Biochemical Pharmacology, Vol. 36, No. 22, pp. 3801–3807, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

GLUTATHIONE AS A SCAVENGER OF FREE HEMIN

A MECHANISM OF PREVENTING RED CELL MEMBRANE DAMAGE

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(*Received* 3 *February* 1987; *accepted* 8 *May* 1987)

Abstract—The interaction of glutathione and hemin was studied at physiological ionic strength and pH conditions. Formation of a glutathione–hemin complex was assessed from the appearance of spectral changes in the visible region. In the presence of excess cyanide and histidine and upon oxidation of the sulfhydryl group, no complex was formed. From these results it was concluded that the thiol group of glutathione serves as a ligand for the heme iron. A binding constant of $3.1(\pm 0.1) \times 10^4\,M^{-1}$ was calculated by use of a Hill plot. The hemolytic effect of hemin on red cell was much reduced in the presence of glutathione. Since hemolysis results from association of hemin with membrane components its binding in the presence and absence of glutathione was studied. It was found that the affinity of hemin for the cytoskeletal membrane proteins as well as for the membrane lipid core was decreased in the presence of glutathione. It was concluded that glutathione competes with the membranes for hemin and by doing so can defend the membrane from injury by hemin.

Unstable hemoglobins which tend to release hemin are involved in various hemolytic anemias [1–3]. Hemin has been shown by us and others to be hazardous to the cell membrane [4–6]. It tends to intercalate into the membrane lipid bilayer as well as bind to membrane proteins [7–10]. These interactions may result in disruption of the cytoskeletal network and cause membrane changes which will finally lead to the hemolysis observed in some anemias [5, 11]. It therefore seems that normal circulating red cells should avoid hemin retention by keeping its steady state level in the membrane as low as possible. One way of doing so is to trap uncommitted hemin by a cytosolic chelating agent and thus prevent hemin load in the membrane whenever a burst of globin released hemin occurs. Enzymes that by binding hemin may serve as hemin traps are the wrong candidates since they exist in small quantities and by binding hemin are inactivated [12, 13]. Thus, it would be expected that the red cell cytosolic system should normally avoid binding of hemin to enzymes by providing an alternative trap for free hemin.

One of the essential metabolites being constantly reproduced in the mature red cell is glutathione which plays a critical role in various detoxification reactions [14]. GSH† serves as a reducing agent responsible for defence of the cell against reactive intermediates such as radicals and peroxides or other electrophiles like quinones and cations. Hemin being a cation as well as an electrophile has the potential to interact with glutathione. Thus, GSH may be suitable to serve as the desired scavenger for hemin in the red cell cytosol.

The objectives of the present study were first to analyze the complex of hemin and glutathione and to determine their binding affinity in order to predict a physiological association of the above participants. Secondly, we studied the possible role of glutathione in preventing damaging interactions of hemin with various membrane components which may ultimately lead to hemolysis.

MATERIALS AND METHODS

Reagents were of analytical grade. GSH, GSSG and hemin were purchased from Sigma. AS was a product of Molecular Probe Inc. For preparation of various membrane constituents, blood bank samples stored up to 7 days were used. For hemolysis experiments fresh blood samples were drawn from healthy donors.

Preparation of complexed hemin. Hemin solutions were prepared prior to each experiment as previously described [5]. To keep the pH, hemin from a stock solution was diluted into a 0.14 M phosphate buffer and consequently the ligand was added to yield the desired concentration. For reduced hemin form the solution was kept under nitrogen and dithionite added to a concentration of 2 mg/ml.

Preparation of hemoglobin-free ghosts and cytoskeletal proteins. Hemoglobin-free ghosts were prepared essentially according to Dodge *et al.* [15], by hypotonic lysis 1:40 in 5 mM phosphate buffer at pH 8.0 as previously described [16]. Cytoskeletal proteins were separated from the membrane lipid core by diluting 1:3 the hemoglobin-free ghosts in 0.1 mM EDTA, 0.5 mM phosphate buffer at pH 8.0 and the mixture was incubated for 30 min at 37°. The

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† Abbreviations used: AS, 9-(1,2-anthroyl)stearic acid; CN, cyanide; GS–hemin, glutathione–hemin; GSH, reduced glutathione; GSSG, oxidized glutathione; hemin–CN, hemin–cyanide; hemin–His, hemin–histidine; His, histidine; RBC, red blood cell.

