Interaction of Hemoglobin Derivatives with Liposomes. Membrane Cholesterol Protects against the Changes of Hemoglobin

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ABSTRACT: Hemoglobin (Hb) was incubated with small unilamellar vesicles of different lipid compositions in physiological or low pH–low ionic strength media, and the alterations in the protein and bilayer structure were analyzed by measuring (i) the sedimentation properties and elution pattern of the vesicles upon gel filtration, (ii) the quenching effect of Hb on the fluorescence of membrane-embedded probes, (iii) the intrinsic fluorescence of Hb, and (iv) the Soret spectra of Hb. The results indicate complex formation between Hb and the membrane, followed by intercalation of the protein into the bilayer. These processes can lead to secondary alterations, including aggregation, peroxidative decomposition of unsaturated fatty acids, unfolding of Hb, oxidation of the heme iron, displacement of the heme relative to globin, and oxidative or nonoxidative deconjugation of the porphyrin ring. Complex formation and intercalation of Hb into the bilayer are primarily due to hydrophobic interaction between the protein and the membrane, whereas displacement of the heme and its nonoxidative deconjugation are elicited by ionic interaction between the heme and negative membrane surface charges. These charges, on the other hand, inhibit the oxidative processes in unsaturated lipid vesicles. The rate and extent of alterations both in the protein and in the membrane show inverse correlation with the stability of the heme–globin linkage. The liquid-crystalline phase state promotes the penetration of Hb into the bilayer, whereas displacement of the heme is most expressed in the case of saturated bilayers in the gel state. Cholesterol inhibits both the hydrophobic and ionic interactions between the protein and bilayer, most remarkably the partition of the heme into negatively charged saturated bilayers. It is suggested that the effects of cholesterol are mediated through changes in membrane fluidity.

The interaction of hemoglobin (Hb)[1] with phospholipid bilayer vesicles (liposomes) has been analyzed in several studies to better understand membrane–protein interactions (Papahadjopoulos et al., 1973, 1975; Kimelberg, 1976), in particular, the relationship between Hb and the inner surface of the red blood cell (RBC) membrane (Shaklai & Ranney, 1978; Shviro, et al., 1982). This interaction is also central in recent works on Hb-containing liposomes (termed "hemosomes" or "neohemocytes"), which are a potential RBC substitute (Gaber et al., 1983; Gaber & Farmer, 1984; Szebeni et al., 1984a,b; Hunt et al., 1985).

In a previous study (Szebeni et al., 1985), we reported that the level of oxyHb decreases significantly faster in hemosomes bearing negative surface charges than in equivalent isoelectric vesicles and that membrane cholesterol inhibits this effect of negative charges. In the present work, we extended these studies to obtain further information on the effects of negatively charged phospholipids and cholesterol on the interaction between Hb and bilayer membranes. We incubated Hb derivatives with small unilamellar vesicles (SUV) and used different techniques to measure the effects of (i) vesicle surface charge, (ii) the degree of saturation of the fatty acids, (iii) the presence of cholesterol in the membrane, and (iv) the ligand status of Hb on the changes of the protein and the lipid membrane. It was assumed that by using SUV, the Hb–bilayer interactions can be analyzed under more defined conditions that in the polydisperse, multilamellar hemosome system, and thus a better insight into the molecular mechanisms can be gained.

Experimental Procedures

Materials. Egg phosphatidylcholine (EPC) and bovine brain phosphatidylserine (BPS) were purchased either from Lipid Products (South Nutfield, U.K.) or from Sigma Chemical Co. (St. Louis, MO). Dipalmitoylphosphatidic acid (DPPA) was either from Sigma or from Bachem Feinchemicalien AG, Bubendorf (Switzerland). All phospholipids were pure by TLC criteria. Dansylated [*N*-5-(dimethylamino)-naphthalene-1-sulfonyl] dipalmitoyl-L-α-phosphatidylethanolamine, triethylammonium salt (D-PE), was from Molecular Probes, Inc. (Junction City, OR). Dipalmitoylphosphatidylcholine (DPPC), 12-(9-anthroyloxy)stearic acid (AS), myoglobin from sperm whale skeletal muscle (type II), and human hemoglobin were obtained from Sigma Chemical Co. (St. Louis, MO). This last preparation (referred to as metHb) was used in the column chromatographic experiments.

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[1] Abbreviations: AS, 12-(9-anthroyloxy)stearic acid; BPS, bovine brain phosphatidylserine; BSA, bovine serum albumin; buffer 1, 5 mM KH_2PO_4/K_2HPO_4 and 0.15 M NaCl, pH 7.4; buffer 2, 5 mM KH_2PO_4/K_2HPO_4, pH 5.6; chol, cholesterol; CNmetHb, cyanomethemoglobin; COHb, (carbonmonoxy)hemoglobin; D-PE, dansylated phosphatidylethanolamine; EPC, egg phosphatidylcholine; DPPA, dipalmitoylphosphatidic acid; DPPC, dipalmitoylphosphatidylcholine; Hb, hemoglobin; metHb, methemoglobin; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, red blood cell; SUV, small unilamellar vesicle(s); TLC, thin-layer chromatography.

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It contained 88% metHb as measured by relating the spectrophotometrically determined metHb content to the dry weight. CL-Sepharose 4B was from Pharmacia Fine Chemicals AB (Zurich, Switzerland). [^{3}H]DPPC was tritiated by EIR (Wurenlingen, Switzerland). Cholesterol (primary standard) was obtained from Eastman Chemical Co. (Rochester, NY) and DL-α-tocopherol (USP-FCC) from Hoffmann-La Roche Inc. (Nutley, NJ). All other reagents were of the highest grade available.

Preparation of Hemoglobin Derivatives. Freshly drawn, heparinized human RBCs were washed 3 times in 0.15 M NaCl, and the packed cells were lysed as described earlier (Szebeni et al., 1984b, 1985, 1986), or (in the experiments analyzing the Soret band) by the method of Drabkin (1946). The lysate was filtered through sterile filters. OxyHb A was separated from the lysate at 4 °C by anion-exchange chromatography as described by Huisman and Dozy (1965), using DEAE-Sephadex A-50. The pooled oxyHb A was dialyzed for 24 h at 4 °C against either 5 mM KH_2PO_4/K_2HPO_4 and 0.15 M NaCl, pH 7.4 (buffer 1), or 5 mM KH_2PO_4/K_2HPO_4, pH 5.6 (buffer 2), and then it was concentrated at 4 °C to 2 mM in an Amicon ultrafiltration apparatus using a Diaflo UM 10 membrane. OxyHb A was stored at −70 °C. Hemoglobin derivatives including MetHb A, COHb A, and CNmetHb A were prepared as described by Di Iorio (1981). MetHb A was filtered through sterile filters or centrifuged at 12000*g* for 5 min before the experiments to remove metHb aggregates. The purity of the above Hb derivatives was verified by UV–vis absorption spectroscopy. Their concentration was determined spectroscopically, using the millimolar extinction coefficients given by Winterhalter (1974). Hemin was prepared by extraction from freshly drawn human blood with strontium chloride/acetone solution as described by Labbe and Nishida (1957). As hemin is insoluble in water, pyridine hemochromogen was used as heme analogue. The heme–pyridine complex was prepared by mixing equimolar amounts of hemin and pyridine in buffer 2. The concentration of pyridine hemochromogen was determined according to DeDuve (1948).

