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Long-term intercalation of residual hemin in erythrocyte membranes distorts the cell

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The effect of long-term incubation of residual globin-free hemin on whole red blood cell and isolated cytoskeletal proteins was studied. Hemin at concentrations found in pathological red cells was inserted to fresh erythrocytes. Increased hemolysis developed in the hemin-containing cells after a few days at 37°C and after about four weeks at 4°C. Since lipid and hemoglobin peroxidation did not depend on the presence of hemin, time-dependent effects on the cytoskeleton proteins were studied. Observations were: (1) spectrin and protein 4.1 exhibited a time-dependent increasing tendency to undergo hemin-induced peroxidative crosslinking. (2) The ability of the serum proteins, albumin and hemopexin, to draw hemin from spectrin, actin and protein 4.1 decreased with time of incubation with hemin. These results were attributed to time-dependent hemin-induced denaturation of the cytoskeletal proteins. Albumin taken as a control for physiological hemin trap was unaffected by hemin. Small amounts of hemo-spectrin (2–5%) were analyzed in circulating normal cells, and this in vivo hemo-spectrin also failed to release hemin. It was concluded that slow accumulation of hemin, a phenomenon increased in pathological cells, is a toxic event causing erythrocyte destruction.

Introduction

Free hemin was shown in previous studies to distort red cells within a few minutes and cause hemolysis [1,2]. To assess the correlation of these findings with a possible in-vivo damage of free hemin, its levels in circulating cells were determined. The amounts determined even in the membranes of the most distorted cells were at least two orders of magnitude smaller than those required to destroy the membrane in vitro [3,4]. Since hemin associates easily with phospholipids [5], the fast distortion of the cell membrane might be related to the detergent-like properties of the hydrophobic hemin molecule.

Despite the high affinity of hemin to lipid, only a negligible fraction of the red cell hemin is expected to be physiologically distributed in the membrane phase for the following reasons; Based on the kinetics of hemin release from globin which is the rate-limiting step of the processes, hemin penetration into the membrane is expected to be rather slow [6]. Nevertheless, efflux of hemin out of phospholipid liposomes was shown to be fast and efficient in the presence of albumin and hemopexin, the hemin-binding serum proteins [7]. Thus, the small amounts detected in the red cell membrane might be a transient, harmless, steady-state, hemin in the process of being drained from the phospholipid bilayer. However, measurements of the efficiency of the serum proteins to clear the biological, red cell membrane, of freshly inserted hemin showed that while albumin and hemopexin are also capable in principle to facilitate hemin extraction from the natural membrane, with time an increasing fraction of the membrane-associated hemin is no longer recovered, even in the presence of the serum proteins [3,8]. These differences between artificial and natural membranes may be attributed to the nonlipid components of the erythrocyte membrane.

The logical implication of these findings is that in vivo toxicity of hemin should be sought in changes inflicted to key proteins by the accelerated accumulation of hemin in pathological erythrocytes. In the current communication we describe the changes inflicted on whole erythrocytes and isolated cytoskeletal proteins by long-term incubation with residual amounts of hemin.

Abbreviations: DMSO, dimethyl sulfoxide; G_3PD, glyceraldehyde-3-phosphate dehydrogenase; UV, ultraviolet; DTT, 1,4-dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; HSA, human serum albumin.

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Experimental

Human blood was drawn in heparin from healthy volunteer donors and used within 2 days. Crystallized globulin and fatty acid free human serum albumin were obtained from Sigma. Resins for column chromatography were diethylaminoethyl (DEAE)-cellulose from Whatman and Sepharose 4B from Pharmacia. All other chemicals of analytical grade were purchased from Sigma.

Preparation of ghosts

Open ghosts were prepared from normal, freshly drawn human blood as described previously [9]. Resealing was carried out immediately after preparation of the open ghosts by the addition of 9 volumes of resealing buffer (145 mM Nacl, 0.2 mM $CaCl_2$ and 2 mM $MgCl_2$ pH 7.3) and incubation for 1 h at 37°C. The ghosts were tested for sealing by evaluating G_3PD activity [10]. The percentage of resealing was determined by comparing the activity of the prepared resealed ghosts with 0.1 % Triton X-100 treated sample. Only prerarations with less than 10% residual activity of G_3PD were considered resealed.

