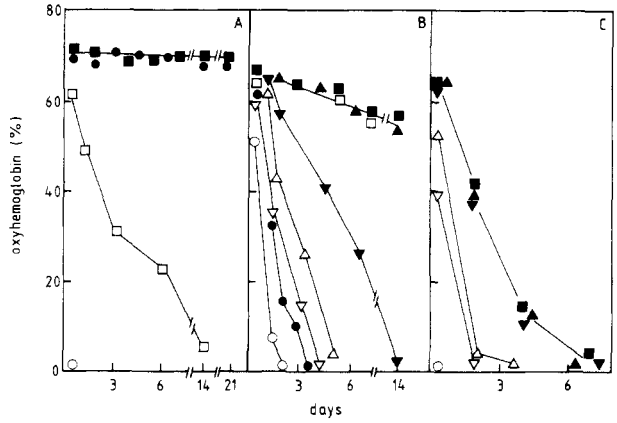

FIGURE 3: (A) Oxy-Hb loss (%) in hemosomes made from saturated phospholipid. Liposomes were prepared from DPPC or DPPC/cholesterol (1:1) (■), DPPC/DPPA/cholesterol (3:1:4) (●), and DPPC/DPPA (3:1) (○) and contained freshly prepared hemolysate except for DPPC/DPPA (3:1) (□), which contained stripped Hb. Hemosomes were made as described under Materials and Methods. The rate of oxy-Hb loss was determined spectrophotometrically at 4 °C. % oxy-Hb = 100[oxy-Hb]/([oxy-Hb] + [met-Hb] + [hemichrome]). Phospholipid mixtures are expressed as mole ratios. (B) Oxy-Hb loss (%) in hemosomes made from EPC and mixtures of EPC and negatively charged phospholipids. Liposomes were prepared from pure EPC (□), EPC/cholesterol (1:1) (■), EPC/EPA/cholesterol (3:1:4) (●), EPC/EPA (3:1) (○), EPC/PI/cholesterol (3:1:4) (▼), EPC/PI (3:1) (▽), EPC/PS/cholesterol (3:1:4) (▲), and EPC/PS (3:1) (△). Hemosomes were made as described under Materials and Methods. Phospholipid mixtures are expressed as mole ratios. (C) Effect of high met-Hb content on the rate of oxy-Hb loss in liposomes. Liposomal preparations and the symbols used are the same as described in (B). The hemolysate that was encapsulated was stored at −20 °C and contained 16% met-Hb.

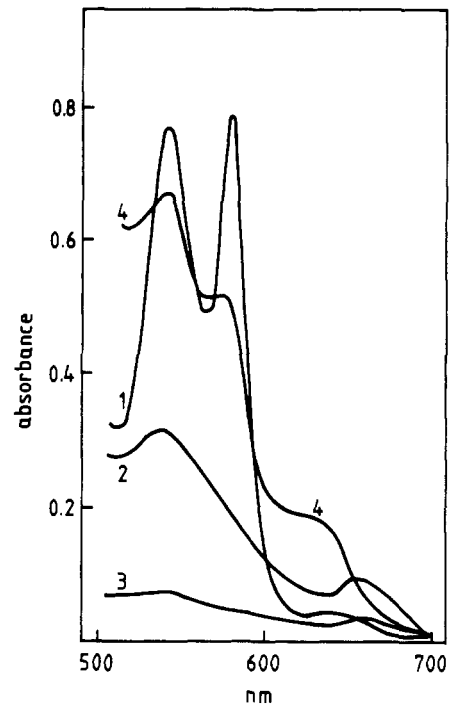

FIGURE 4: Visible absorption spectra of hemosomes prepared from different phospholipids and fresh hemolysate. DPPC/DPPA/cholesterol (3:1:4) recorded about 3 weeks after preparation (1), DPPC/DPPA (3:1) recorded immediately after preparation (2) and 1 day later (3), and EPC/PG (3:1) recorded 3 days after preparation (4). Phospholipid mixtures are expressed as mole ratios.