Preparation of Small Unilamellar Vesicles (SUV). Unless otherwise stated, 2 mg of the lipid mixture in $CHCl_3/CH_3OH$ (2:1 v/v) was dried in a round-bottom glass flask either by rotary evaporation or by blowing of the solvent by nitrogen, and further dried for several hours under high vacuum. The lipid film was dispersed by handshaking with a few glass beads, in 4 mL of buffer 2. The dispersion was sonicated under N_2 at 4 °C in a Branson B12 sonifier (microtip, 50% duty cycle) for 20 min, or in a bath sonicator cooled to 4°C, for several hours until it became entirely translucent. These methods gave identical results. Following probe sonication, SUV were centrifuged at 12000*g* for 5 min to remove titanium and lipid aggregates. Liposomes were stored at 4 °C and were used within a week of preparation. Fluorescent and ^{3}H-labeled liposomes were prepared as above, except that 10 μg of label [D-PE, AS, or [^{3}H]DPPC (840000 cpm)] was codissolved with the lipids before drying. In the case of ^{3}H labeling, 40 mg of lipid was dispersed in 4 mL of buffer 1 and sonicated for 1 h. Liposomes with the following lipid compositions, expressed as mole ratios were made: EPC; DPPC; EPC/chol 1:1; DPPC/chol 1:1; EPC/BPS 3:1; DPPC/DPPA 3:1; EPC/BPS/chol 3:1:4; and DPPC/DPPA/chol 3:1:4.

Sedimentation and Column Chromatography of SUV. ^{3}H-Labeled SUV of different composition in buffer 1 were incubated with 0.4 mM oxyHb A or metHb at 4 °C with shaking. At indicated times, 1-mL aliquots were centrifuged at 12000*g* for 5 min. The pellets were redissolved in 10% Triton X-100 and were analyzed for [^{3}H]DPPC, metHb, and oxyHb content. The supernatants of the samples incubated for 15 h were applied to a CL-Sepharose 4B column (28 cm × 1.2 cm) equilibrated with buffer 1. The column was presaturated with the appropriate phospholipid dispersion. Elution was carried out with buffer 1, at 4 °C at a rate of 0.2 mL/min. Routinely, 1-mL samples were collected and analyzed for metHb or oxyHb A content and ^{3}H activity. The latter was corrected for color quenching by Hb. The amount of protein in the pellets and in the eluted samples (after solubilizing the lipids by Triton X-100) was estimated by measuring the Soret absorbance.

Measurement of SUV and Hb Fluorescence. Fluorescence measurements were done at room temperature, in an Aminco SPF 500 recording spectrofluorometer at right-angle illumination, under constant stirring. Unless otherwise stated, the excitation and emission bandwidths were 2 and 10 nm, respectively. Further instrumental details are given in the figure legends.

Measurement of the Absorption Spectra of Hb. A Beckman DU-7 spectrophotometer, with data storage and accumulation facilities, and SUV as blank were used. Two- to six-microliter aliquots of Hb derivatives were added to 0.5 mL of SUV to give 2 μM final protein concentration. After gentle shaking by hand for 3–4 s, the spectra were recorded at room temperature and accumulated. The content of the cuvette was gently shaken between the recordings.

RESULTS

Sedimentation of SUV Incubated with Hb Derivatives. Centrifugation of the vesicle dispersion after various incubation times with metHb at 4 °C yielded increasing amounts of lipids in the pellet with all types of unsaturated liposomes. The increase was greatest with EPC/BPS vesicles, where in a typical experiment the amount of lipids recovered in the pellet increased from zero (before adding metHb) to 26, 42, and 51% of the total lipids after 1-min, 3-h, and 15-h incubation, respectively. The corresponding values at 15 h for EPC, EPC/chol, and EPC/BPS/chol vesicles were 38, 18, and 31%, respectively. OxyHb A caused significantly less aggregation (after 15 h, 3% of EPC/BPS vesicles were recovered in the pellet).

MetHb cosedimented with the lipids in significant amounts with all types of vesicles (11–18% of the total Hb after 15 h), but a direct correlation between the amount of sedimented lipids and metHb could not be established. OxyHb A was not detectable in the lipid sediment with any type of vesicles even after 15-h incubation at 4 °C.

These findings indicate that protein–lipid aggregation is significantly greater with metHb and negatively charged vesicles than with oxyHb and isoelectric liposomes and that cholesterol inhibits the aggregation process both in charged and in uncharged vesicles.

Gel Filtration on Sepharose-4B. The Hb-induced morphological alterations of liposomes were further analyzed by chromatographing the supernatants obtained from the 15-h samples in the above-described sedimentation studies. As shown in Figure 1, all types of vesicles incubated with metHb were enlarged, as concluded from the increased amounts of lipid eluting in the void volume. This change was most pronounced with EPC/BPS liposomes, where the ratio of large vesicles (i.e., those excluded from the gel) increased from 10% (no metHb added) to 70% in the presence of metHb (Figure 1B). Incorporation of cholesterol in the membrane reduced the BPS-induced size increase of liposomes (Figure 1C).

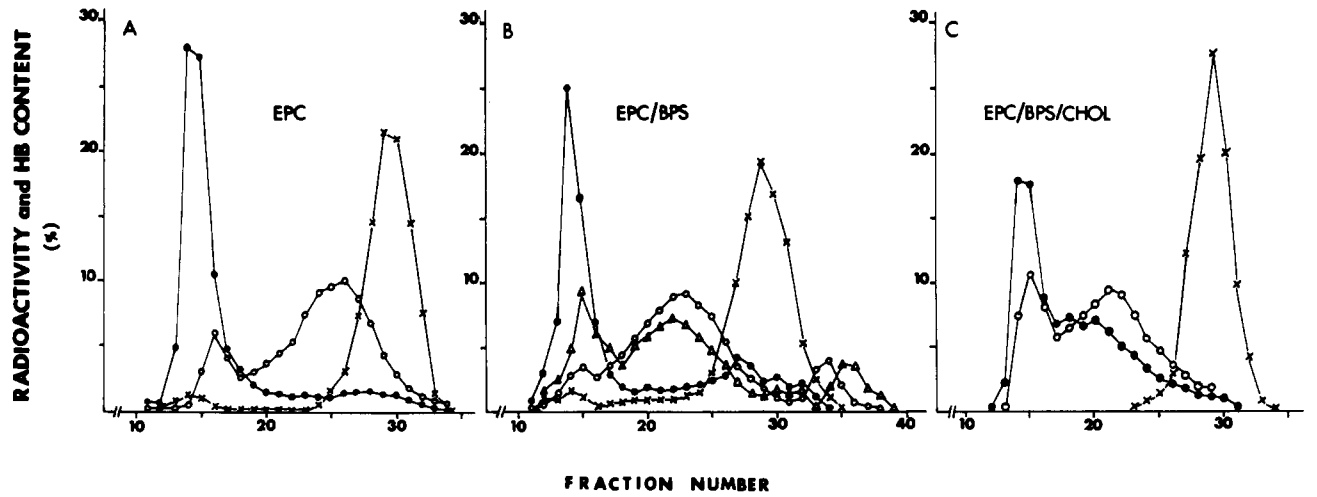

FIGURE 1: Size exclusion chromatography of liposomes on Sepharose-4B, after incubation with Hb derivatives. Liposomes in the supernatant of the samples incubated with oxyHb or metHb for 15 h at 4 °C were subjected to gel filtration as described under Experimental Procedures. The compositions of vesicles, expressed as a mole ratio, were EPC (panel A), EPC/BPS 3:1 (B), and EPC/BPS/chol 3:1:4 (C). (●) ^{3}H-Labeled liposomes (10 mg of lipid/mL) were incubated with 0.4 mM metHb at 4 °C for 15 h with shaking; (○) the same liposomes treated similarly without addition of metHb (control); (△) liposomes were incubated with 0.4 mM oxyHb for 15 h under the same conditions as with metHb (only on panel B); (×) the elution profiles of metHb, after 15-h incubation with liposomes. Each run was reproduced 2–3 times, and the reproducible chromatograms are presented.

Incubation of SUV with oxyHb A caused much less change in the elution patterns (shown for EPC/BPS liposomes in Figure 1B). In vesicles lacking cholesterol, a minor fraction of metHb (but no oxyHb A), coeluted with the main liposomal peak (Figure 1A,B). These findings are consistent with the sedimentation pattern, in that they show (i) the binding of Hb to the bilayer with subsequent morphological alteration (enlargement), (ii) the higher propensity of metHb to bind to the membrane and induce morphological changes as compared to oxyHb, (iii) the enhancing effect of negative surface charge on the binding process and on the ensuing morphological changes, and (iv) the protective effect of cholesterol against these changes.