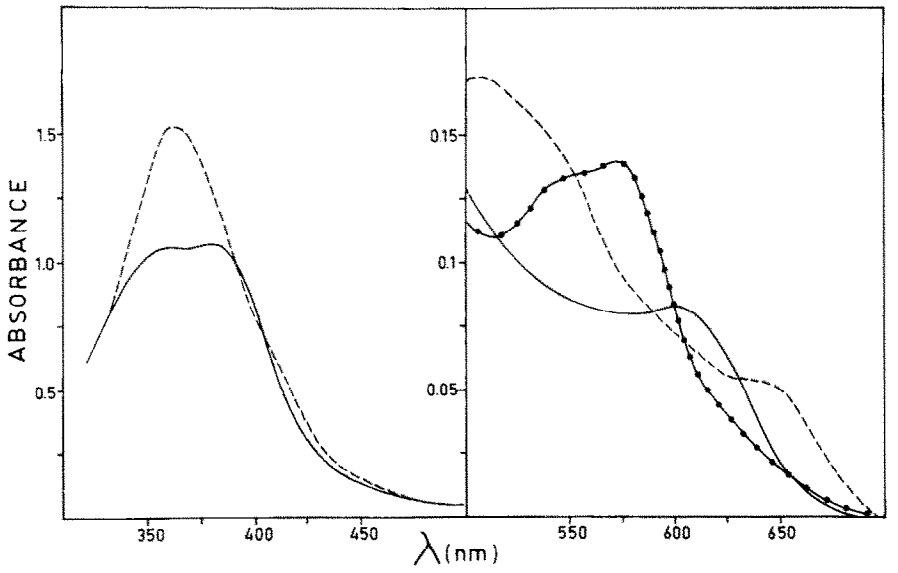

Fig. 1. Spectral features of hemin in the presence and absence of glutathione as compared to the reduced form. All hemin solutions were prepared in 0.14 M phosphate buffer pH 8.0, 20 μM hemin solution aerated or under nitrogen (——). The same hemin solution in the presence of 8.7 mM GSH either aerated or under nitrogen (– – –). Hemin in the presence of 2 mg/ml dithionite under nitrogen (■——■). (Since excess of dithionite contributes to the absorption below 450 nm, the last spectrum is shown in the visible region only.)

suspension was centrifuged at 43,000 *g* for 90 min and the supernatant was separated from the pellet. The concentration of cytoskeletal proteins was determined using an $E^{1\%}_{[280]} = 10$.

Lipid core preparation. For further separation of the proteins from the lipid core the pellet was rediluted in EDTA–phosphate solution and incubation over night at 4° and then centrifuged. The final pellet will be referred to as the membrane lipid core.

Preparation of AS labelled membranes. Membranes were labelled by addition of an aliquot of AS dissolved in ethanol to a ghost solution, in a 1:100 w/w ratio of probe to membrane lipid [16]. The sample was stirred well and incubated for 30 min at 37°. The excess probe was removed by washing twice with the appropriate experimental solution.

RESULTS

Interaction of glutathione with hemin

When water soluble hemin was incubated with glutathione spectral changes were observed similar to those reported previously by Silver and Lukas [17]. The interaction between glutathione and hemin was pH independent in the range of 7–8.5. In Fig. 1 the spectrum of hemin in the absence and presence of glutathione is demonstrated. Figure 1 shows the typical hemin spectrum in the soret (left) and visible (right) bands. Upon addition of glutathione a shift of the peak to the blue is observed and followed by an increase of the maximal extinction coefficient. Typical spectral changes were also observed in the visible region. To analyze the nature of the product we compared the spectrum of hemin–glutathione with that of dithionite reduced hemin. As seen in the visible region, the spectrum of hemin in the presence of glutathione is entirely different from the typical reduced heme form. The binding parameters were assessed from the spectral changes which resulted upon addition of GSH to hemin. GSH concentration of free GSH was considered the same as the concentration of its total. Binding data were analyzed by relating the difference in absorption at 365 nm to the fraction of hemin transformed into the GSH bound form. Figure 2 demonstrates a Hill plot of typical titration curve. It is seen that the binding is noncooperative (n = 1) and the association constant was calculated from five different experiments as $K_a = 3.1 \pm 0.01 \times 10^4\,M^{-1}$.

