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Serum proteins as mediators of hemin efflux from red cell membranes: specificity of hemopexin

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The involvement of the serum heme-binding proteins hemopexin and albumin in the clearance of erythrocyte membranes from toxic hemin was compared. In the presence of hemopexin initial rates of hemin efflux from resealed ghosts were faster and the amount of extracted hemin larger. When hemin-containing ghosts were treated with a protein mixture of 1:45 hemopexin to albumin, as present in serum, most of the hemin was extracted in the form of heme-hemopexin. It was concluded that hemopexin is the serum protein responsible for heme extraction from cell membranes.

Hemin; Hemopexin; Albumin; Erythrocyte membrane

1. INTRODUCTION

In states of increased hemolysis, in which extracorpuscular hemoglobin in the plasma exceeds the binding capacity of haptoglobin for hemoglobin, hemin released from hemoglobin is bound to plasma proteins and eventually degraded in the liver [1]. Hemin added in vitro to serum associates first with albumin and is then slowly transferred to hemopexin [2]. The precise role of these two serum proteins in the delivery of circulating hemin to hepatocytes is currently unclarified. While uptake of hemin bound to hemopexin by receptor-mediated endocytosis has been documented [3], it was recently recognized that only albumin facilitates entry of heme into cultured primary rat hepatocytes [4].

By interacting with lipids as well as structural proteins or enzymes, hemin becomes toxic to red cell membranes [5–7]. Most of the hemin embedded in red cell membranes, is expected to be released as long as the erythrocyte circulates. A likely mechanism for hemin release from membranes invokes participation of serum heme binding proteins. Indeed, protein-mediated heme efflux from artificial phospholipid vesicles was demonstrated by kinetic studies [8,9]. Albumin, the major protein in the serum, was shown to facilitate hemin exocytosis from red cell membranes although the yield of extracted hemin was lower than that found in liposomes [10].

The kinetics of heme transport through membranes should not depend on the protein used as a trap, since the rate limiting step in heme efflux is heme transport within the membrane. In support of this view, the kinetic parameters of heme efflux from liposomes induced by hemopexin and albumin were the same, yet the extent of heme efflux in the rapid phase was larger in hemopexin than in albumin [8,9]. In the present study we aimed to clarify the role of albumin and hemopexin in the clearance of hemin from erythrocyte membranes.

2. EXPERIMENTAL

All chemicals were of analytical grade. Human fresh blood was obtained from healthy donors and resealed erythrocyte ghosts were prepared as previously described [10].

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2.1. *Labeled membranes*

Dansyl was used as a covalently bound stable probe as previously described [10]. In brief, ghosts were dansylated by incubation in 5 mM bicarbonate containing 0.5 mM dansyl chloride for 40 min at 0°C (ice-water slash). To avoid non-specific adsorption of the dye, labeled ghosts were resuspended in bicarbonate containing 50 mM lysine and maintained on ice for an additional 30 min before the final wash.

2.2. *Resealing*

Ghosts (labeled or unlabeled) were resealed by dilution into 9 vols of isotonic buffer, pH 7.3, which contained 5 mM phosphate, 145 mM NaCl, 0.2 mM $CaCl_2$ and 2 mM $MgCl_2$ and were kept at 37°C for 1 h.

2.3. *Proteins*

Hemopexin was prepared as described [11] from human serum of healthy individuals and its concentration was determined spectrophotometrically (E_{mM} = 185) at 280 nm. Human albumin and albumin free of globulin or fatty acid were purchased from Sigma; they gave identical results. Total protein in membrane preparations was determined by the method of Lowry using BSA as a standard [12].

2.4. *Hemin*

Stock hemin solutions were made on the day of the experiment by dissolving it into 20 mM NaOH to make 1.0–1.7 mM solution and was kept on ice. Hemin concentration was measured in NaOH using an E_{mM} at 385 nm of 58.5 [9].

3. RESULTS AND DISCUSSION

3.1. *Hemin efflux from RBC membranes. Initial rates*

The fluorescence intensity of dansylated resealed membranes was monitored before and after addition of hemin. Reduction in fluorescence intensity upon addition of hemin at very low concentrations was indicative of radiationless energy transfer resulting from binding of hemin to membranes [13]. The membranes with intercalated hemin were incubated for 60 min at 37°C and then exposed to albumin or hemopexin. Addition of each of the above proteins resulted in a time-dependent restoration of fluorescence intensity due to release of hemin, the acceptor of fluorescence energy, from the membrane compartment. Fig.1 compares the initial time course of fluorescence recovery upon addition of albumin or hemopexin. It appears that the rate of hemin efflux from red cell membranes depends on the heme binding protein involved, hemopexin causing a faster efflux of hemin than albumin. Since fluorescence changes are not readily translated into quantitative data, the yield of heme efflux was further assessed by employing additional techniques.