(3:1:4) decreased only slightly over a period of about 20 days (Figure 3A). This is contrasted by the behavior of DPPC/DPPA hemosomes. In the absence of cholesterol, the oxy-Hb content decreased to zero almost instantaneously after the encapsulation procedure. Similar results were obtained when pure DPPA was used instead of the DPPC/DPPA mixture or when DPPC was replaced by dimyristoyl- or distearoylphosphatidylcholine. However, encapsulating stripped hemolysate instead of one containing organic phosphates in DPPC/DPPA liposomes significantly slowed down the loss of oxy-Hb. The stabilizing effect of cholesterol was also observed with hemosomes made from EPC and negatively charged phospholipids. The loss of oxy-Hb content depended on the nature of the charged phospholipid and was in the order of EPA > PI ≃ PG > PS. In all cases studied the rate of oxy-Hb loss was reduced in the presence of about 50 mol % cholesterol (Figure 3B). Figure 3C shows that, when hemolysate was used containing 16% met-Hb as opposed to fresh hemolysate with less than 2% met-Hb, the rate of oxy-Hb loss was usually increased.

Visual inspection of various hemosome preparations confirmed the conclusions drawn from Figure 3. Hemosomes made of saturated PCs and cholesterol retained the red color characteristic of oxy-Hb for about 20 days. In contrast, all EPC hemosomes, particularly those containing negatively charged phospholipids, usually turned brown within days except for EPC/PS/cholesterol (3:1:4) hemosomes in which the encapsulated Hb appeared to be stabilized at least over a period of 2 weeks. The stabilizing effect of cholesterol was most obvious in DPPC/DPPA hemosomes. While in DPPC/DPPA/cholesterol (3:1:4) hemosomes the red color was well preserved (for at least 3 weeks), these hemosomes without cholesterol became colorless almost immediately after preparation.

Consistent with the results mentioned above, the visible spectrum of DPPC/DPPA/cholesterol (3:1:4) hemosomes characteristic of oxy-Hb with a minor peak at 630 nm from met-Hb remained practically unchanged when the sample was stored at 4 °C for about 20 days (Figure 4, spectrum 1). In contrast, the visible spectra of DPPC/DPPA hemosomes without cholesterol showed small peaks at 530 and 650 nm when recorded immediately after the preparation (Figure 4, spectrum 2). After 1 day, an almost flat base line was obtained with the same sample (Figure 4, spectrum 3). The visible spectrum recorded from EPC/PG (3:1) hemosomes (Figure 4, curve 4) shows the presence of large amounts of met-Hb at least after a few days. This is entirely consistent with the data in Figure 3B and the brown appearance of the sample.

The concentration dependence of the stabilizing effect of cholesterol was studied by recording visible spectra of the hemosome preparation as a function of cholesterol content. It could be shown that in DPPC/DPPA (3:1 mole ratio) hemosomes cholesterol at concentrations greater than 20 mol % had the maximum stabilizing effect. In contrast, with EPC/EPA (3:1 mole ratio) hemosomes, 50 mol % cholesterol was required to achieve maximum stabilization. At 33 mol % cholesterol, Hb oxidation was significant, and at 20 mol %, spectral changes occurred indicative of hemichrome formation, i.e., denaturation of Hb.

Discussion

Hb present in fresh hemolysate was encapsulated in liposomes of different lipid composition. The resulting hemosomes are mainly multilamellar liposomes with the majority of particles having a diameter between 0.1 and 1 μm. Gaber et al. (1983) reported the encapsulation of Hb in large unilamellar vesicles of a mean diameter of 0.24 μm and of a narrow size distribution. Their vesicles were produced by successive extrusion of Hb together with the phospholipid dispersion

through polycarbonate filters of decreasing pore size (0.6, 0.4, and 0.2 μm). No effort was made here to produce well-defined unilamellar vesicles since the questions we set out to answer can equally well be tackled in multilamellar liposomes of a wide size range. This paper is addressed essentially to two questions: (1) is an intact phospholipid bilayer a diffusion barrier for small molecules such as O_2 or CO and (2) what is the stability of the encapsulated Hb?