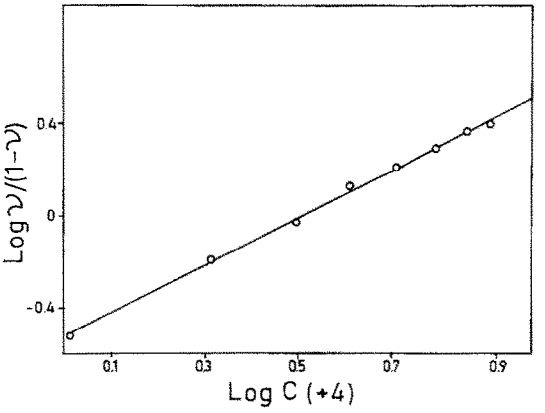

Fig. 2. Binding affinity of hemin and glutathione represented as a Hill plot. Buffer conditions: 0.14 M phosphate, pH 8.0, temperature 25°. Hemin concentration: 17.1 μM. Slope (n) = 1; $K_a = 3.1 \times 10^4\,M^{-1}$.

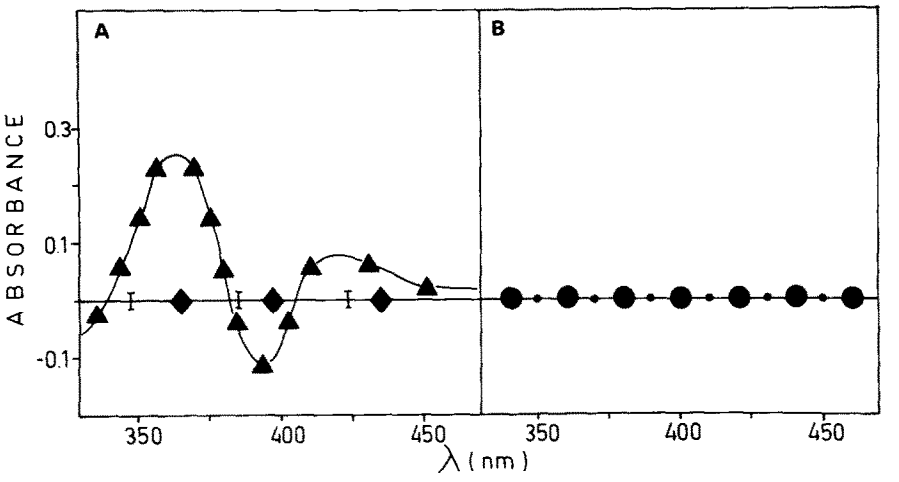

Fig. 3. Difference spectra of liganded hemin. (A) The reference cuvette contained hemin and the sample cuvette contained hemin in the presence of the following: no additions (I), GSH (▲), GSSG (◆). (B) hemin–CN in both cuvettes and GSH in the sample cuvette only (●). Hemin–His in both cuvettes and GSH in the sample cuvette only (•). Concentrations: hemin, 17.1 μM; GSH, 2 mM; GSSG, 2 mM; CN, 0.3 M and His, 0.5 M.

To characterize the hemin–glutathione complex, difference spectra of hemin and various possible ligands were analyzed. Figure 3A demonstrates a typical difference spectrum in the Soret of hemin in the absence and presence of glutathione. When GSH was replaced by its oxidized form, GSSG, no difference spectrum was observed. We further formed hemin complexed with either CN or His by incubation of hemin with a large excess of the above ligands. As shown in Fig. 3B, in the presence of CN and His no complex between hemin and GSH could be formed.