Preparations of proteins

Spectrin tetramers were extracted [11] and isolated by gel filtration chromatography on Sepharose 4B column [3]. Protein 4.1 was purified from membranes of fresh erythrocytes using previous procedures [4,5]. Actin was prepared from rabbit skeletal muscle according to established procedures [6]. Hemopexin was prepared from human serum of healthy individuals [7]. Protein concentrations were determined spectrophotometrically using an absorption coefficient ϵ_{280} of 0.8 $(mg/ml)^{-1}$ cm^{-1} for protein 4.1, 1.0 $(mg/ml)^{-1}$ cm^{-1} for spectrin and 185 mM^{-1} cm^{-1} for hemopexin. ϵ_{290} of 0.63 $(mg/ml)^{-1}$ cm^{-1} was used for actin.

Hemin preparation

Hemin stock solutions were made on the day of the experiment by dissolving it into 20 mM NaOH or 80% DMSO. Hemin concentration was determined spectrophotometrically using an absorption coefficient ϵ_{385} of $58.4 \cdot 10^3$ M^{-1} cm^{-1} in NaOH and an ϵ_{403} of $170 \cdot 10^3$ M^{-1} cm^{-1} in 80% DMSO.

Determination of hemin in cell membranes

Hemin concentrations were determined as described before [8]. In brief, hemin was converted to porphyrin and the amount of porphyrin determined by monitoring its fluorescence intensity compared to standards.

Degree of hemolysis and cell oxidation markers

Hemolysis was determined as previously described [1]. The degree of hemoglobin oxidation was calculated using the following equation: $A_0 - A_t/A_0 - 0.836$ where $A = OD_{576}/OD_{540}$ [17]. Malondialdehyde was determined according to established procedures [18].

Instruments

Spectrophotometric measurements were carried out with a Cary 219 or Shimadzu UV-160 spectrophotometer.

Results and Discussion

1. Long term incubation of red cells with residual hemin

Previous studies demonstrated that circulating red cells accumulate some residual hemin in their membrane in amounts that increase with cell age, with old cells containing about double the amount carried by young cells [3]. In pathological cases, despite the younger age of circulating cells, a higher level of membraneous hemin was recorded, and the amount found to correlate with the severity of the clinical state [3,4]. Thus, considering that in vivo circulating cells are exposed to hemin for days, direct correlation between hemin accumulation and cell damage requires in vitro exposure of the cells to residual hemin for long-term periods of incubations. The best way to perform such experiments and avoid affecting the erythrocyte state would be to insert hemin from without and allow it to distribute naturally in the membrane [1]. But, externally introduced hemin may be distributed differently than cytosolic, globin-free hemin which reaches the membrane physiologically from within. To determine whether hemin distribution in the membrane depends on the side of insertion, the following experiments were carried out.

1.1. Distribution of globin-free hemin in the erythrocyte membrane

Ghosts were prepared from fresh blood cells and one part was resealed (see Methods). Hemin to a final concentration of 1–5 μM was added to 0.25 mg/ml membranes and the reaction mixture further incubated at 37°C for 1 h. The membranes were washed to remove unbound hemin and the main cytoskeletal proteins separated from the lipid core (containing the lipids and integral proteins) by low ionic strength extraction; hemin content was determined in both fractions. Comparison of open and resealed ghosts (Table I) indicates that the distribution of hemin in the membrane compartments is independent of the direction of hemin insertion. Thus, the final equilibrium of hemin distribution in the membrane depends only on its affinity for the various membrane components. Since the amounts of protein in the cytoskeleton and in the membrane lipid core are about equal, it can be estimated from Table I that almost half of the membrane hemin is associated with the cytoskeletal proteins in each mem-

TABLE I

Distribution of hemin in the red cell membrane

5 μM hemin added to 0.25 mg/ml membranes. Average of approx. $3 \cdot 10^6$ hemin copies per cell. The amount of hemin is expressed as μmol hemin per μg protein $\times 10^5$.