CO binding to encapsulated Hb is faster than that to red blood cells, ligand concentration dependent, and cooperative (Table II). A detailed explanation of this behavior is beyond the scope of this work and would be extremely complex, if at all possible. However, the fast CO binding, taken together with its autocatalytic nature (Table II), is a major asset when considering hemosomes as a blood surrogate. In discussing CO binding to Hb encapsulated in liposomes, the question of the bilayer integrity is crucial. ^{31}P NMR indicates that the structure and motion of phospholipid molecules is by and large unaffected by the presence of Hb and is characteristic of an intact phospholipid bilayer. The determination of encapsulated Hb according to Drabkin provides further evidence for the integrity of the lipid bilayer. In the presence of Drabkin's reagent containing $K_3Fe(CN)_6$ and KCN in phosphate buffer, pH 7.4, accessible Hb is converted to CN-met-Hb which is readily identified spectrophotometrically. Encapsulated Hb was unaccessible to Drabkin's reagent except when the phospholipid bilayers were lysed with sodium cholate (2%) or Triton X-100 (0.1%) [cf. Gaber et al. (1983)]. This indicates that the phospholipid bilayers are impermeable to ions in the presence of Hb.

The effect of adding negatively charged phospholipids to PC is to increase the encapsulation of Hb. However, the effect varies within wide limits, the observed increase being smaller with synthetic saturated phospholipid bilayers in the gel state than that observed with liquid-crystalline bilayers of naturally occurring phospholipids. This increase in encapsulation efficiency may be explained at least qualitatively in terms of electrostatic repulsion between phospholipid bilayers, leading to a volume increase of the aqueous compartments entrapped between the bilayers.

Our study of the stability of encapsulated Hb confirms previous results [cf. Gaber et al. (1983) and Szebeni et al. (1984a,b)]. Replacing EPC by DPPC and EPA by DPPA improved the stability of Hb encapsulated provided an equimolar amount of cholesterol was present in the bilayer (cf. Figure 3A,B). Further, results presented in Figure 3 indicate that Hb encapsulated in either saturated or unsaturated negatively charged phospholipids is significantly less stable than Hb encapsulated in neutral (isoelectric) phospholipids. The effect is drastic with DPPC/DPPA (3:1). In general, the presence of cholesterol in negatively charged lipid bilayers stabilizes encapsulated Hb; however, the degree of stabilization varies widely, whereby the most dramatic effect was observed with saturated phospholipids (Figure 3A). Equimolar quantities of cholesterol in DPPC/DPPA produced a long-term stabilization while in bilayers consisting of unsaturated EPC and negatively charged phospholipids the stabilizing effect varies from long-term as in EPC/PS to negligible as in EPC/EPA (Figure 3B).

These results suggest that the stability of Hb encapsulated in phospholipid liposomes depends on several factors. We assume that the encapsulated Hb interacts primarily at the phospholipid–water interface and that this interaction in turn leads to Hb denaturation. It is clear from the results presented that Hb interacts preferentially with negatively charged phospholipid bilayers. The nature of the negatively charged phospholipid polar group is important in promoting the interaction with Hb, phosphatidic acid having the biggest effect, and hence, Hb encapsulated in PA containing liposomes is least stable. However, the stabilization effected by the presence of cholesterol indicates that another important factor is the lipid acyl chain unsaturation and related to it the lipid bilayer fluidity. Cholesterol seems to be most effective in preventing the interaction between Hb and negatively charged lipid bilayers if the lipid acyl chains are saturated.