Effect of glutathione on the hemolysis of red cells induced by hemin

Since glutathione binds hemin and previous studies indicated that hemin by itself causes red cell hemolysis [4–6], the linkage of glutathione binding and the hemolytic effect of hemin was further studied. Fresh washed red cells were suspended in isotonic phosphate buffer at pH 7.3 to yield a 0.05% cell suspension. The cells were incubated in the dark at 37° and a defined volume of cell suspension was mixed with hemin solution in the absence or presence of an additional ligand. To keep all hemin complexed, ligand was added in a concentration which was shown to yield maximal absorption change. Final concentrations of interacting species were the following: hemin—10 μM, GSH—1 mM, GSSG—1 mM, CN—10 mM and His—54 mM. The cell suspension mixture was shaken for an additional 5 min and then centrifuged for 20 min at 43,000 *g*. The supernatants were collected and hemoglobin concentration was determined by measuring the absorbance at 578 nm (the visible peak of hemoglobin where minimal interference from liganded hemin exists). The percentage of hemolysis was determined by comparing the hemoglobin concentration with that of cells totally lysed in a hypotonic solution. The results (Table 1) demonstrated that hemin alone (described as OH ligand), brought about a much larger hemolysis than in the presence of GSH, CN and His. On the other hand, the oxidized form of GSH, GSSG, did not significantly prevent the lytic effect of hemin.

Association of hemin with red cell membrane

Hemin was shown to associate with membrane components [9–11]. To discover whether the reduced hemolysis relates to changes in the above associations, we analyzed the relative affinity of hemin and cell membrane components in the absence and presence of GSH. RBC ghosts were separated into cytoskeletal proteins and membrane lipid core by EDTA treatment, and the lipid core was labelled with the fluorescent probe AS [10]. The relative

Table 1. Hemolysis of intact erythrocytes induced by hemin in the presence of various ligands

Ligand	OH^-	GSH	GSSG	CN^-	His
Percent of hemolysis	62 ± 5	7 ± 0.5	56 ± 5	7 ± 2	2 ± 1

0.05% cell suspension in 0.14 M phosphate buffer pH 7.3. Temp 37°. Concentrations: hemin, 10 μM; GSH, 1 mM; GSSG, 1 mM; CN, 10 mM; His, 54 mM. The values represent an average of six different experiments.

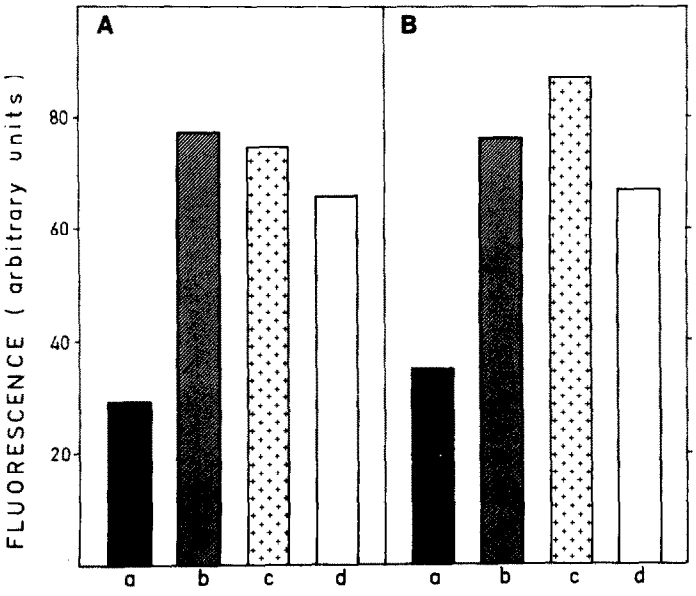

Fig. 4. Association of liganded hemin with red cell membrane components. (A) Membrane lipid core, (B) cytoskeletal proteins. Binding is assessed by fluorescence intensity set as 100% of either a AS probe for the lipid core or intrinsic for proteins. Isotonic phosphate buffer (0.14 M) was used at pH 8.0. Reactants concentrations; membranes $\sim 10^7$ cells/ml (1:600 dilution of packed lipid core membranes), cytoskeletal proteins 1.98×10^{-3} mg/ml and hemin 1.4 μM (each point is an average of 10 measurements). (a) No ligand added; (b) in the presence of 4 mM, GSH; (c) in the presence of 58 mM, His; (d) in the presence of 53 mM, CN. Excitation wavelength 360 nm for A and 280 nm for B. Emission wavelength 440 nm for A and 340 for B.