Effects of Hb Derivatives on the Fluorescence of Lipophilic Probes Incorporated in SUV. To shed light on the molecular mechanism and kinetics of Hb–bilayer interaction, we incorporated fluorescent probes in the vesicle membrane; dansylated phosphatidylethanolamine (D-PE), monitoring the polar head-group region, or anthroylstearic acid (AS), labeling the paraffin core of the membrane (Waggoner & Stryer, 1970). The Hb and lipid concentrations were lowered, and a pH 5.6 phosphate buffer without NaCl (buffer 2) was used to promote the ionic interactions between Hb and negatively charged vesicles (Shviro et al., 1982).

Figure 2A shows the fluorescence emission spectra of D-PE-labeled EPC/BPS liposomes as a function of incubation time with MetHb A. Addition of the protein to the vesicles caused significant fluorescence quenching over some minutes, without spectral shift. When the same probe in different liposomes, and other heme-containing molecules in addition to metHb A, is used as quencher, the time course of fluorescence changes is shown in Figure 3. In general, the rates of fluorescence decay do not obey single-exponential functions. The curve shapes are suggestive of a complex mechanism for quenching, involving several different processes.

Panel A of Figure 3 shows that the quenching effect of metHb A on D-PE fluorescence was initially faster in negatively charged vesicles (curves d and f) than in isoelectric ones (curves c and e) and on the long run quenching was significantly inhibited by cholesterol both in isoelectric and in negatively charged liposomes (curves c and d). In saturated, negatively charged vesicles, the quenching effect of metHb A showed a remarkably different pattern. After the protein was added to the vesicles the fluorescence of D-PE sharply dropped by 10–15% and then remained at this level without further decrease (curve a). Surprisingly, cholesterol in these liposomes increased the quenching effect of metHb A (curve b), so that the decay of D-PE fluorescence became similar to that observed with cholesterol-containing unsaturated liposomes (curves c and d).

Bovine serum albumin had no effect on the fluorescence of D-PE in negatively charged (EPC/BPS) vesicles (curve b in Figure 3B), suggesting that quenching is specific to Hb and is due to the heme in the protein. This conclusion is also supported by the strong quenching effect of the water-soluble heme derivative pyridine hemochromogen (curve h), but not of pyridine (curve c in Figure 3B).

Experiments exploring the quenching efficiencies of different Hb derivatives in EPC/BPS vesicles showed metHb A (Figure 3B, curve g) to be a significantly more effective quencher of D-PE fluorescence than CNmetHb A (curve e), COHb A (curve d), or (on the short run) oxyHb A (curve f). Considering that the strength of the heme–globin bond is significantly lower in metHb than in the other derivatives (Winterhalter & Derauleau, 1967), this observation suggests an inverse correlation between the stability of the heme–globin complex and the efficiency of quenching. This, in turn, points to a displacement of the heme relative to globin, as part of the quenching process.

To assess the role of the electrostatic interactions in the above fluorescence changes, the quenching effect of metHb A in EPC/BPS vesicles was analyzed as a function of pH and ionic strength in the medium. Figure 3C shows that in the presence of 0.15 M NaCl (curve b) or at pH 7.4 (curve a), the initial rate of quenching was slightly lower than at pH 5.6 without NaCl (curve c) but the differences disappeared on the long run. This implies that electrostatic interaction plays only a minor role in the quenching process. At high salt concentrations (1 and 2 M NaCl, curves d and e), however, the quenching effect of metHb A was increased. Considering that high ionic strength promotes the dissociation of Hb to dimers and monomers (Antonini & Brunori, 1971), the latter observation suggests that Hb binds to the membrane as a tetramer and dissociated Hb is a more effective quencher than

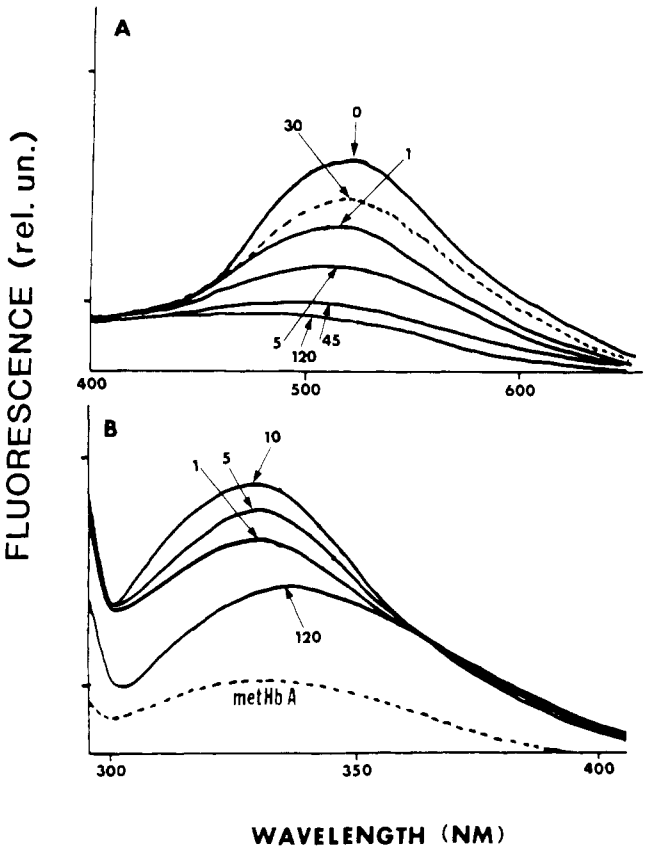

FIGURE 2: (A) Effect of methemoglobin on the fluorescence of dansylphosphatidylethanolamine incorporated in the membrane of negatively charged liposomes. To 1 mL of D-PE-labeled EPC/BPS 3:1 liposomes, prepared and suspended in 5 mM phosphate, pH 5.6, was added 2 μM metHb A (10 μL of a filtered, 0.2 mM stock solution), with stirring in the cuvette. With excitation at 338 nm, the emission spectrum of the probe was recorded at the indicated times (in minutes). The spectrum of control liposomes (without addition of metHb A) is shown at 30 min (dashed curve). (B) Effect of negatively charged liposomes on the intrinsic (tryptophan) fluorescence of metHb A. To 1 mL of (nonlabeled) EPC/BPS 3:1 vesicles was added 2 μM metHb A similarly as in (A), and the emission spectrum of the protein was recorded at the indicated times (in minutes). Excitation was at 283 nm. The dashed curve represents the emission spectrum of 2 μM metHb A recorded in the absence of liposomes. The spectra were corrected for the apparent fluorescence (light scattering) of the buffer and liposome solutions. Lipid compositions are expressed as a mole ratio. Other conditions are the same as in panel A.

the tetramer. This notion was further strengthened by the fact that the quenching effect of the monomeric molecule, myoglobin, was not increased by 2 M NaCl (data not shown).

The pattern of metHb A elicited fluorescence decay in different unsaturated vesicles containing AS resembled that observed with D-PE: the quenching was increased in negatively charged vesicles (Figure 3D, curves c and d) relative to that in isoelectric liposomes (curves a and b), and cholesterol decreased the rate of quenching in both cases (curves a and c). In sharp contrast to D-PE-labeled liposomes, however, the fluorescence of AS dropped to 70–90% of the control (unquenched vesicles) level immediately after addition of metHb A; i.e., the initial decay was too fast to be detected on the time scale of minutes.

Effect of SUV on the Intrinsic Fluorescence of Hb Derivatives. Parallel to monitoring the changes in liposome fluorescence, we also analyzed the ultraviolet fluorescence of Hb derivatives due to the aromatic amino acids phenylalanin, tyrosine, and (mainly) tryptophan (Teale, 1960; Burstein et al., 1973). Normally, this intrinsic fluorescence is almost completely quenched by the heme groups. Its increase indicates decreased quenching, which may be due to a change in the position and/or the environment of the heme groups (Brunori et al., 1972; Grossmann et al., 1979).

