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Interaction of hemin with erythrocyte membranes: alterations in the physical state of the major sialoglycoprotein

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Hemin has been shown to disrupt erythrocyte membrane skeletal protein–protein interactions, initially those involving band 4.1 (Shaklai et. al. (1986) Biochem. Int. 13, 467–477). We have used electron spin resonance (ESR) spin labels specific for cell-surface carbohydrates, skeletal membrane proteins, or bilayer lipids to find: (1) simultaneous reaction of the protein-specific spin label, MAL-6, which binds to skeletal protein SH residues, and 10 μM hemin suggested that hemin decreased skeletal protein–protein interactions; (2) 10 μM hemin markedly decreased (> 60%, $P < 0.001$) the rotational motion of spin-labeled erythrocyte membrane cell-surface sialic acid residues, 70% of which are located on the major transmembrane sialoglycoprotein, glycophorin A; and (3) 10 μM hemin caused a small, but significant ($P < 0.02$), decrease in the motion of a lipid bilayer specific spin label (5-NS) in the erythrocyte membrane. Since glycophorin A is reportedly linked to the erythrocyte membrane skeletal protein network by band 4.1, it is conceivable that hemin-induced disruption of skeletal protein interactions, particularly those of band 4.1, could subsequently lead to the alterations in the motion of cell-surface sialic acid presented in this report.

Introduction

Hemin, a hemoglobin breakdown product, is elevated in aged red cells and in both the erythrocyte lipid bilayer and the protein skeletal network in patients with sickle-cell anemia and β-thalassemia [1]. In the latter conditions, hemin levels are reported to correlate with disease severity [1]. Hemin is also found associated with Heinz bodies, which in sickle-cell disease induce a clustering of the major transmembrane proteins, band 3 and glycophorin A, with ankyrin [2], the attachment site for a fraction of band 3 proteins to the erythrocyte membrane skeletal protein network [3]. Hemichromes, the precursor to hemin and Heinz bodies, are reported to bind the cytoplasmic pole of band 3 with a greater affinity than hemoglobin [4].

Considerable evidence to suggest that hemin disrupts skeletal protein–protein interactions has been accumulated [5,6]. Among other skeletal membrane alterations reportedly induced by hemin are: altered conformation of the major skeletal protein (spectrin), band 3 and band 4.1 [5]; decreased interaction between spectrin chains and spectrin dimers [7] and between spectrin and band 4.1 [5]; diminished mechanical stability of extracted skeletons [5]; and inhibition of the polymerization of actin [8]. The results of Shaklai et. al. [6] indicate that hemin reacts with and disrupts the membrane skeletal protein network in a temperature-, concentration-, and time-dependent manner by a mechanism in which the release of band 4.1 from the skeleton is the initial step, followed by dissociation of spectrin tetramers to dimers and eventual complete separation of skeletal components.

The sulfhydryl compounds, glutathione [9], cysteine [10] and mercaptoethanol [10], bind hemin and decrease the latter's toxic effects on erythrocyte membranes. This finding is consistent with the observation that glutathione apparently acts as an in vivo hemin scavenger [9].

70% of the sialic acid residues on the erythrocyte membrane are located on the major sialoglycoprotein, glycophorin A [11]. This transmembrane protein is also thought to be an attachment site for the skeletal protein network to the membrane bilayer via the skeletal pro-

Abbreviations: PBS, phosphate-buffered saline; 5-NS, 5-doxylstearic acid; 5P8, sodium phosphate buffer (pH 8).

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tein, band 4.1 [3,12]. Since hemin has been shown to disrupt skeletal protein–protein interactions [5,6], in particular the spectrin–band 4.1 interaction, it is conceivable that such disruption of the band 4.1 interaction with spectrin could lead to alterations in the conformation of glycophorin A and subsequently the environment of terminal sialic acid residues attached to this sialoglycoprotein.

To test this idea, we have employed ESR using a sialic-acid-specific spin-labeling procedure developed in our laboratory [13]. Other spin labels specific for either the skeletal network of proteins or the lipid bilayer were also employed [14]. The results suggest that hemin has a pronounced effect on the motion of cell-surface sialic acid.