The interaction of the Hb with lipid bilayers may lead to an acceleration of the Hb oxidation and in turn to protein denaturation. In this context it is interesting to note that stripping the hemolysate free from organic phosphates increases the stability of hemoglobin. Organic phosphates are known to shift the conformational equilibrium of the protein toward its low-affinity state. The reduced stability of the encapsulated protein in the presence of organic phosphates may be related to the faster autooxidation rate of Hb under these conditions (Mansouri & Winterhalter, 1974) or to a different surface charge distribution of the protein in the low-affinity state. That the interaction of Hb with charged lipids in the absence of cholesterol produces heme loss from the protein is evident from the bleaching of the Hb upon encapsulation, and a number of other observations support this interpretation. With all charged hemosomes containing no cholesterol, a blue shift in the Soret band from 420 to 407–411 nm and a reduction in absorption of this band were observed. In the presence of equimolar concentrations of cholesterol there was either no shift (e.g., in DPPC/DPPA/cholesterol, 3:1:4) or a reduced shift to 415–416 nm in all EPC-containing mixtures. This shift indicates a decrease in the ground energy level of heme and was suggested by Shviro et al. (1982) to arise from the dissociation of heme from globin. These authors studied the interaction of Hb with PS vesicles and concluded that Hb binds to the PS bilayer, leading to the dissociation of the heme–globin complex and in turn to the partitioning of the heme into the lipid bilayer.

Another indication that the heme group interacts strongly with charged lipid bilayers and that the presence of cholesterol in the bilayer inhibits this interaction is evident from the following experiment. When cytochrome *c* was encapsulated in DPPC/DPPA liposomes (3:1 mole ratio) in the same way as Hb, rapid bleaching of the sample occurred with a concomitant loss of the visible absorption spectrum, including the Soret band. Equimolar concentrations of cholesterol in the bilayer prevented these changes and stabilized the encapsulated cytochrome *c*. The interaction of the cytochrome heme with the lipid bilayer is most remarkable considering that the heme is covalently bound to the protein by two thioether links (Timkovich, 1979).

In summary the disappearance of the visible spectrum between 500 and 700 nm, the blue shift and the substantial reduction in absorbance of the Soret band, and the appearance of a small peak at 650 nm characteristic of iron-free porphyrin taken together all suggest a dissociation of the heme–globin complex and subsequent removal of iron from the porphyrin ring.

Since biological membranes all bear a net negative charge, the observation that cholesterol has a stabilizing effect on Hb seems to be of biological relevance. It is interesting to note that proteolytic activities with different specificities are located both in the membranes as well as in the cytoplasm of the erythrocyte (Vettore et al., 1983). Hence, globin released as the result of the interaction of Hb with the lipid bilayer can be envisaged to be degraded in the first step by membrane-

bound proteases. The experiments presented here suggest that cholesterol inhibits or at least moderates the interaction of Hb with the negatively charged lipid bilayer, which entails the denaturation of Hb. It is conceivable that this inhibiting effect is produced by cholesterol inserting between phospholipid molecules and thus preventing the partitioning of heme into the lipid bilayer. Results presented here show that the inhibiting effect of cholesterol depends on both the nature of the charged polar group and the degree of unsaturation in the lipid hydrocarbon chains. To our knowledge such a possible functional role of cholesterol in the plasma membrane has not been proposed before. It is clear that the interaction of Hb with lipid bilayers is complex and factors such as surface charge density, nature of the lipid polar group, membrane fluidity, and curvature may be important. A systematic examination is desirable showing how well-defined changes in the lipid polar head group and in the acyl chain composition, and hence bilayer fluidity, effect Hb stability. More work is also required in order to prove that there is a direct competition between cholesterol and the heme ring for available binding sites in biological membranes. Future work will be directed toward these questions and toward an understanding of the molecular events accompanying the interaction of Hb with negatively charged phospholipid bilayers.

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