As shown in Figure 2B, the fluorescence emission spectrum of metHb A has a broad maximum at about 330 nm, corresponding to the depressed intrinsic fluorescence. Addition of metHb A to EPC/BPS vesicles resulted in a 3-fold increase in fluorescence intensity around 330 nm within 10 min. The reaction rate was similar to the kinetics of the fluorescence decay of D-PE in equivalent liposomes, suggesting a common underlying process for the two phenomena. Figure 2B also shows that substantially longer incubation (2 h) of metHb A with EPC/BPS vesicles led to a decrease and a red-shift of the peak. This may arise from increased polarity around the fluorescent residues as a consequence of protein unfolding (Brunori et al., 1972; Grossmann et al., 1979). Recording the fluorescence intensity of metHb A at 330 nm as a function of incubation time with different vesicles (Figure 4) revealed that EPC/BPS/chol liposomes elicited slower and smaller enhancement of intrinsic fluorescence than EPC/BPS vesicles and that isoelectric (EPC and EPC/chol) liposomes had no effect at all (Figure 4A, curves b, a, e, and d, respectively). In contrast to the fluorescence decay of D-PE, the increase in intrinsic fluorescence showed a clear inverse correlation with the concentration of NaCl in the medium (Figure 4B). There was also an inverse relationship between the pH and the rate of fluorescence enhancement in the range of 5.6–7.4 (data not shown).

DPPC/DPPA vesicles induced a higher increase in the intrinsic fluorescence of metHb A than EPC/BPS ones (Figure 4C, curve a). Cholesterol in these liposomes completely eliminated the liposome-elicited fluorescence enhancement (curve c). Pure DPPC or DPPC/chol vesicles caused no changes (curves c and d).

The fluorescence enhancement caused by DPPC/DPPA vesicles in different Hb derivatives increased in the order CNmetHb A = COHb A < oxyHb A < metHb A (Figure 4D, curves c, d, b, and a, respectively). Since the quenching efficiency of the same derivatives on D-PE fluorescence increased in a similar order (Figure 3B), these observations strengthen the notion of a common underlying process for the quenching of liposome fluorescence and enhancement of protein fluorescence. Furthermore, the inverse correlation between the latter processes and the stability of the heme–globin complex implies that both processes are linked to a displacement of the heme relative to globin.

Changes of the Soret Band of Hb Interaction with Liposomes. To obtain further information on the changes in Hb following its interaction with liposome bilayers, the Soret spectra (Falk, 1964) of both oxyHb A and metHb A were studied under experimental conditions identical with those in the fluorescence studies.

The spectra in Figure 5A show the changes of the Soret band of oxyHb A incubated with EPC vesicles. The first recording (5 s) was unchanged. One minute later, the maximum was shifted from 414.5 to 405 nm, and its intensity increased. From about 1 min on, the absorption gradually decreased with a further blue-shift of 0.5 nm. The Soret band of control oxyHb A (no vesicles added) displayed 0.5-nm blue-shifting after 10 min, without detectable change in intensity (not shown). With metHb A, there was no spectral shift, but the decrease in peak intensity was much faster than with oxyHb A (Figure 5B). As metHb has its Soret band at 405 nm and a higher molar extinction coefficient than oxyHb at 415 nm (Winterhalter, 1974), the blue-shift of the Soret