Type of ghosts	(A) Hemin in hydrophobic core	(B) Hemin in EDTA extract	(C) A/B ratio
Open	4.3 ± 0.8	3.4 ± 0.5	1.26 ± 0.05
Resealed	4.9 ± 0.6	3.6 ± 0.2	1.36 ± 0.08

brane form. We conclude from these results that: (1) introduction of hemin to red cell membranes from without mimics the state in situ where free hemin reaches the membrane from within; and (2) the cell cytoskeleton includes a major portion of the membrane hemin.

1.2. Changes in cell parameters induced by membrane intercalated residual hemin

Finding that hemin can be equally distributed in red cells when added from without, we next examined whether the amounts of residual free hemin estimated in circulating red cells of severe hemoglobinopathies promote distortion of normal erythrocytes.

Red cells separated from fresh blood were resuspended under sterile conditions in isotonic phosphate buffer (pH 7.4) containing 10 mM glucose. Erythrocytes concentration varied in the range of $1 \cdot 10^8$–$2 \cdot 10^9$ cells/ml and hemin was added to yield the amount found in membranes of pathological cases ($6 \cdot 10^6$ monomers per cell [3]); the cells were kept at 37°C or 4°C. Samples were withdrawn at different intervals to measure the degree of hemolysis and oxidation parameters. Malondialdehyde was measured as the probe for membrane lipids, and methemoglobin formation as the representative of the cytosol oxidation state. In each case the same cells treated identically but without the addition of hemin served as controls. It was found that the levels of the oxidation markers malondialdehyde and oxidized hemoglobin, although increased with time, did not differ in hemin-treated and control cells. On the other hand, as compared to controls, increased hemolysis (time and temperature dependent) developed in the hemin-treated samples (Fig. 1). Which membrane components are candidates for hemin-induced injury? In the cell, free cytosolic hemin encounters both phospholipids and cytoskeletal proteins, but since the results of Fig. 1 illustrate that peroxidation of phospholipids is unaffected by hemin, subsequent experiments focused on the cytoskeletal proteins.

2. Preincubation with hemin activates peroxidative crosslinking of cytoskeletal proteins

Previous studies illustrated that in various hemolytic anemias the red cell membranes contained spectrin aggregates and their formation was mediated by oxidized hemoglobins [19]. Similar aggregates could also be formed in spectrin solutions containing hemin or hemoglobin by the addition of minor amounts of hydrogen peroxide [20]. We recently observed that both spectrin and protein 4.1 but not actin form covalent crosslinked aggregates in the presence of peroxidized free hemin [21]. To determine whether crosslinking of cytoskeletal proteins is involved in long-term effects of heamin, the following experiments were carried out.

Hemin was added to protein 4.1 or spectrin dimers (1 μM hemin to 5.5 μM of each protein) and the hemoprotein formed was preincubated under sterile conditions at 37°C. At time intervals H_2O_2 was added and the protein mixture further incubated for 70 min. The reactions were terminated by addition of DTT containing boiled SDS solution, and the protein profile was analyzed by SDS-PAGE. The data (Fig. 2) show that prolonged preincubation of spectrin or protein 4.1 with hemin prior to H_2O_2 addition resulted in increased covalent protein crosslinking and parallel diminution of the monomeric protein bands.

In the case of spectrin, some high molecular weight aggregates were present when peroxidation was performed immediately after hemo-spectrin formation, and the pattern of the reaction mixture was unchanged up to 6 h of preincubation (Fig. 2a, lane B). However, prolonged preincubation caused an increase in the amount and size of aggregates in a time (of preincubation)-dependent manner (lanes C and D). Under the reaction

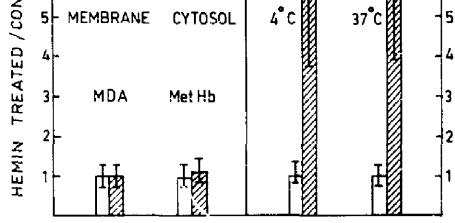

Fig. 1. Hemin-induced hemolysis and oxidation in long-term incubated erythrocytes. Freshly drawn erythrocytes were incubated with and without hemin and parameters measured periodically. The measurements of each parameter were normalized to 1.0 at time zero of each experiment (hemin-lacking cells). Hypotonic lysis of the cell suspension was considered 100% hemolysis. □ (empty boxes), the relative values of each parameter as measured after one day of incubation. ▨ (hatched boxes), the relative parameters after 7 days of incubation at 37°C or 28 days at 4°C.