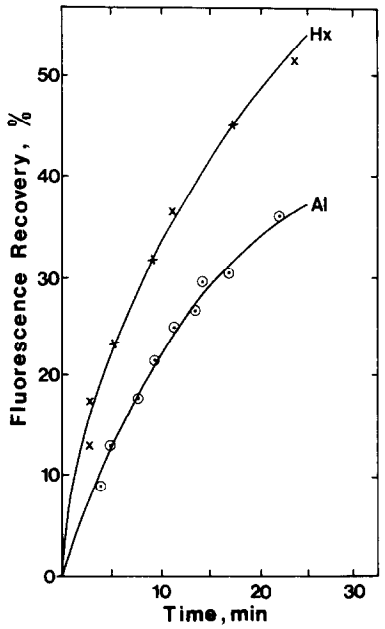

Fig.1. Initial rates of hemin efflux as measured by monitoring fluorescence intensities. Rate of hemin efflux from RBC membranes induced by albumin and hemopexin. Isotonic buffer pH 7.3, 37°C. Concentrations: hemin, 0.2 μM; membranes, 9.2 μg protein/ml; albumin, 1.6 μM; hemopexin, 1.67 μM. The intensity of fluorescence quenched was set as 100% and the fraction of quenched fluorescence that was recovered was expressed accordingly. Each symbol represents the mean of three experiments. (⊙—⊙) In the presence of albumin; (×—×) in the presence of hemopexin.

3.2. *Relative efficiency of the proteins as traps for membrane intercalated hemin*

Hemin was equilibrated at 37°C with red cell membranes for 5 (short-term) or 120 (long-term) min. In the short incubation period hemin resides mainly in the outer layer while after 120 min at 37°C hemin equilibrates in the two membrane bilayers reaching even the cytoskeleton (unpublished results). At this stage, albumin or hemopexin in an amount sufficient to bind all hemin in the membrane was added and the reaction mixtures were further incubated for 5 or 120 min. The membrane suspensions were immediately centrifuged for 10 min at 45000 × *g* in the cold (2°C) and the absorption of the supernatant at the heme-protein Soret maximum determined. Based on these measurements the fraction of membrane dissociated hemin was calculated. The results as summarized in fig.2 show the following: more than

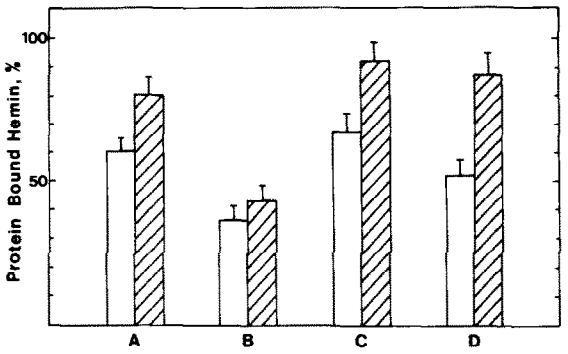

Fig.2. Recovery of membrane-associated hemin in a protein bound form. Empty columns, as heme-albumin; hatched columns, as heme-hemopexin. Isotonic buffer, pH 7.3, 37°C. Concentrations: hemin, 1 μM; membranes, 30 μM protein/ml; albumin or hemopexin, 1.05 μM. (A) 5 min incubation with hemin and an additional 5 min incubation with protein. (B) 120 min incubation with hemin and an additional 5 min incubation with protein. (C) 5 min incubation with hemin and an additional 120 min incubation with protein. (D) 120 min incubation with hemin and an additional 120 min incubation with protein.

50% of hemin in the outer layer (short term incubations, A,C) was already recovered from the membrane after 5 min and a small additional amount after 120 min (C,D). In each case, the efficiency of hemopexin was higher than that of albumin. Column C demonstrates that within experimental error hemopexin induced complete extraction of membraneous hemin after 120 min. The amount of hemin extracted by albumin after 120 min was only 70% (compare A,C). When hemin was embedded in the inner as well as in the outer bilayer (results of columns B,D), both proteins (hemopexin being somewhat more efficient) extracted a smaller portion of hemin after 5 min (B), indicating that hemin from the inner bilayer had not reached the membrane surface. Two hours after the proteins were added, additional hemin was extracted from the membrane by both proteins, but the fraction of hemin extracted by hemopexin was much larger (compare B,D). The small fraction of hemin that remains in the membrane in the presence of hemopexin could not even be extracted by up to 4 h incubation and may correlate with degraded or irreversibly protein-bound hemin. Unlike the conditions found in serum, where the molarity of albumin is about 45-fold that of hemopexin, in the experiments presented in figs 1 and 2 equal concentrations for both proteins were used. Subsequent experiments were performed with increased albumin concentrations, and the results are shown in the upper three lines of table 1. No increase was found in the fraction of hemin delivered to albumin at 20 μM and 50 μM as compared to 1.1 μM.

3.3. *Hemin efflux induced by protein mixtures*

To test whether the difference in heme release from the membrane by the two heme-binding proteins has physiological significance, a series of experiments were performed for the long-term incubation period (fig.2D). Resealed ghosts were incubated for 120 min with 1 μM hemin and an additional 120 min with the protein mixture containing 1.1 μM hemopexin and increasing amounts of albumin. The fraction of each heme-protein was calculated from the absorption observed in the supernatants at 403 nm and 413 nm [2]. Under these conditions heme-protein complexes in the supernatant were entirely in the form of heme-hemopexin.