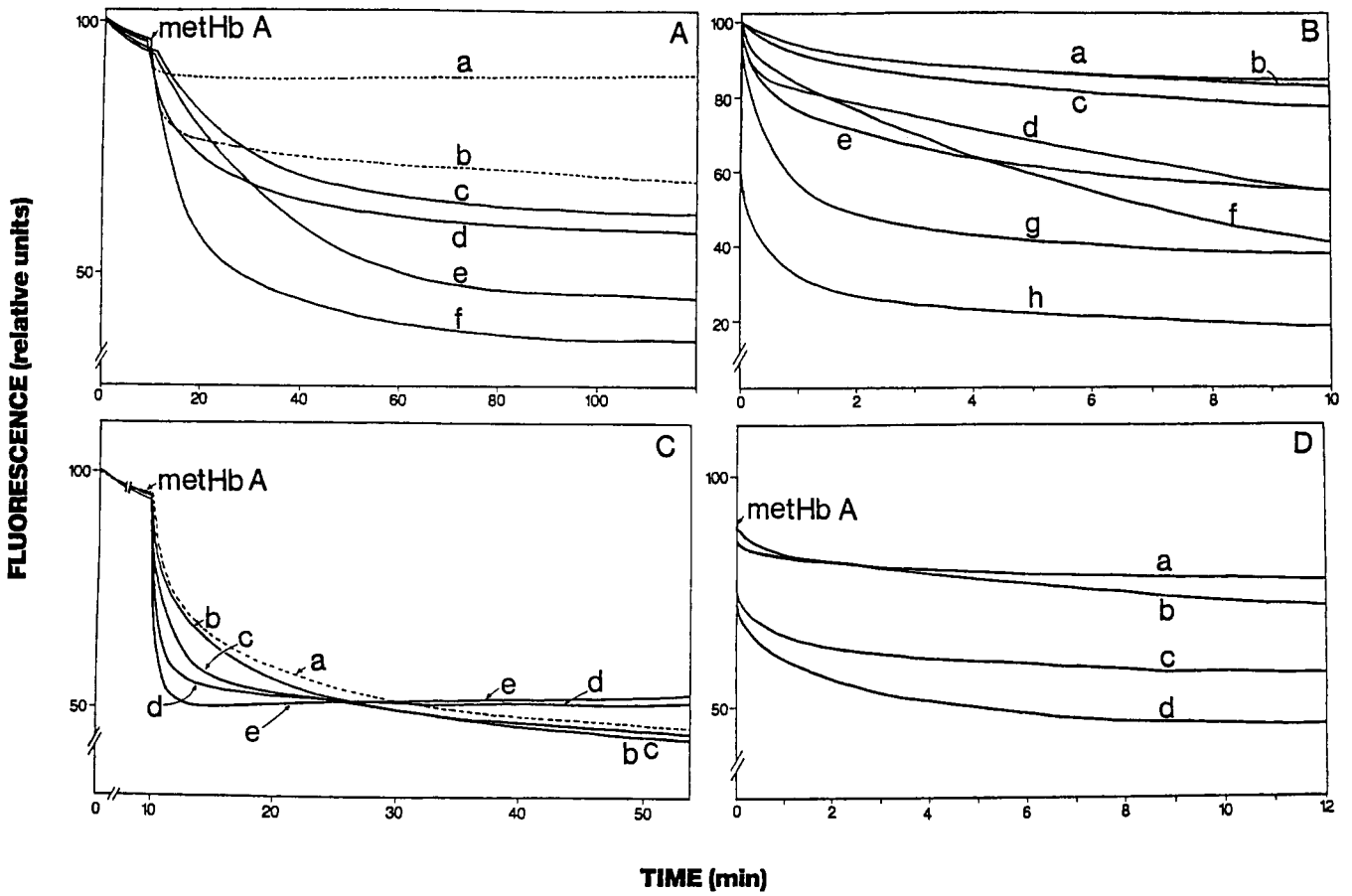

FIGURE 3: Time course of the quenching effects of hemoglobin derivatives on the fluorescence of membrane probes, embedded in liposome bilayers. (A) To 1 mL of D-PE-labeled liposomes, prepared from the lipids shown below, was added 2 μM metHb A as described in Figure 2A (arrow). With excitation of the samples at 338 nm, the fluorescence intensity was recorded at 514 nm. The composition of vesicles was (a) DPPC/DPPA 3:1, (b) DPPC/DPPA/chol 3:1:4, (c) EPC/chol 1:1, (d) EPC/BPS/chol 3:1:4, (e) EPC, and (f) EPC/BPS 3:1. The dashed curves indicate saturated vesicles. Other conditions are the same as in Figure 2A. (B) To D-PE-labeled EPC/BPS 3:1 liposomes were added 2 μM samples of the following proteins: (b) BSA; (d) COHb A; (e) CNmetHb A; (f) oxyHb A; and (g) metHb A. Curves a, c, and h show the control (appropriate volume of buffer added) and the effects of 8 μM pyridine or 8 μM pyridine hemochromogen, respectively. Other conditions were as for Figure 2A. (C) To EPC/BPS 3:1 liposomes was added 2 μM metHb A. The vesicles were prepared, and the experiment was carried out in the following media: (a) 5 mM phosphate, pH 7.4; (b) 5 mM phosphate/0.15 M NaCl, pH 5.6; (c) 5 mM phosphate, pH 5.6; (d) 5 mM phosphate/1 M NaCl, pH 5.6; (e) 5 mM phosphate/2 M NaCl, pH 5.6. Other conditions were the same as in Figure 2A. (D) To 1 mL of AS-labeled liposomes, prepared from the lipids shown below, was added 2 μM metHb A. With excitation of the samples at 365 nm, the fluorescence intensity was recorded at 443 nm. The composition of vesicles was (a) EPC/chol 1:1, (b) EPC, (c) EPC/BPS/chol 3:1:4, and (d) EPC/BPS 3:1. Other conditions are the same as in Figure 2A. Lipid compositions are expressed as a mole ratio.

band indicates oxidation of oxyHb A to metHb A. The minor blue-shifting observed in the control oxyHb A is probably due to the acidic medium, promoting the oxidation of the protein (Mansouri & Winterhalter, 1974). Diminution or disappearance of the Soret band is known to occur if the conjugation of the porphyrin ring is interrupted for some reason (Falk, 1964).

Figure 5C shows that incorporation of α-tocopherol in the membrane of EPC vesicles significantly inhibited the spectral changes of oxyHb A. The spectral changes in metHb A were similarly inhibited by α-tocopherol (data not shown). These observations point to lipid peroxidation as a significant contributor to the oxidation of Hb and deconjugation of the porphyrin macrocycle.

The Soret band of oxyHb A added to EPC/BPS liposomes showed biphasic changes (Figure 5D); an initial decrease was followed by an increase and slight blue-shift (4.5 nm after 10 min). This indicates that in the presence of BPS, the oxidative processes are depressed. The changes of the Soret peak of metHb A added to EPC/BPS liposomes were similarly biphasic (Figure 5E).

In sharp contrast to unsaturated lipid vesicles, DPPC/DPPA liposomes elicited a rapid, monophasic decrease of the Soret peak of oxyHb A (Figure 5F). This was even faster with metHb A, where the Soret band was almost completely flattened in 1 min (the curve at 1 min corresponded to that at 10 min in Figure 5F).

The decrease of the soret peak of oxyHb A added to EPC/chol liposomes (shown in Figure 5G) was significantly less than with pure EPC vesicles (shown in Figure 5A), suggesting a direct or indirect inhibitory effect of cholesterol on the oxidative deconjugation of the porphyrin ring.

Saturated (DPPC) vesicles caused a minor (4–5 nm in 10 min) blue-shifting of the Soret band of oxyHb A, a process which was significantly increased by cholesterol. As a consequence, the spectral changes of oxyHb A added to EPC/chol and DPPC/chol vesicles became very similar (shown for EPC/chol in Figure 5G). In EPC/BPS liposomes, cholesterol did not affect significantly the spectral changes (shown in Figure 5D).

In keeping with other observations on the protective effect of cholesterol against the changes of the heme, flattening of the Soret band was not observed when oxyHb A was added to DPPC/DPPA/chol vesicles (Figure 5H). The spectral changes were similarly biphasic as observed with EPC/BPS ± chol vesicles (Figure 5D), although with less blue-shifting.

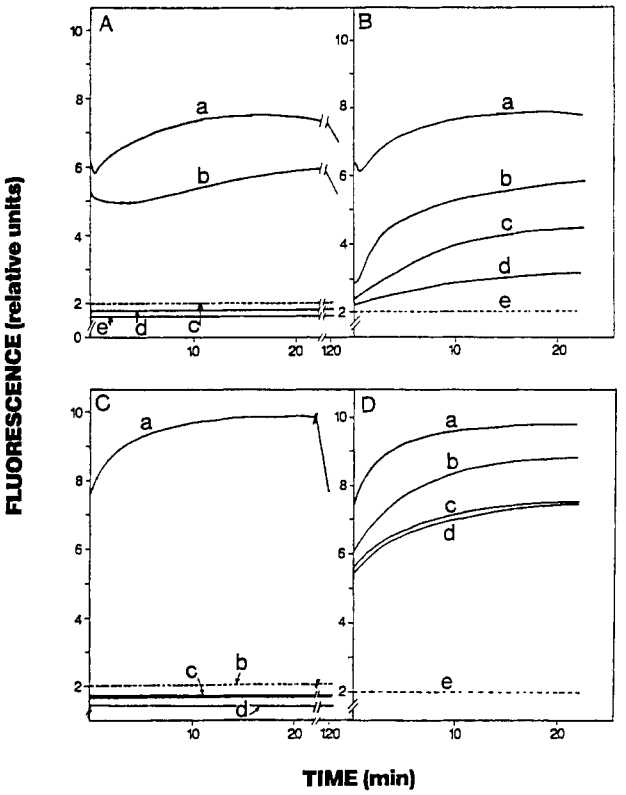

FIGURE 4: Changes in the intrinsic fluorescence of hemoglobin derivatives as a function of incubation time with different liposomes. Excitation was at 283 nm, and the emission was recorded at 330 nm. Other experimental conditions were the same as in Figure 2B. (A) Unsaturated liposomes were incubated with 2 μM metHb A. The composition of vesicles was (a) EPC/BPS 3:1, (b) EPC/BPS/chol 3:1:4, (d) EPC/chol 1:1, and (e) EPC. Curve c shows the intrinsic fluorescence of metHb A in the absence of liposomes. (B) EPC/BPS 3:1 liposomes were incubated with 2 μM metHb A. The vesicles were prepared, and the experiment was carried out in 5 mM phosphate, pH 5.6, in the presence of the following amounts of NaCl: (a) no NaCl; (b) 20 mM NaCl; (c) 68 mM NaCl; (d) 135 mM NaCl. Curve e shows the intrinsic fluorescence of metHb A in the absence of liposomes. (C) Saturated liposomes were incubated with 2 μM metHb A. The composition of vesicles was (a) DPPC/DPPA 3:1, (c) DPPC and DPPC/DPPA/chol 3:1:4 (these were identical), and (d) DPPC/chol 1:1. Curve b shows the intrinsic fluorescence of metHb A in the absence of liposomes. (D) DPPC/DPPA 3:1 liposomes were incubated with 2 μM samples of the following hemoglobin derivatives: (a) metHb A; (b) oxyHb A; (c) CNmetHb A; (d) COHb A. The intrinsic fluorescence in various Hb derivatives, without adding liposomes, did not differ significantly (shown for metHb A, curve e). Lipid compositions are expressed as a mole ratio.

Discussion

In a previous study on the stability of liposome-encapsulated Hb, we observed that negative phospholipids in the membrane tend to destabilize the encapsulated protein, and membrane cholesterol interferes with this effect of negative charges (Szebeni et al., 1985). In the present study, we report on our continued investigations concerning the above phenomena. "Hemosomes", in which they occured, are multibilayer structures with the protein sandwiched between the bilayers. Under these conditions, the membrane surface available for direct contact with Hb largely depends on structural features of the vesicles, such as the number of lamellae and the relative amount of Hb localized in the interbilayer aqueous spaces. In an attempt to reduce the influence of these variables, we used here SUV and added Hb from the outside of the vesicles.

Processes Involved in the Hb–Bilayer Interaction. In spite of the relative simplicity of the system, the interaction of Hb with liposome bilayers turned out to be rather complex, with several independent processes being combined under different experimental conditions. In the following sections, these processes are discussed separately. It should be stressed, however, that they can proceed simultaneously and are closely interrelated.