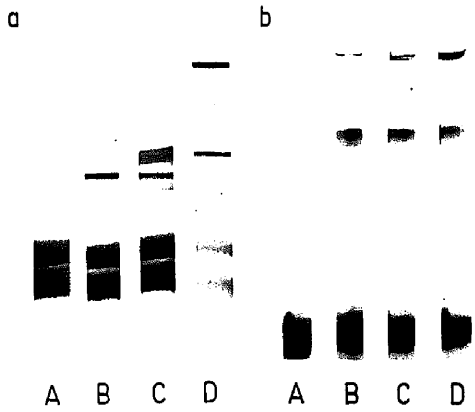

Fig. 2. Peroxidative crosslinking of cytoskeletal proteins; dependency on time of preincubation with hemin. Reaction conditions: incubation temperature 37°C; solutions buffered with 0.1 M phosphate buffer (pH 7.2); H_2O_2 1.8 mM; hemin 1 μM; protein concentration 5.5 μM. The reaction was started by addition of H_2O_2 and incubated for 70 min. The reaction was terminated by addition of a boiled SDS-containing sample buffer. (a) Reactions with spectrin. Lane A, controls containing protein only at time zero or after 24 h incubation and reaction mixtures containing protein with either hemin or H_2O_2. Lane B, preincubation with hemin for 5 min to 6 h. Lane C, preincubation with hemin for 12 h. Lane D, preincubation with hemin for 24 h. (b) Reactions with protein 4.1. Lane A, controls containing protein only at time zero or after 24 h incubation and reaction mixtures containing protein with either hemin or H_2O_2. Lane B, preincubation with hemin for 5 min. Lane C, preincubation with hemin for 60 min. Lane D, preincubation with hemin for 6 h.

conditions (Fig. 2), after 24 h of preincubation most of the spectrin monomers were crosslinked in very high aggregates, appearing in the gel origin. In the case of protein 4.1 (Fig. 2b), crosslinked material including very high aggregates appeared immediately after formation of the hemo-protein 4.1 complex (compare Fig. 2b, lanes A and B). Propagation of the reaction was noticed already in the first six hours of preincubation where about half the material was consumed (compare Fig. 2b, lanes A and B). Comparison of Fig. 2a, lane B and Fig. 2b, lane D illustrates that in addition to protein 4.1 being more sensitive than spectrin to the time of preincubation with hemin, it tends to form very high aggregates, leaving the amount of transient high molecular aggregates minimal. From the amount of material missing in the monomeric position in Fig. 2b lane D, it appears that some of the aggregates were too large to even penetrate the gel origin.

The time-dependent growing tendency of both spectrin and protein 4.1 to undergo crosslinking may reside in changes of the proteins backbone. Indeed, hemin-mediated time-dependent changes in the secondary structure of cytoskeletal proteins was already observed in our laboratory [22]. Hemin added to actin and spectrin at the same concentration range as in the present study induced a gradual loss of α-helix content of the proteins [22]. Since both spectrin and actin are highly ordered proteins, actin being a combination of α-helix and β-pleated sheet [23] and spectrin mostly α-helix [24], it is logical that changes induced by bound hemin would affect their secondary structure. The finding that hemo-protein 4.1 undergoes peroxidative crosslinking in a time-dependent manner indicates that this protein undergoes hemin-mediated time-dependent conformational changes as well. It should be mentioned that, unlike spectrin and actin, these conformational changes were not reflected in the far UV CD spectrum of protein 4.1: this is not surprising in view of the fact that only approx. 17% of the sequence of protein 4.1 exists in a α-helix form, and non as β-pleated sheet [25].

That time-dependent changes in cytoskeletal proteins upon hemin binding accelerate the hemin-induced peroxidative crosslinking may be a crucial event in situ, namely in integer membranes, as well. Indeed, in a recent paper we were able to demonstrate that detainment of hemin in red cell membranes results in increased efficiency of cytoskeletal proteins peroxidative crosslinking [21].