Materials and Methods

Blood was obtained from healthy human volunteers by venipuncture into heparinized tubes, immediately placed on ice, and processed within 30 min of collection. Intact cells were isolated by centrifugation at 4°C at 600 × *g* and a subsequent three resuspensions and washings in PBS buffer (150 mM NaCl/5 mM sodium phosphate (pH 8.0)). The buffy coat was carefully removed. Erythrocyte ghost membranes were obtained by hypotonic lysis with 5P8 (5 mM sodium phosphate buffer (pH 8.0)) employing 1 vol. of cells to 20 vol. of 5P8, and subsequent centrifugation at 4°C and 27000 × *g*. The ghosts were resuspended in ice-cold 5P8 and this process was continued until the membranes were free of residual hemoglobin. Protein content was estimated by the method of Lowry et al. [15].

Erythrocyte membranes were spin-labeled with either a skeletal protein-specific spin label, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) [14,16,17], a lipid-specific spin label, 5-doxylstearic acid (5-NS) [14], or a cell-surface carbohydrate-specific spin label, Tempamine [18,19], as previously described in publications from our laboratory. Tempamine was employed to label either terminal sialic acid [13] or terminal galactose residues [20] (depending on which was desired) at the erythrocyte cell surface by reductive amination. All ESR spectra were recorded at a temperature of 20 ± 0.5°C on a Varian E-109 spectrometer with computerized data acquisition.

50 μM hemin solutions were prepared fresh daily by dissolving approx. 50 mg hemin chloride (Sigma) in a minimal volume 0.01 M NaOH (approx. 2 ml) and diluting to 1 liter with 5P8. The precise hemin concentration was determined spectrophotometrically using a millimolar absorption coefficient of 58.4 $mM^{-1} \cdot cm^{-1}$ at 385 nm [21]. In direct addition experiments, 4 vol. of spin-labeled ghosts were held for 15 min at room temperature and incubated 30 min with one vol. of 5 or 50 μM hemin, and the ESR spectrum was recorded. The final membrane protein concentration was 2.5 mg/ml and the final hemin concentrations were 1 or 10 μM.

To determine whether hemin and MAL-6 competed for similar SH binding sites, erythrocytes were labeled with 40 μM MAL-6 in the presence of 10 μM hemin at 4°C for 16 h. Following incubation, the excess spin-label and hemin were removed by six 5P8 washes in the cold. The labeled ghosts were adjusted to a protein concentration of 2.5 mg/ml, warmed to room temperature for 30 min and the ESR spectrum was recorded.

Results

The effect of hemin on the physical state of the erythrocyte membrane was examined by employing ESR spin-labeling techniques specific for membrane skeletal proteins, terminal-sialic acid residues, terminal-galactose residues or the lipid bilayer.

Previous ESR studies from our laboratory demonstrated that disruption of skeletal-protein–protein interactions of the erythrocyte membrane skeletal network by increasing the proportion of dimeric spectrin [17], or by polyphosphates [17,22], resulted in altered conformation of skeletal proteins. Since hemin is known to cause disruption of skeletal protein–protein interactions, it is concievable that such interaction would lead to changes in the conformational state of skeletal proteins. To see the effect of hemin on skeletal protein interactions we employed the protein-specific spin-label, MAL-6. This spin label binds covalently to membrane protein SH groups with 70–90% of MAL-6 bound to skeletal proteins (particularly spectrin [23]) with the remainder bound to the cytoplasmic pole of band 3 [14,16,17,23–25]. A typical spectrum of MAL-6 labeled erythrocyte membranes is given in Fig. 1. At least two classes of MAL-6 binding sites, characterized by their motion, are observed: weakly immobilized and strongly immobilized SH sites. W sites are relatively polar, as judged by the electron-nuclear hyperfine coupling of the spectral

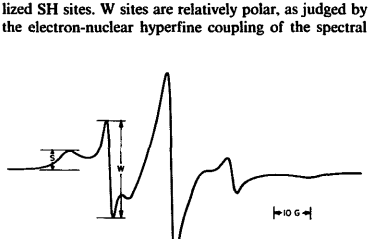

Fig. 1. Typical spectrum of MAL-6 attached to erythrocyte membrane proteins. The spectral amplitudes of the low-field strongly and weakly immobilized spin-label binding sites are given by *S* and *W*, respectively. Instrument setting: MAL-6, 50 G scan width, 0.32 G modulation amplitude, and 16 mW power incident on the resonant cavity.