(*A*) *Protein–Lipid Complex Formation.* The aggregation and cosedimentation of metHb with unsaturated liposomes, together with the enlargement and coelution of the protein with the vesicles, present evidence for the formation of lipid–protein complexes. Such complexes have been described earlier and analyzed by gel filtration by Ushakova et al. (1982).

(*B*) *Penetration of Hb into the Bilayer.* The progressive quenching effect of Hb on the fluorescence of membrane-embedded probes suggests that following the binding, the protein intercalates into the hydrophobic region of the membrane. That the observed quenching is not simply a reflection of Hb binding to the membrane is substantiated by the following facts: (i) The electrostatic binding of cytochrome *c* to liposomes (Vankerkooi et al., 1973) or of Hb to RBC ghost membranes (Shaklai et al., 1977) is an instantaneous process on a time scale of minutes, whereas in our case the half-time for the metHb-elicited decay of D-PE fluorescence in EPC/BPS vesicles (which electrostatically bind Hb) is 0.5–1 min. (ii) The rate of fluorescence decay of D-PE in our experiments, taking place in the course of minutes, is very similar to the reported rate of penetration of cytochrome *c* into phospholipid monolayers (Teissie, 1981) and into bilayer membranes (Vanderkooi et al., 1973). (iii) Among the tested liposome preparations, those consisting of DPPC/DPPA caused the most expressed changes in metHb A fluorescence through an instantaneous process in our time resolution. If the same process (i.e., electrostatic binding) was the main cause of the quenching effect of metHb A on D-PE fluorescence, one would expect a strong, immediate quenching with the same, i.e., DPPC/DPPA, vesicles. The case was, however, the opposite: metHb A caused only a relatively small and nonprogressive quenching of D-PE in these liposomes.

The proposal of Hb penetration into the bilayer matrix is in keeping with the findings of Papahadjopoulos et al. (1973), indicating the expansion of monolayers following the addition of Hb.

(*C*) *Displacement of Heme in Hb.* Evidence for this process is provided by the increased intrinsic fluorescence of the protein upon interacting with EPC/BPS vesicles. Similar observations were made previously by Ushakova et al. (1981), and by Shviro et al. (1982) with liposomes consisting exclusively of acidic phospholipids. The reason for implying displacement instead of detachment of the heme lies in the fact that the increased intrinsic fluorescence of the protein was not coupled with a significant decrease or shift of the Soret band (apart from a transient decrease). This suggests that the heme group remained in the heme pocket of Hb, although with altered position or orientation.

(*D*) *Detachment of the Heme from Globin with Nonoxidative Deconjugation of the Porphyrin Ring.* The combination of complete flattening of the Soret band with an extreme increase in intrinsic protein fluorescence in the case of oxy- and metHb A interacting with DPPC/DPPA vesicles suggests that deconjugation of the porphyrin ring is preceded or accompanied by detachment of the heme from globin. This observation is in keeping with our observation reported previously, on complete bleaching of Hb in DPPC/DPPA multilamellar liposomes (Szebeni et al., 1985). In the absence of polyunsaturated fatty acids, i.e., oxidative lipid–heme interaction, the mechanism of the loss of Soret absorption is not

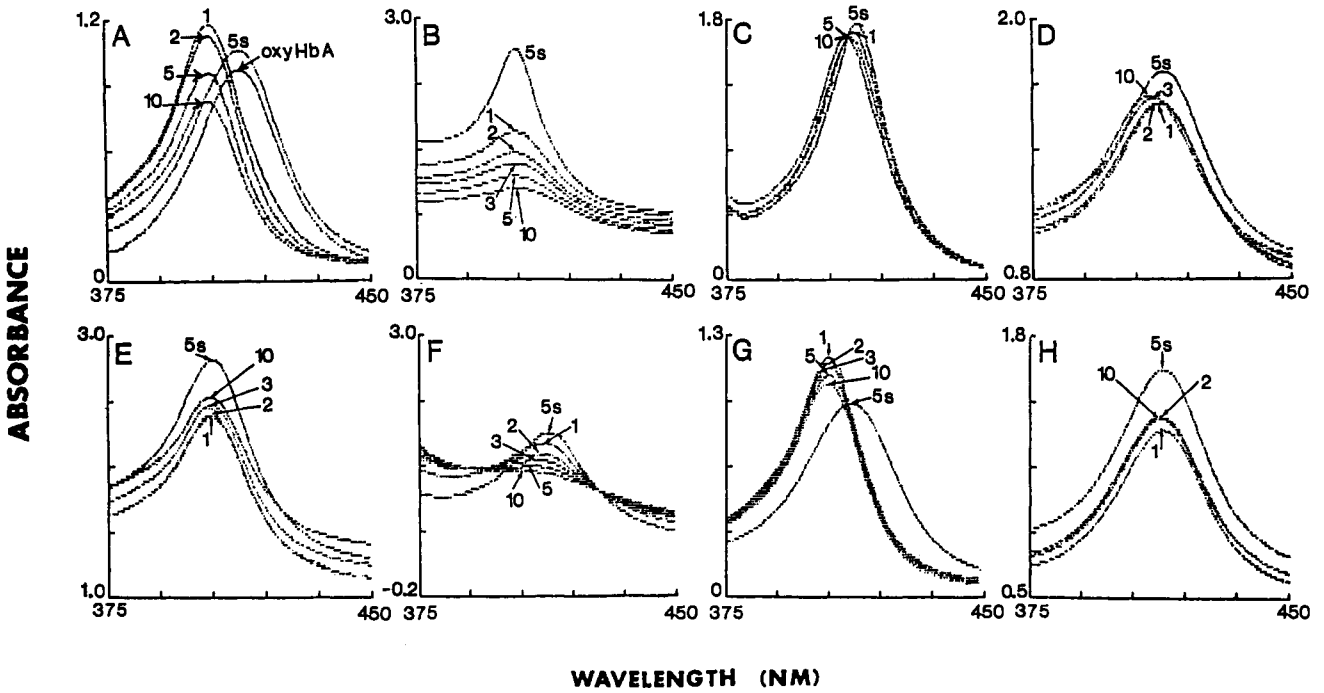

FIGURE 5: Time-resolved electronic spectra in the Soret region of oxyHb A and metHb A, incubated with small unilamellar liposomes. The protein was added to 0.5 mL of vesicles, to give 2 μM final tetramer concentration, and the spectra were recorded at the indicated times against the corresponding liposomes as blank. The numbers pointing to the peaks are minutes, except for the first recording, which was made 5 s after the addition of Hb to liposomes (5 s). The Hb species, the composition of vesicles, and the mole ratio of lipids were as follows: (A) oxyHb A and EPC; (B) metHb A and EPC; (C) oxyHb A and EPC/α-tocopherol (20:1); (D) oxyHb A and EPC/BPS (3:1) or EPC/BPS/chol (3:1:4); (E) metHb A and EPC/BPS (3:1); (F) oxyHb A and DPPC/DPPA (3:1); (G) oxyHb A and EPC/chol (1:1) or DPPC/chol (1:1); (H) oxyHb A and DPPC/DPPA/chol (3:1:4). The spectra shown were typical out of two to three identical experiments. Other experimental details are described under Experimental Procedures.

clear and requires further investigation.

(*E*) *Unfolding of Hb.* The EPC/BPS vesicle-elicited increase in the intrinsic tryptophan fluorescence of metHb A was followed by a decrease and red-shift of the emission maximum, suggesting that the protein undergoes a conformational change and ultimately unfolds (Brunori et al., 1972; Grossmann et al., 1979). Unfolding of Hb upon interaction with a monomolecular lipid film was earlier reported by Fromherz et al. (1972) and is also supported by significant changes in the circular dichroism spectra of Hb encapsulated in different liposomes.[2]

(*F*) *Oxidation of Ferrous to Ferric Heme.* Evidence for this process comes from the blue-shift of the Soret peak upon the interaction of oxyHb A with EPC liposomes. The same phenomenon was described by Bossi et al. (1975) in their study investigating the effects of Hb on liposome permeability. Our results showing that metHb formation is significantly less with saturated liposomes, which do not undergo peroxidation, together with that α-tocopherol, a known inhibitor of lipid peroxidation, inhibited the spectral changes suggest that lipid peroxidation plays a key role in the oxidation of Hb. This is consistent with the well-known catalytic effect of heme proteins on lipid peroxidation, during which the catalyst undergoes oxidation (Haurowitz et al., 1941; Tappel, 1955; Tappel et al., 1961).