3. Is hemin binding to cytoskeletal proteins a reversible process?

The serum hemin-binding proteins play a major role in clearance of hemin from the membrane [3,7,8]. As time seems to be the crucial factor in hemin inflicted changes to the red cell, it was considered important to determine whether protein changes affect the potential of the serum proteins to clear the membrane of hemin associated with the cytoskeleton.

Spectrin, actin and protein 4.1 were isolated and hemin added in reduced stoichiometry to minimize free hemin in the mixture. The hemo-proteins were preincubated at 37°C under sterile conditions, and albumin or hemopexin added at set time intervals, at concentrations expected by mass action to transfer hemin entirely to the added protein. Following further incubation, the spectrum of the mixture in the Soret region was recorded for analysis of hemin dissociation from each cytoskeletal protein.

3.1. Actin-bound hemin

Hemo-actin was formed at 37°C by addition of hemin to actin (see figure legends for details). Excess albumin was added to samples withdrawn at time intervals, and the reaction mixture further incubated for 1 h at 37°C, at which time absorbance was recorded. For comparison, record was also made of the spectra of the hemo-actin alone and hemo-albumin expected to be formed by full transfer of the heme from actin to albumin. Fig. 3a illustrates that addition of albumin to hemo-actin immediately after formation resulted in a complete transfer of hemin from actin to albumin.

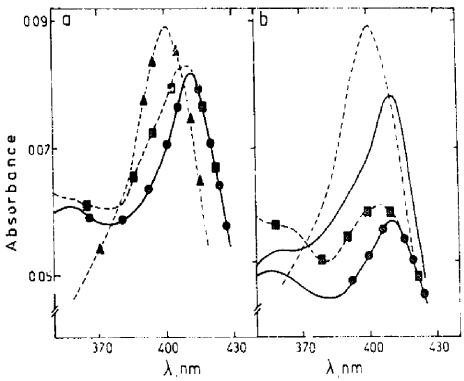

Fig. 3. Actin and protein 4.1 lose their ability to release bound hemin with time. Hemin was added to each protein and formation of a complex assessed by the characteristic spectrum in the Soret region. The complex was incubated at 37°C and albumin added at time intervals (5 min is reffered to as time zero). The spectrum of the mixture was recorded after 1 h. (a) Actin. Reaction conditions: actin concentration, 17 μM; human serum albumin (HSA) concentration 1.5 μM; hemin concentration 1 μM; solutions buffered with 5 mM Tris (pH 7.3). ———, hemo-actin at time zero. ●———●, hemo-actin after 24 h after formation. - - - - - -, hemo-albumin at time zero or 24 h after formation. ▲--▲, hemo-actin to which albumin was added at time zero. ■- -■, hemo-actin to which albumin was added after 24 h. The data represent an average of three experiments. (b) Protein 4.1. Reaction conditions: protein 4.1 concentration 4.6 μM; HSA concentration 2 μM; hemin concentration 1 μM; solutions buffered with 50 mM phosphate (pH 7.3). ———, hemo-protein 4.1 at time zero. ●———●, hemo-protein 4.1 6 h after formation. - - - - - -, hemo-albumin at time zero or 24 h after formation. ■- -■, hemo-protein 4.1 to which albumin was added after 6 h. The data represent an average of three experiments.

However, as indicated from the Soret spectrum, 24 h later, while the hemo-actin was unchanged, addition of albumin resulted in only a partial transfer of hemin to albumin. From a comparison of absorption at 403 nm (the Soret maximum of hemo-albumin) of the reaction mixtures containing fresh and '24-h-old hemo-actin', it was estimated that only about 40% of the hemin could transfer to the albumin, while the rest was undissociable.