TABLE I

Effect of hemin on the physical state of erythrocyte membrane skeletal proteins as monitored by the W/S ratio of MAL-6 [a]

n.s., not significant.

Sample	$W/S_{subject} - W/S_{control}$	P[c]
Direct addition [b]		
Control	0.00	–
Hemin 1 μM	−0.05 ± 0.06	n.s.
Hemin 10 μM	+0.02 ± 0.08	n.s.
Competitive addition [b]		
MAL-6/5P8	0.00	–
MAL-6/hemin 10 μM	1.05 ± 0.30	< 0.02

[a] Mean ±S.E. of difference of $W/S_{subject} - W/S_{control}$. This difference is expected to be zero should hemin exert no influence on the physical state of red blood cell membrane skeletal proteins.
[b] $N = 6$.
[c] P determined by two-tailed Student's t-test.

lines of this group [14], and S sites are largely accessible to solvent as evidenced by ascorbate-decay studies [14].

The relevant ESR parameter measured is the ratio of the ESR spectral amplitude of the $M_I = +1$ low-field weakly immobilized line (W) and that of the $M_I = +1$ low-field strongly immobilized line (S) (the W/S ratio). This ratio has been shown by us and others to be extremely sensitive to alterations in the conformation of and interactions between erythrocyte membrane skeletal proteins, and is reflective of the segmental motion of protein binding sites (reviewed in Refs. 14, 16, 26). We showed that, following labeling with 40 μM MAL-6, no difference in the state of spectrin aggregation, the ability to convert spectrin tetramers to dimers, or the reversibility of this conversion relative to unlabeled membranes, could be demonstrated [17]. Consequently, this low level of SH group modification does not appear to alter protein–protein interactions. Others have shown that our MAL-6-labeling procedures yield highly reproducible spectra that can be used with confidence [24].

Addition of 1 or 10 μM hemin to MAL-6-labeled membrane ghosts did not alter the W/S ratio relative to control (Table I). This surprising result appeared inconsistent with previous results of biological studies by others which indicated that hemin disrupted skeletal-protein–protein interactions [5–7]. Polyphosphates, which also are known to disrupt skeletal-protein–protein interactions, have been shown to cause an alteration in the W/S ratio of labeled membrane skeletal proteins [17,22].

A possible, though unproven, explanation for this apparent contradiction is that hemin may exhibit its biological effect by binding to specific SH sites on skeletal proteins or the cytoplasmic pole of band 3 [1,4,42], sites to which MAL-6 is also bound [14]. Consistent with SH group binding by hemin are recent reports which suggest that reduced glutathione binds hemin and that the hemin-glutathione complex is nontoxic, implying that glutathione may act as an intracellular hemin chelator [9]. Other SH compounds have also been shown to bind hemin, including cysteine [10]. However, in MAL-6-labeled ghosts, the putative specific SH binding sites, located on skeletal protein – or band 3 – cysteine residues to which hemin binds, would be already occupied by the spin label, presumably preventing hemin binding and the attendant disruption of skeletal protein–protein interactions.