(*G*) *Oxidative Deconjugation of the Porphyrin Ring.* We detected gradual flattening of the Soret band in oxy- and metHb A interacting with EPC vesicles. The inhibition of this process by α-tocopherol points again to lipid peroxidation as the major underlying mechanism. As to how lipid peroxidation can affect the heme group, an important recent observation is that lipid hydroperoxides can release free iron from heme-containing containing proteins (Gutteridge, 1985).

Dual Nature of the Hb–Bilayer Interaction. There is no consensus in the literature with regard the forces involved in the interaction between Hb and phospholipid bilayers. The works from Papahadjopoulos et al. (1973, 1975) attest to the hydrophobic nature of this interaction, whereas the studies of Shaklai et al. (1977) and of Shviro et al. (1982) highlight the electrostatic element. Our findings suggest the involvement of both ionic and hydrophobic forces, although their relative contributions to the different secondary changes in the protein and the membrane are substantially different. While the binding and intrusion of Hb into the membrane seem to be governed primarily by hydrophobic forces, the heme changes appear to be due mainly to ionic interactions. The conclusion on hydrophobic interactions is based on several observations: (i) Both isoelectric and negatively charged liposomes displayed structural changes following the interaction with Hb (aggregation, enlargement of the diameter). (ii) The Hb-elicited fluorescence decay of D-PE and AS was observed both with isoelectric and with negatively charged liposomes. (iii) Increased ionic strength and pH had only marginal effects on the quenching effect of metHb A on D-PE fluorescence, irrespective of membrane surface charge.

The effect of ionic forces on the binding and intrusion of Hb into the bilayer seems to be restricted to a modulation of the rate or extent of the hydrophobic interaction, as apparent from the more expressed morphological changes and fluorescence quenching in the case of negatively charged liposomes as compared to isoelectric ones.

The conclusion with regard the electrostatic nature of the heme changes is based on the fact that the fluorescence enhancement in Hb was observed only with negatively charged vesicles and it was significantly decreased by increasing the ionic strength or pH in the medium.

[2] Unpublished experiments.

The concept outlined above on a combined hydrophobic and electrostatic interaction is in agreement with a study of Fromhertz et al. (1972) demonstrating Hb to undergo both hydrophobic and hydrophilic interactions with fatty acid films. The hydrophobic reactivity of Hb is probably due to externally accessible hydrophobic regions of the protein (Tilton et al., 1984), while the ionic interaction obviously involves the negative surface charges on the vesicles, the positively charged iron in the heme, and (at pH 5.6) the positive charges on the protein surface (the pK of Hb is around 6.8).

Mechanism of Hb's Quenching Effect on Fluorescent Membrane Probes. The lack of significant shift or distortion in the emission band of D-PE rules out light scattering as an artifactual cause of the decay of its fluorescence. The fact that the decrease of fluorescence intensity was gradual precludes a significant role of light absorption by Hb (inner filter effect), too (Lakowicz, 1983). Considering penetration of Hb into the bilayer as the primary cause of quenching, the decrease of D-PE fluorescence is probably due, at least in part, to "static" quenching, i.e., formation of a nonfluorescent, ground-state complex between Hb and the fluorophores (Lakowicz, 1983). Resonance energy transfer between the heme and D-PE may be a minor contributor to quenching, since evidence for this process, namely, an instantaneous drop in fluorescence intensity on a time scale of minutes, was apparent only in the case of AS-labeled liposomes. The implication of energy transfer in the quenching of AS fluorescence, rather than for the case of D-PE, is in apparent contradiction with that AS is located deeper in the membrane than D-PE; hence, D-PE is closer to bound Hb than AS (AS is anchored at the 12th carbon position of stearic acid in the paraffin region, while D-PE is located in the head-group region of the bilayer). This discrepancy can be, however, rationalized if one considers the significantly higher quenching efficiency of metHb A on AS than on D-PE fluorescence. The difference arises from a 5 times higher overlap between the emission spectrum of AS and the absorption spectrum of metHb (and the derived separation distance for 50% energy transfer) as compared to the corresponding parameters of the D-PE–heme couple (Vanderkooi et al., 1973; Shaklai et al., 1977).[3] Nevertheless, other factors may also play a role in the initial drop of AS fluorescence in liposomes after adding metHb A, such as binding of monomeric AS to Hb or preferential localization of AS in the outer monolayer of the membrane (Vanderkooi et al., 1973).

Deteriorative and Protective Effects of Phosphatidylserine. A remarkable observation in this study was the dual effects of BPS in EPC vesicles, eliciting the displacement of the heme and at the same time inhibiting the oxidative processes (Hb oxidation and oxidative deconjugation of the heme). These observations confirm our previous reports on the destabilizing effect of BPS on liposome-encapsulated Hb (Szebeni et al., 1985), and on its inhibitory effect on Hb-induced lipid peroxidation in the same vesicles (Szebeni et al., 1986). As discussed above, the deteriorative effects of BPS are mainly due to electrostatic interaction with the heme. The mechanism of its antioxidant influence is far less clear. In hemosomes, it was shown not to be linked to the polyunsaturated fatty acid content of BPS (Szebeni et al., 1986).

Effects of Membrane Cholesterol on the Hb–Bilayer Interaction. The protective effect of cholesterol against (i) Hb-induced morphological changes of unsaturated vesicles, (ii) EPC/BPS liposome-elicited enhancement of intrinsic Hb fluorescence, and (iii) the oxidative deconjugation of heme is in keeping with our earlier reports on decreased decay of oxyHb level (Szebeni et al., 1985) and decreased lipid peroxidation (Szebeni et al., 1986) in cholesterol-containing hemosomes as compared to those lacking cholesterol. These findings are also in agreement with those of Papahadjopoulos et al. (1973) showing decreased ability of Hb to enhance the permeability of EPC liposomes to Na^+ and to expand monolayers if the membrane contains cholesterol. The above effects may be explained in terms of cholesterol's well-known condensing–stabilizing effect in unsaturated bilayers (Papahadjopoulos et al., 1973; Chapman, 1975).

Mechanism of Cholesterol's Protective Action against Heme Loss from Hb Interacting with DPPC/DPPA Liposomes. The interaction of Hb with DPPC/DPPA bilayers resulted in the detachment of the heme from globin, although the quenching effect of the protein on D-PE fluorescence, which assumably reflects penetration in the membrane, was relatively small. Cholesterol in these membranes increased the quenching effect of Hb and at the same time inhibited the detachment of the heme. These observations are most easily rationalized by taking together the effects of cholesterol on the phase state of the membrane with the individual effects of the latter on the membrane–globin and membrane–heme interactions. It is known that cholesterol decreases the order of acyl chain packing in saturated bilayers in the gel state (fluidizes the membrane) (Chapman, 1975). Increased membrane fluidity, in turn, seems to influence the intercalation of hydrophobic proteins and of hemin into lipid membranes in opposite directions; it promotes the former (Mollay & Kreil, 1973; Pownall et al., 1974; Faucon et al., 1976) and hinders the latter process (Ginsburg & Demel, 1983). Consequently, a working hypothesis for the protective effect of cholesterol against the drastic changes of Hb interacting with DPPC/DPPA vesicles may be outlined as follows. In the absence of cholesterol, the heme is pulled out from the globin as a consequence of electrostatic attraction between the iron and negative membrane charges. Since the gel phase favors the movement of heme into the bilayer but hinders that of the globin, the heme ultimately detaches from globin and partitions into the membrane. In the presence of cholesterol, i.e., fluidized bilayer, the partition of heme into the membrane is hindered, and the globin has more freedom to move together with the heme (and to accommodate to steric strains). As a result, the heme does not detach from globin. The biphasic changes in the Soret intensity of oxyHb A interacting with DPPC/DPPA/chol (and EPC/BPS/±chol) vesicles may be envisaged as a consequence of transient changes in the polarity or electronic equilibrium around the heme (Falk, 1964), upon the initial stage of heme displacement.