3.2. Protein 4.1 bound hemin

Similar experiments were carried out with protein 4.1. Hemo-protein 4.1 was incubated at 37°C, excess albumin was added to reaction mixtures and withdrawn immediately or after increasing time intervals. Spectra of the hemo-protein 4.1 complex alone and after incubation with albumin were recorded and compared to hemo-albumin as in 3.1. As illustrated in Fig. 3b, time-dependent changes occurred in the hemo-protein 4.1 complex itself. As incubation time increased, the absorption at shorter wavelength due to the contribution of light scattering and a reduction in the Soret peak were observed. The reduced absorption can be attributed to loss of light due to scatter but contribution from changes in the hemo-protein 4.1 complex is possible. An example of the spectrum of hemo-protein 4.1 after six hours is illustrated in Fig. 3b. Here protein 4.1 differs from actin, where no changes in the complex spectral characteristics could be observed during incubation periods up to 24 h (Fig. 3a). In the presence of HSA the ability of hemin to dissociate from protein 4.1 depended on the duration of incubation of the hemo-4.1 complex, but loss of ability to dissociate hemin from the complex was faster than in the hemo-actin case. Addition of albumin to hemo-4.1 immediately after formation induced transfer of most of the hemin to albumin (not shown). Since additional absorption at 403 nm resulted from light scatter developed during incubation, it was difficult to calculate the amount of hemin transferred to albumin; however, even when its contribution is ignored, only 50% of hemin was estimated to dissociate after 6 h of preincubation. From the above it was concluded that hemin-bound protein 4.1 undergoes conformational changes and concomitant aggregation, and the ability of hemin to dissociate from this material is reduced.

3.3 Spectrin-bound hemin

Hemo-spectrin was prepared and incubated at 37°C and the spectrum of the complex periodically measured. Within 24 h of incubation, unlike protein 4.1 and similar to actin, the spectrum of hemo-spectrin was unchanged. The spectra of samples taken from the reaction mixtures after incubation with albumin are illustrated in Fig. 4. It is seen that the amount of hemin transferred to albumin was reduced with time of hemo-spectrin preincubation: 84% after 2 h, 60% after 4 h, and 45% after 6 h were dissociable. After 24 h no transfer of hemin from the hemo-spectrin complex could be achieved. It is noteworthy that loss of dissociabilty in the hemo-specrin complex explains the difficulties in analyzing the isotherms of hemin binding to spectrin reported by Gratzer and co-workers [26].

In summary: All three cytoskeletal proteins undergo slow denaturation when associated with hemin, which is hardly dissociated from the denatured protein complexes. protein 4.1 that showed no changes in its helix region appears to be the most sensitive protein. The fact that with spectrin and actin, unlike protein 4.1, no light scatter followed the protein conformational changes shows that aggregation, if any, is limited to small size aggregates. The large aggregates or precipitates formed by protein 4.1 are probably the cause of accelerated covalent crosslinking demonstrated in hemin-induced peroxidation (section 3.2). Thus, in general, association of cytoskeletal proteins with hemin could actually be treated as equilibrium systems only shortly after exposure to hemin. Since time is the important factor in this system, the efficiency of hemin efflux out of the

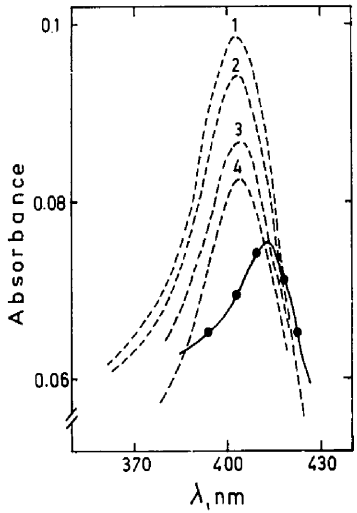

Fig. 4. Reversibility of the hemo-spectrin complex: time dependency. Reaction conditions: spectrin concentration 4 μM; albumin concentration 1.5 μM; hemin concentration 1 μM; solutions buffered with 50 mM phosphate (pH 7.3); Incubation temperature 37°C. Hemin was added to a spectrin solution to form hemo-spectrin. Aliquotes of albumin were added at time intervals (5 min will be reffered to as time zero) and the spectrum of the mixture recorded after 1 h. ———, hemo-spectrin at time zero and 24 h after formation. 1, hemo-albumin at time zero and 24 h after formation, and hemo-spectrin to which albumin was added at time zero. 2, hemo-spectrin to which albumin was added after 2 h. 3, hemo-spectrin to which albumin was added after 4 h. 4, hemo-spectrin to which albumin was added after 6 h. ●———●, hemo-spectrin to which albumin was added after 24 h. The data represent an average of three experiments.

membrane is the dominating factor in hemin-induced membrane changes.