binding of hemin was assessed from fluorescence quenching data as described previously [16]. Hemin was mixed with excess GSH to form GS–hemin and this complex was added to AS labelled membrane lipid core or alternatively to the cytoskeletal proteins. The same experiments were performed with hemin complexed with either CN or His. As seen in Fig. 4A under the same conditions hemin alone could quench about 70% of the fluorescence intensity while GS–hemin, hemin–CN and hemin–His all quenched only a minor amount of the fluorescence intensity. These results imply that in the presence of GSH the affinity of the membrane lipid core for hemin has been reduced. Figure 4B demonstrates that the ability of the various hemin forms to quench the intrinsic fluoresence of the cytoskeletal proteins is affected in a similar manner.

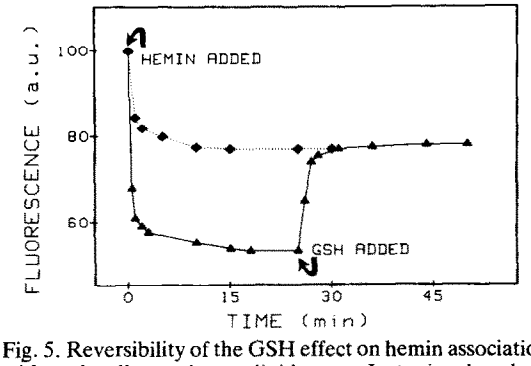

Fig. 5. Reversibility of the GSH effect on hemin association with red cell membrane lipid core. Isotonic phosphate (0.14 M) at pH 8.0 was used. —▲—▲—, GSH added after hemin addition; ···◆··◆···, GSH added before hemin addition. Each point represents an average of six measurements deviated from the reported value by 5%. Fluorimetric parameters as in Fig. 4A. Concentrations of reactants: hemin, 0.43 μM; GSH, 4 mM; cytoskeletal proteins, 3.23×10^{-3} mg/ml.

In order to find out whether the above changes are reversible, hemin with and without GSH was incubated with the membrane lipid core. After 25 min, where no further changes in fluorescence intensity could be observed, GSH was added to the sample lacking GSH and the fluorescence intensity monitored for 25 min. The results in Fig. 5 demonstrate that addition of GSH to the hemin associated lipid core was followed by a fast increase of the fluorescence intensity. Within 5 min the sample reached the same level of fluorescence intensity as the mixture preincubated with GSH, indicating that the process is reversible. A similar experiment carried out with a mixture of cytoskeletal proteins is demonstrated in Fig. 6. As depicted in these data, unlike the lipid core, by addition of GSH to a mixture of hemin and cytoskeletal proteins the sample failed to reach the fluorescence intensity of the GSH preincubated sample.

Further experiments were designed for two purposes: (a) to find out whether or not hemin associated with the membrane in the presence of GSH is complexed; (b) to determine the relative amount of hemin bound to the membrane under various conditions. Membrane lipid core was incubated with hemin in

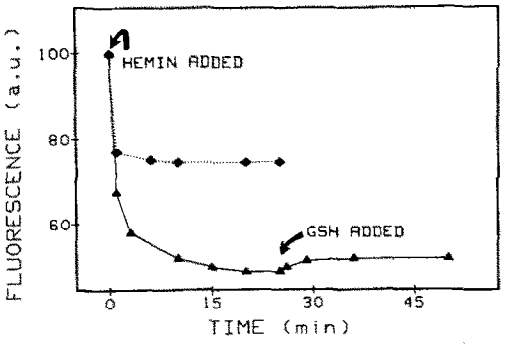

Fig. 6. Reversibility of GSH effect on hemin association with cytoskeletal proteins. Fluorimetric parameters as in Fig. 4B. Reactant concentrations: hemin and GSH as in Fig. 5. Cytoskeletal proteins, 3.23×10^{-3} mg/ml. —▲—▲—, GSH added after addition of hemin; ···◆··◆···, GSH added before addition of hemin. Each point represents an average of six measurements deviated from the reported value by 5%.