Concluding Remarks. Considering that Hb is a typical water-soluble globular protein, the fact that is undergoes substantial changes when added to liposomes is unusual and, as Kimelberg (1976) stressed earlier, points to Hb having unique properties in its interaction with lipid membranes. It seems clear that the basis of this phenomenon is the hydrophobic affinity of the protein. By highlighting the significant contribution of hydrophobic forces to the interaction between

[3] The spectral overlap integral (J) values for the AS-heme (with Hb heme) and D-PE–heme (with cytochrome c heme) pairs are 2.5×10^{-13} (Shaklai et al., 1977) and 4.6×10^{-14} M^{-1} cm^3 (Teissie, 1981), respectively. The separation distance at 50% energy transfer (R_0) values for the AS–heme and D-PE–heme couples are 4.6 (Shaklai et al., 1977) and 0.9 nM, respectively. The calculation of R_0 for the D-PE–heme couple is based on the assumptions that the hemes in Hb and cytochrome c are equivalent with respect to fluorescence quenching and that other factors determining R_0 (refractive index, emission quantum yield, and dipole orientation factor) are identical for the AS–heme and D-PE–heme couples.

Hb and model membranes, our study points to an essential role of this interaction in the deterioration of RBCs in some pathological states and upon senescence (Schwartz et al., 1985).

As it is known, the inner monolayer of the RBC membrane is negatively charged due to its high PS content (Schwartz et al., 1985). The potential for negative charge elicited detachment from globin and subsequent partition of heme into the membrane is therefore present in RBCs. Our findings suggest a cardinal role for membrane cholesterol in preventing these negative charge elicited changes. On the other hand, our results also highlight the beneficial roles of negative membrane charges and of cholesterol in protecting against the oxidative interaction between Hb and the membrane.

The alterations of Hb reported here are quantitatively different from those obtained with Hb encapsulated in large multilamellar hemosomes. In addition to the structural differences, this is also due to the experimental conditions in this study, which have been chosen to favor, rather than inhibit, the interaction of Hb with the membrane. These conditions include the use of purified Hb, the lower Hb to phospholipid mole ratio as compared to that in hemosomes [for example, 10–20 times lower than in "hand-shaken" hemosomes (Szebeni et al., 1985)], and the use of a low ionic strength–low pH medium in the spectroscopic studies. Apart from a tendency of increased oxidation, Hb has been found to be fairly stable in hemosomes devised for RBC substitution [Djordjevich & Miller, 1980; Gaber et al., 1983; Gaber & Farmer, 1984; Szebeni et al., 1984a,b; Arakawa et al., 1984; Hunt et al., 1985; Beissinger et al., 1986; Pirkl et al., 1986; see review by Dellacherie et al. (1987)]. It was our purpose to point to potentially harmful reactions and, in this sense, to contribute to a more conscientious design of a blood replacement fluid based on the hemosome concept.

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Registry No. Hb A, 9034-51-9; COHb A, 9072-24-6; oxyHb A, 9062-91-3; metHb A, 12646-21-8; CNmetHb A, 39340-60-8; DPPA, 19698-29-4; DPPC, 2644-64-6; cholesterol, 57-88-5; pyridine hemochromogen, 15629-11-5; hemin, 16009-13-5; pyridine, 110-86-1; heme, 14875-96-8.

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Rip Locus: Regulation of Female-Specific Isozyme (I-P-450$_{16\alpha}$) of Testosterone 16α-Hydroxylase in Mouse Liver, Chromosome Localization, and Cloning of P-450 cDNA†

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ABSTRACT: The constitutive expression of phenobarbital-inducible mouse cytochrome P-450 (I-P-450$_{16\alpha}$) at the mRNA level and its associated testosterone 16α-hydroxylase activity in liver microsomes was a female characteristic in many inbred mice, including BALB/cJ, A/HeJ, and C57BL/6J. This sex-dependent constitutive expression of the mRNA and enzyme activity was severely reduced in females of mouse strain 129/J. The distribution patterns of the mRNA and activity levels in individual offspring of F1, F2, and F1 backcrosses to progenitors, generated from crosses between 129/J and BALB/cJ mice, indicated that the female-specific expression of I-P-450$_{16\alpha}$ is an autosomal dominant trait under the regulation of a sex-limited single locus. It was found that the genotypes of this locus exhibited concordance with that of the coumarin hydroxylase locus (Coh locus) in eight out of nine 9×A recombinant inbred strains, suggesting the localization of this sex-limited locus on chromosome 7. We propose Rip (*r*egulation of sex-dependent, constitutive expression of phenobarbital-*i*nducible *P*-450) as the name of this sex-limited locus. With the use of the rat P-450e cDNA probe, a cDNA library from liver poly(A+) RNA of BALB/cJ was screened, and three distinct cDNAs (pf3, pf26, and pf46) were selected on the basis of their restriction patterns. Nucleotide sequences of the cDNAs revealed that pf3 and pf46 are clones overlapped, with the exception that the 27-bp DNA is inserted in the coding region of pf46. The nucleotide sequence (named pf3/46) obtained from the overlapping sequences of pf3 and pf46 contained 1473 or 1500 bp of open-reading frame, and the deduced amino acid sequence shared 93% similarity with those of rat P-450b. The 27-bp insertion resulted in nine extra amino acids just in front of the cysteine residue, the fifth ligand for heme binding. The mRNA with 27-bp insertion was ubiquitously present in other inbred mice such as A/HeJ and C57BL/6J, but not in 129/J. S-1 nuclease analysis estimated a ratio of p46 and pf3 to be 1:50. Nucleotide and deduced amino acid sequences of the 1473-bp open-reading frame in pf26 possessed 83% similarity to those of pf3/46. Hybridizations of oligonucleotide probes (pf26-cu and pf3/46-cu) specific to either pf26 or pf3/46 with liver poly(A+) RNA from males and females of BALB/cJ, 129/F, and F1 offspring demonstrated that the expression of pf26, but not pf3/46, mRNA was associated with the autosomal dominant inheritance of I-P-450$_{16\alpha}$. The levels of the hybridization of pf26cu to mRNA from F1 backcross to 129/J correlated well with those of the high and the low I-P-450$_{16\alpha}$-dependent testosterone 16α-hydroxylase activities at a 1:1 ratio. Therefore, it was concluded from the genetic evidence that pf26 represents cDNA encoding of testosterone 16α-hydroxylase I-P-450$_{16\alpha}$.

Cytochrome P-450 (P-450)[1] represents a group of terminal oxidases of a membrane-bound monooxygenase system that consists of NADPH–cytochrome P-450 reductase, cytochrome b_5, and NADH–cytochrome b_5 reductase (Sato & Omura, 1978). This monooxygenase system in hepatic microsomes is involved in metabolic inactivation of many endogenous compounds, such as steroid hormones. But in some cases, toxicity and carcinogenicity of certain xenobiotics are enhanced by P-450-dependent metabolism. Since metabolism is influenced by the presence of multiple forms of P-450 (Lu & West,

† A portion of this work has been presented at the Annual Meeting of the American Society of Biological Chemists in 1986 (Noshiro et al., 1986a).

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[1] Abbreviations: Rip, *r*egulation of sex-dependent, constitutive expression of phenobarbital-*i*nducible *P*-450; PB, phenobarbital; P-450, cytochrome P-450; 20× SSC, 3 M sodium chloride and 0.3 M sodium citrate; 6× NET, 0.9 M sodium chloride, 6 mM ethylenediaminetetraacetate, and 0.09 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; 5× Denhardt's solution, 0.1% each of poly(vinylpyrrolidone), bovine serum albumin, and Ficoll 400.