4. The time-dependent ability of hemin to dissociate from its physiological carrier, albumin

Albumin is used physiologically as the hemin trap in selective transport of circulating free hemin in the serum, and in the next step hemin transfers to hemopexin, the vehicle for transportation to final degradation in the liver [27,28]. The fact that albumin-bound hemin can be completely transferred to hemopexin has already been documented, but the time-dependent stability of the hemo-albumin complex is unknown. Having demonstrated that the cytoskeletal proteins lose their ability to release hemin, it was of interest to determine whether this malfunction is indeed avoided in a protein that should function as a hemin reservoir. We therefore compared the ability of hemopexin to withdraw hemin from hemo-albumin and hemo-spectrin incubated up to 24 h at 37°C.

Reactions were carried out in the same manner as described above except that the hemin scavenging protein, heamopexin, was incubated with each hemo-protein for 24 h to allow complete transfer of all dissociable hemin. The results, illustrated as spectra of the reaction mixtures in the Soret region, are shown in Fig. 5. As seen, immediately after formation of the hemoprotein complexes, hemin could completely transfer from both albumin and spectrin to hemopexin. However, 24 h later, in correlation with the same experiments using albumin as a trap, hemin could no longer dissociate from hemo-spectrin. In contrast, albumin's ability to deliver hemin to hemopexin was conserved even after 24 h. The difference between albumin and the cytoskeletal proteins may reside in the fact that albumin provides a built-in hydrophobic cavity as a site for hemin, while cytoskeletal proteins probably associate hemin via semi-hydrophobic regions on their surface and as a result lose their stability.

5. The state of spectrin in vivo, correlation to the findings in vitro

We have previously demonstrated higher detectable levels of hemin in old circulating cells as compared to

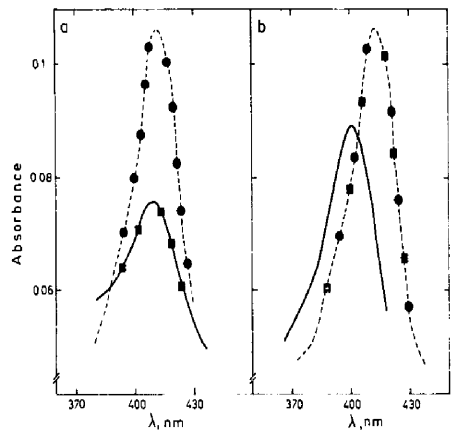

Fig. 5. Dissociation of hemin from hemo-spectrin and hemo-albumin. Reaction conditions; Concentration of proteins; spectrin 4 μM dimers; albumin 1.5 μM; hemopexin 1.7 μM; hemin concentration 1 μM; solutions buffered with 50 mM phosphate (pH 7.3); incubation temperature 37°C. Hemin was added to spectrin or albumin to form hemo-spectrin or hemo-albumin, respectively. Each hemoprotein was incubated for either 5 min (which will be reffered to as time zero) or 24 h at 37°C before adding hemopexin. The absorption spectrum in the Soret region was recorded 24 h after addition of hemopexin. (a) Hemo-spectrin. ———, hemo-spectrin at time zero or 24 h after formation. – – – – – –, heme-hemopexin at time zero or 24 h after formation. ●– –●, hemo-spectrin to which hemopexin was added at time zero. ■———■, hemo-spectrin to which hemopexin was added after 24 h. (b) Hemo-albumin. ———, hemo-albumin at time zero or 24 h after formation. – – – – – –, heme-hemopexin at time zero or 24 h after formation. ●– –●, hemo-albumin to which hemopexin was added at time zero. ■– –■, hemo-albumin to which hemopexin was added after 24 h. The data represent an average of three experiments.