the absence or presence of GSH, CN and His. The membranes were shaken in the dark at 37° for 5 min, and then centrifuged for 20 min at 43,000 *g*. The supernatants were separated from the pellet, and the pellet was then resolubilized in the same buffer. Figure 7 shows the Soret absorption spectra of the above solutions normalized to the same peak height. The right dotted line in Fig. 7A represents the spectra of hemin precipitated with the lipid core in the presence of the three ligands, all represented by one since they were identical. This spectrum is typical to monomeric hemin associated with lipids [18]. The supernatants demonstrated in Fig. 7B are typical spectra of hemin in the Soret band associated with the respective ligand which was present in the reaction mixture. The fractions of hemin intercalated

Table 2. Distribution of liganded hemin between the supernatant and membrane lipid core

Ligand	OH^-	GSH	CN^-	His
Percent of residual hemin in the membrane	89 ± 2 75 ± 2*	51 ± 1 13 ± 2*	69 ± 2 —	62 ± 4 —

Solutions buffered by 0.14 M phosphate pH 8.0. Temp 37°. Concentrations: hemin, 3.4 μM; CN, 5 mM; His, 46 mM; GSH, 0.4 mM. *Hemin, 34 μM; GSH, 4 mM.

Each value represents an average of five different experiments.

in the membrane lipid core as calculated from the absorbance of the supernatant are listed in Table 2. It is seen that the presence of all ligands reduced the association of hemin with the membrane. Moreover, the effect of GSH is more pronounced at a physiological concentration of GSH [~3 mM]. In a separate experiment membrane lipid core was added to the GS–hemin complex in a phosphate buffer and the spectra as recorded before and after addition of membranes are shown in Fig. 7A. The left spectrum is the typical GS–hemin spectrum already demonstrated in Fig. 1, and the right one represents the spectral features after addition of membranes. It is seen that the latter spectrum coincides with the typical monomeric hemin in the lipid core.

DISCUSSION

Our study shows that hemin and GSH can associate under physiological conditions. It has been known from early studies that the vinyl groups of

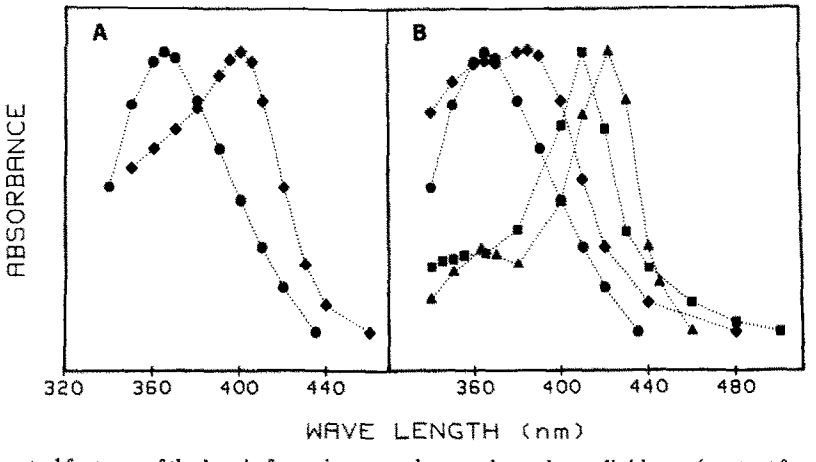

Fig. 7. Spectral features of the hemin forms in water phase and membrane lipid core (see text for detailed explanation). All spectra are normalized to the same height. (A) ···●··●···, GS–hemin in 0.14 M phosphate buffer pH 8; ···◆··◆···, the same GS–hemin solution in the presence of membrane lipid core and resuspended pellets equilibrated in separated samples in the presence of CN, His or GSH. The above spectra coincided and therefore one symbol has been used. (B) The spectral features of the supernatants of hemin liganded with the following: ···●··●···, GS–hemin; ···◆··◆···, hemin; ···■··■···, hemin–His; ···▲··▲···, hemin–CN.

hemin interact readily with various thiols in a slow reaction which requires extreme conditions of pH and temperature [19]. In contrast, the GS–hemin complex in this study was reached under mild conditions within the mixing time of a few minutes and we therefore conclude that a different active hemin group was involved in the reaction described in this study. From the data of Fig. 3B which indicate that by binding hemin to the typical iron chelators cyanide and histidine the difference spectrum of hemin and GS–hemin failed to form, one must conclude that GSH is also linked to hemin through its iron.