In order to investigate whether MAL-6-labeling blocked hemin binding, experiments in which MAL-6 and hemin were simultaneously reacted with membranes were performed. Ghosts were labeled with 40 μM MAL-6 solutions dissolved in either 5P8 or in 5P8 containing 10 μM hemin as described in Materials and Methods. The results showed a significant increase in the W/S ratio for ghosts incubated with MAL-6 and hemin relative to ghosts incubated with MAL-6 only (Table I). This result is consistent with the idea that MAL-6 and 10 μM hemin may bind similar SH sites on the skeletal proteins or the cytoplasmic ploe of band 3 [42] and could explain why no alteration in W/S ratio was observed by addition of 1 or 10 μM hemin for 30 min at room temperature to ghosts previously labeled with MAL-6. Furthermore, the increased W/S ratio, shown in Table I, for the hemin-MAL-6 competition experiment is consistent with hemin causing a decrease in protein–protein interactions within the erythrocyte skeleton as shown by others [5–8]. In separate experiments, hemin and MAL-6 were incubated together in 5P8 in the absence of membranes. Over a 1 h period, no change in the MAL-6 spectrum occurred, suggesting that these two agents do not react with each other under these conditions.

To test the possibility that hemin-induced disruption of skeletal-protein interactions, particularly those involving band 4.1 [6] which is reportedly linked to glycophorin A [12], could lead to altered motion of extracellular sialic acid, the physical state of Tempamine-labeled terminal sialic acid residues in erythrocyte membranes was investigated. A typical resulting spectrum of perdeuterated, [^{15}N]Tempamine covalently attached to sialic acid is given in Fig. 2. As shown in recent publications from our laboratory [26,36,40], this spectrum is characterized by an apparent rotational correlation time (τ), which can be conceptualized as the time required for the spin label, covalently attached to sialic acid, to rotate one radian through space. An increase in τ is indicative of decreased motion of the spin-labeled sialic acid. The final hemin concentrations employed were 1 or 10 μM. As shown in Table II, the results suggest that hemin caused a significant, concentration-dependent increase in τ of spin-labeled sialic acid residues, reflecting decreased sialic acid motion in the presence of 1 and 10 μM hemin relative to control. Note that the SH groups

TABLE II

Effect of hemin on the motion of tempamine-labeled cell-surface sialic acid and galactose residues and 5-NS-labeled lipid bilayers of erythrocyte membranes

	[Hemin] (μM)	% of control [a]	N	P [b]
Cell surface				
Sialic acid (τ)	0.0	100	8	–
	1.0	136 ± 5.9	8	< 0.001
	10.0	164 ± 11.4	8	< 0.001
Galactose (τ)	0.0	100	2	–
	10.0	108 ± 0.4	2	< 0.05
Lipid bilayer				
(HWHH)	0.0	100	5	–
	1.0	98.5 ± 0.6	5	n.s.
	10.0	96.4 ± 0.6	5	< 0.02

[a] Mean ± S.E. for percent of respective control.
[b] P values determined by two-tailed Student's t-test.

bound by MAL-6 in protein-spin-labeling studies were not blocked in Tempamine-labeled erythrocytes.

At least two possible origins of the hemin-induced decrease in sialic acid motion can be postulated. The first has been mentioned and involves a secondary effect caused by hemin-induced disruption of the band 4.1–glycophorin A interaction. A second possibility is that hemin associates directly with extracellular carbohydrates, resulting in decreased sialic acid motion possibly due to steric interference.

In order to help gain insight into these two alternatives, two types of experiment were performed. In the first, the SH-group-specific agent, *N*-ethylmaleimide (NEM) at 80 μM was added to ghosts prior to spin-labeling sialic acid residues with Tempamine. Addition of 10 μM hemin to these ghosts, in which SH groups of skeletal proteins were blocked by NEM, yielded a modest increase in τ relative to ghosts to which buffer only was added (mean ± S.D. ($N = 5$): $\tau_{hemin}/\tau_{control} = 118 \pm 9.95$ ($P < 0.02$)). This 18% change is to be compared to the 64% change in τ in ghosts without SH group blockage (Table II), consistent with the idea that hemin disruption of band 4.1–glycophorin A interactions is a major cause of the large change in physical state of cell-surface sialic acid. The second type of experiment also is consistent with this idea. The effect of hemin on the physical state of another selectively spin-labeled cell-surface carbohydrate was investigated. Terminal galactose residues are located primarily on band 3, band 4.5 and glycophorin A. These galactose residues can be selectively spin-labeled with Tempamine by a reductive amination procedure also developed in our laboratory. This method, similar to the one employed in sialic acid-labeling, involves the enzymatic production of the initial aldehyde on terminal galactose [20]. The resulting spectrum of Tempamine-labeled galactose is similar to that of Tempamine-labeled sialic acid (Fig. 2). The motion of labeled galactose residues was assessed by τ. The preliminary results suggested that 10 μM hemin does not appreciably alter Tempamine-labeled galactose motion, as evidenced by the small and marginally-significant increase in τ (Table II). This finding, consistent with the hemin–MAL-6 competition experiments noted above, is consonant with the concept that the effect of hemin on sialic acid motion (Table II) may have been caused primarily by hemin-induced disruption of spectrin-band 4.1–glycophorin A interactions as shown by others [5–7].