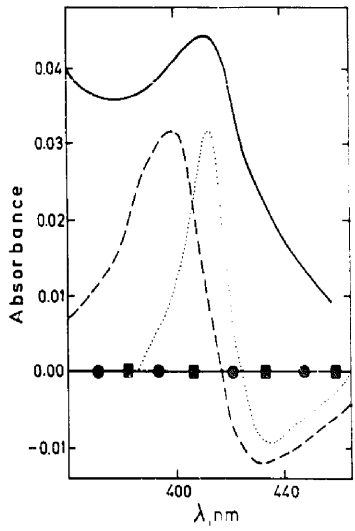

Fig. 6. Characterization of the in vivo hemo-spectrin. Reaction conditions: Proteins concentration: spectrin 7 μM; hemopexin 2.5 μM; albumin 2.5–75 μM; hemin concentration 0.4 μM; solutions buffered with 50 mM phosphate (pH 7.3); incubation temperature 37°C. Difference spectra of hemin bound to spectrin and hemin bound to either albumin or hemopexin was recorded in the Soret region. Tendom (two compartment) cuvettes were used to cancel light scattering contributions derived from proteins at high concentrations. In the reference cuvette, one compartment contained hemo-spectrin and the other contained albumin or hemopexin. In the sample cuvette each compartment contained the mixture of hemo-spectrin and albumin or hemopexin. ———, spectrum of isolated spectrin from in vivo circulating cells in the Soret region. Note the curved baseline resulting from the contribution of the 280 nm protein band(not shown). ●– –●, difference spectrum generated by the addition of 2.5–75 μM albumin to in vivo hemo-spectrin. ■———■, difference spectrum generated by the addition of 2.5 μM hemopexin to in vivo hemo-spectrin. – – – – –, difference spectrum generated by the addition of albumin to freshly prepared hemo-spectrin (5 min after formation). ....., difference spectrum generated by the addition of hemopexin to freshly prepared hemo-spectrin (5 min after formation). The difference spectrum was recorded after 24 h incubation of albumin or hemopexin with hemo-spectrin.

young [3]. The results of the current study show that cytoskeletal proteins complexed in vitro with hemin lose their tendency to release bound hemin with time. Based on the above it was reasoned that accumulation of irreversible bound hemin occur in vivo as well. It was therefore decided to analyze whether cytoskeletal proteins extracted from circulating cells contain bound hemin. Spectrin was chosen as the representative protein based on its large quantity in the membrane to allow best spectral detection of residual hemin. The protein was extracted and purified from normal red cells and its absorption spectrum recorded in the Soret region as demonstrated in Fig. 6. As seen, the purified spectrin indeed has some absorption at the Soret band which is typical to hemo-spectrin. That existence of hemo-spectrin in circulating normal RBCs is a general phenomenon was validated in 10 blood samples derived from healthy donors. From the concentration of spectrin (determined by the Lowry method or by absorption at 280 nm) and based on the estimation of 20 heme sites per spectrin dimer [26], the average fraction of saturated heme sites in circulating spectrin was calculated to be 5 ± 2 percent. Since the collection of circulating cells represents a total spectrum of the erythrocytes age, this averaged number possibly demonstrates a higher fraction of heme saturation in older as compared to younger cells.

The ability of the in vivo formed hemo-spectrin complex to release its bound hemin was examined by addition of the physiologically hemin scavengers albumin and hemopexin. Samples of spectrin solution containing in vivo formed hemo-spectrin were incubated for 24 h at 37°C with albumin (2.5–75 μM) or hemopexin (2.5 μM), and the transfer of hemin from spectrin to the appropriate protein was measured by difference spectra at the Soret region. Fig. 6 shows the expected difference spectra considering that all hemin in hemo-spectrin had been transferred to albumin or hemopexin. However, the in vivo isolated hemo-spectrin failed to release any of its hemin in the presence of hemopexin or albumin (up to 75 μM).

In summary: The results of the current study demonstrate that globin-free hemin intercalated in the erythrocyte membrane for long periods become toxic by inducing irreversible structural changes in the key cytoskeletal proteins. Prolonged association of hemin with the membrane is expected under conditions of delayed clearance. Unlike the serum, where albumin serves as primary free hemin scavenger and hemopexin as the transporter, for membrane intercalated hemin hemopexin was found to be the direct scavenger [8]. Since hemopexin is liquidated in the process of hemin catabolism, its amount in the serum (which is normaly low) may reduce to near zero in severe hemolytic anemias [29]. In the serum, under such conditions the bulk of albumin serves to maintain the extra hemin in a nonactive form, but the red cell membrane is no longer protected. Thus, accumulation of hemin in the membrane seems to be the major event in limitation of the erythrocyte life span in hemolytic anemias.

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