The results in table 1 show that (1) in the absence of hemopexin, the concentration of albumin did not influence the amount of hemin that could be extracted from the membranes under all conditions employed, and (2) in the presence of hemopexin albumin at any concentration had no effect on the extractable hemin. As hemin is readily transported from albumin to hemopexin [2], these results may be interpreted as demonstrating either a direct

Table 1

Percent of hemin extracted from red cell membranes incubated with different mixtures of hemopexin (Hx) and albumin (Al)

Protein (μM)		A[a]	B	C	D
Hx	Al				
0	1.1	60	36	67	51
0	20.0	65	37	70	47
0	50.0	64	35	72	47
1.1	0	n.d.	n.d.	n.d.	87
1.1	1.1	n.d.	n.d.	n.d.	84
1.1	20.0	n.d.	n.d.	n.d.	83
1.1	50.0	n.d.	n.d.	n.d.	76

[a] For incubation periods under each letter see legend to fig.2

Concentrations: membranes, 50 μM protein/ml; hemin, 1.0 μM. Temperature 37°C. Each value represents the mean of three experiments

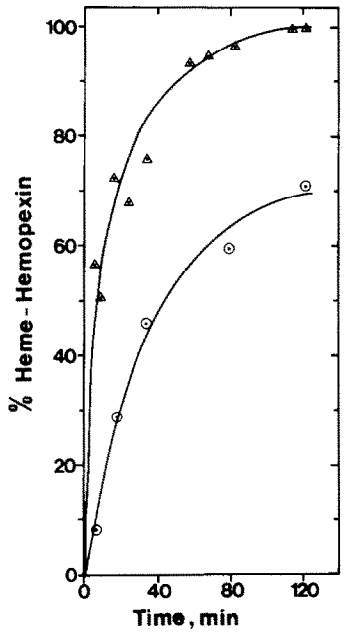

Fig.3. Time course of hemin transfer to hemopexin: comparison to heme-albumin and heme-membranes. The interacting species were kept in isotonic pH 7.3 phosphate buffer and the solution was thermostatted at 37°C. Concentrations: 50 μM membrane protein/ml; 1 μM hemin; 1.1 μM hemopexin and 50 μM albumin. The fraction of hemopexin in percent of total heme-associated protein was calculated from the molar extinction coefficients of both proteins at 413 nm (hemopexin) and 403 nm (albumin). (△) Heme transfer from red cell membranes. The reference cuvette contained a filtered solution with the apoprotein mixtures. The sample cuvette contained the supernatant solution at the indicated times after addition of the proteins. Each symbol represents the mean of three experiments. (○) Heme transfer from heme-albumin was obtained by incubation (60 min) of 1 μM hemin with 50 μM albumin. At time zero, an aliquot of hemopexin was added to a final concentration of 1.1 μM. At time intervals the absorbance at 413 nm and 403 nm was measured and the percent of heme-hemopexin determined. The reference cuvette contained a mixture of the apoproteins. Each symbol represents the mean of three experiments.

transfer of membranous hemin to hemopexin or, indirectly, via a heme-albumin complex.

To distinguish the route by which membrane hemin reaches hemopexin, the time-dependent pattern of hemin efflux was studied. Membranes were preincubated with hemin for 120 min and then a mixture of 50 μM albumin and 1.1 μM hemopexin was added, designated as zero time. At timed intervals aliquots were filtered directly into a cuvette and the absorbance at 403 nm and 413 nm determined. For comparison, the rate of heme transfer from heme-albumin to hemopexin was measured without the presence of membranes. 1 μM hemin and 50 μM albumin were preincubated for 60 min and 1.1 μM hemopexin was added at zero time. Absorption was again measured at time intervals and the fraction of heme-hemopexin was determined. Fig.3 (upper curve) shows that after a 10 min incubation of membranes and proteins most of the heme-protein (~70%) in solution was already in the form of heme-hemopexin. Based on the large difference in heme binding affinity between albumin (k_D 10^{-8} M) and hemopexin (k_D 10^{-13} M) [11], heme that had reached the cell surface could have first been bound by albumin and then by hemopexin. However, the data in the lower curve in fig.3 indicate that within 10 min only a negligible amount of heme could have been transferred to hemopexin via heme-albumin. Thus, heme-hemopexin could only have been formed by direct interaction of extracorpuscular hemin and hemopexin.

The inefficiency of albumin to trap heme may be explained by the fact that it is not easily detached from phospholipid membrane surfaces [14,15]. Heme-albumin may remain with the surface much longer than non-specifically attached heme-hemopexin molecules. This difference in the ability of the two proteins to accept heme from membranes could not be demonstrated in previous studies employing fast kinetics [8,9].

In summary: this study shows that despite the much smaller amount in serum, as compared to albumin, hemopexin is an efficient vehicle for draining toxic hemin from cell membranes. Albumin, on the other hand, appears not to be required as a mediator in the transport of membrane intercalated hemin to the aqueous milieu.

[1] Ferriprotoporhyrin IX is used interchangeably as hemin and heme.

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