Kirschner-Zilber et. al. [27] and Ginzburg and Demel [28] have provided evidence for an interaction between hemin and the phospholipid membranes of erythrocytes. To gain insight into whether such an interaction altered the order and motion of the erythrocyte lipid bilayer, we employed the lipid-specific spin label, 5-NS. The order and motion of the membrane lipids were assessed by measuring the half-width at half-height (HWHH) of the $M_I = +1$ line of the spectrum of 5-NS [29], as illustrated in Fig. 2. Mason and co-workers [29] demonstrated that the HWHH was considerably more sensitive than measurement of spectral extrema to small alterations in the order and motion of the erythrocyte membrane lipid bilayer as monitored by 5-NS. This increased sensitivity is due to the fact that with decreased (increased) lipid order and increased (decreased) lipid motion, linewidth broadening (narrowing) occurs before alterations in extrema separation take place. Additionally, unlike measurements of extrema separation, HWHH is independent of the polarity of

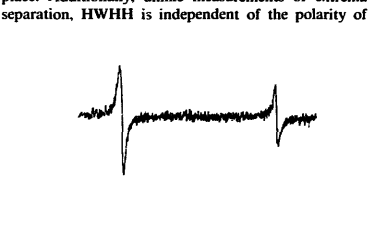

Fig. 2. Typical ESR spectra of [^{15}N]Tempamine-labeled cell-surface sialic acid residues and a typical spectrum of the low-field line of 5-NS-labeled lipid bilayers in erythrocyte membranes. Instrument setting: Tempamine, 50 G scan width, 0.32 G modulation amplitude, and 16 mW power incident on the resonant cavity; 5-NS, 100 G scan width, 0.32 G modulation amplitude, and 18 mW power. The rotational correlation time for perdeuterated [^{15}N]Tempamine was calculated by well-established equations [40], employing the single crystal anisotropic T-tensor values of perdeuterated [^{15}N]MAL-6 [41].

the nitroxide moiety microenvironment within the lipid bilayer [29]. Similar to the linewidth changes associated with chemical exchange, changes in the rate of reorientation of the principal axis of 5-NS, between parallel and perpendicular orientations relative to the normal to the erythrocyte membrane surface, lead to altered linewidth of the low-field line of the spin-label spectrum. The observations for the motion of 5-NS in erythrocyte membranes are consistent with theoretical models [30,31].

The present results suggest that 10 μM hemin caused a small, but significant ($P < 0.02$), decrease in lipid motion in ghosts, as reflected by a decreased HWHH (Table II), while 1 μM hemin did not significantly alter lipid motion in these studies. The effect of 10 μM hemin may be consistent with results from other laboratories, which suggest hemin intercalates into lipid bilayers [27,28] or reportedly enhances lipid peroxidation [32], both of which conceivably could lead to decreased motion within the lipid bilayer [14].

Discussion

The evidence is considerable that hemin disrupts erythrocyte skeletal protein–protein interactions [5–8]. The initial step in hemin-induced disruption of the cytoskeleton has been reported to be the release of band 4.1 by spectrin, followed by spectrin dimerization, and complete dissociation of skeletal interactions [6].