What is the oxidation state of the hemin iron after association with glutathione? As shown in Fig. 1, the typical spectral features of the GS–hemin complex are completely different from those of the dithionite reduced hemin. Consequently, no iron reduction could be involved in this reaction as was also concluded from the Mossbauer spectroscopy [17]. Which group on the GSH is responsible for its link to the hemin iron? Since reduction is not involved, other suggested groups besides sulfhydryl, namely amino and carboxyl ones, could potentially participate in the glutathione–hemin complex as suggested previously [17]. However, the finding of the current study that blocking of the sulfhydryl by formation of GSSG prevented production of the hemin–glutathione complex, leads to the unavoidable conclusion that glutathione is linked to hemin through the thiol group.

Being a chelator does not necessarily turn glutathione into an ultimate metabolite for preventing the damage of hemin to the membrane. On the contrary, even the opposite can be true, namely, chelated hemin may become more harmful to the membrane as has been shown in the case of hemin complexed with chloroquine [20]. We therefore compared the effects of hemin on the cell, in the presence and absence of GSH. The damage of hemin to the membrane is grossly represented by its hemolytic activity [4–6]. As demonstrated in Table 1, glutathione as well as other iron ligands almost completely prevented the hemolysis caused by hemin. The fact that GSSG, which was unable to bind hemin, also had no influence on the hemolytic activity of hemin, correlates binding of GSH to hemin with its antihemolytic effect. Earlier studies from this and other laboratories have postulated that hemin causes hemolysis of red cells due to its dual ability to intercalate in the phospholipid bilayer and to associate with the main cytoskeletal proteins spectrin and actin [7–9, 11]. The data of Fig. 4 clearly show that the presence of glutathione as well as other chelators diminishes markedly the ability of hemin to associate with the membrane lipid core as well as to bind to the cytoskeletal proteins.

It is clear that by binding GSH the hydrophobicity of hemin is much reduced and the size of the complexed molecule becomes considerably larger as compared to monomeric hemin. As a result, the complex may have a lower affinity but will still associate with the membrane as such. The results in Fig. 7 clearly show that in the presence of all ligands the membrane-associated hemin is ligand free and the diminished amount of membrane-bound hemin resulted from competition of the ligands and the membrane for hemin. The results in Table 2 can also be explained by a competition of the membranes and GSH for hemin. The increase in free GSH shifted the equilibrium so that a much smaller fraction of hemin could associate with the membrane. Once associated with membrane components glutathione could potentially shift the equilibrium by binding hemin. However, our results show that this is true only for lipid intercalated hemin but not for hemin which has already been bound to membrane cytoskeletal proteins (compare Figs 5 and 6).

From the binding data in Fig. 2 one can estimate that at the normal concentration range of GSH in the red cell [1–3 mM], at least 97% of the cytosolic hemin can be complexed with glutathione. Based on this calculation we suggest that GSH may serve physiologically as the cytosolic chelator for free hemin. In some pathological cases, hemolysis is linked to reduced GSH in the red cell reaching concentrations as low as 0.01 mM [21]. From the affinity of the GS–hemin complex it turns out that at this GSH concentration only less than half of the hemoglobin free hemin will be GSH conjugated, leaving the rest free to injure the cell membrane. Glutathione has long keen known to act as a powerful agent for clearing the cell from various toxic molecules. Unlike other glutathione involved reactions, the association with hemin does not consume the GSH reservoir. Only a negligible fraction is occupied to begin with, and even this amount becomes available once hemin is transported out of the cell [10]. Our findings also point at another indirect defense of the red cell by glutathione. Being occupied, hemin can no longer cause inhibition of the cytosolic key enzymes which are responsible for maintenance of the red cell function [12, 13]. We may therefore conclude that glutathione plays a key role in protection of the red cell from hemin damage.

Acknowledgements—This work was supported by Binational Science Foundation Grant No. 8500096. We thank Dr I. Kirschner-Zilber for many helpful discussions.

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