Addition of hemin to protein spin-labeled ghosts did not seem to alter the segmental motion of the MAL-6 binding sites of skeletal proteins (Table I). This result, appearing at first to be a contradiction with the observations which indicate that hemin disrupts skeletal protein–protein interactions [5–7], is likely due to the fact that the protein-specific spin label, MAL-6, which binds skeletal SH groups, blocks the interaction of hemin with the skeleton.

In support of this idea, glutathione, an intracellular sulfhydryl compound, has been shown to bind hemin and may act as an intracellular hemin scavenger in erythrocytes [9]. Additionally, hemin has been shown to bind other SH compounds [10]. As would be predicted from the above, hemin does not appear to react with disulfide bonds [9]. The competition study, in which we spin-labeled ghosts with MAL-6 in the presence of 10 μM hemin, led to an increase in the W/S ratio compared to control (Table I). This result is suggestive of decreased skeletal protein–protein interactions, as would be predicted by previous studies by others [5–7], and is also consistent with the concept that hemin may bind to skeletal protein SH groups that are also bound by MAL-6. In agreement with this idea, Beaven and Gratzer [33] have suggested that spectrin dimers possess 15–20 hemin binding sites, a number equal to the number of SH residues (15–25) available for MAL-6 binding [34].

Band 4.1 is thought to provide one of the linkages between the red cell transmembrane glycoproteins and the skeletal-protein network by linking the major sialoglycoprotein, glycophorin A, and the major skeletal protein, spectrin [12]. Glycophorin A contains 70% of the total terminal sialic on the red cell extracellular surface [11]. Since the initial effect of hemin-induced degradation of skeletal protein interactions has been reported to involve disruption of the associations of band 4.1 with other skeletal proteins [6] and possibly glycophorin A, it is conceivable that such disruption could lead to alterations in the conformation of glycophorin A and subsequently the motional environment of its terminal sialic acid residues. Little is known about the topology of glycophorin at the cell surface, but model studies suggest that the glycoconjugates of this sialoglycoprotein are hydrogen-bonded to lipid bilayer headgroups [35]. The motion of Tempamine-labeled terminal sialic acid residues was significantly reduced by 1 and 10 μM hemin compared to control (Table II). One possible interpretation of this result is that a disruption of skeletal protein–protein interactions on the cytoplasmic side of the membrane could secondarily lead to a decrease in the motion of terminal sialic acid residues on glycophorin A on the extracellular side of the membrane. Such communication between the skeleton and cell-surface sialic acid has been demonstrated previously in our laboratory by the addition of the polyamine, spermine, to erythrocytes, which caused a primary increase in skeletal–skeletal and skeletal–bilayer protein interactions and secondarily-induced increased motion of spin labeled-sialic acid residues [36].

Hemin has been reported to associate with phospholipid bilayers via an apparent hydrophobic interaction, which is independent of ionic strength [27]. Such association of hemin with the erythrocyte membrane lipid bilayer may explain the apparent decrease in the motion of 5-NS in hemin-exposed bilayers (Table II).

Intracellular hemin concentrations are elevated in a number of pathological states associated with unstable hemoglobins [1,37,38]. Elevated hemin levels induce hemolysis, possibly due to disruption of skeletal protein interactions [5,6]. In normal erythrocytes, hemin levels are maintained by two possible mechanisms [1,9]. The first involves passage of hemin through the lipid bilayer and the subsequent binding of extracellular hemin by serum albumins [1]. This mechanism requires hemin to exhibit a hydrophobic nature and intercalate into lipid bilayers as has been demonstrated by others [1,27,28,39] and is consistent with the results of the present study (Table II). The second mechanism proposed requires glutathione binding to hemin through a sulfhydryl linkage, thereby decreasing the free hemin concentration available to disrupt skeletal protein interactions [9]. The ESR spin-labeling results in this report are also consistent with this mechanism. In addition, our results

suggest that hemin selectively decreases the motion of cell-surface terminal sialic acid, compared to galactose, which may be due to a hemin-induced disruption of the interaction between band 4.1 and glycophorin A. While the physiological significance of this interaction is yet unknown, it is possible that such conformational changes could act as a transmembrane signal by altering the motional environment of cell-surface sialic acid.

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