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IDENTIFICATION OF THE HEMOGLOBIN BINDING SITES ON THE INNER SURFACE OF THE ERYTHROCYTE MEMBRANE

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The hemoglobin binding sites on the inner surface of the erythrocyte membrane were identified by measuring the fraction of hemoglobin released following selective proteolytic or lipolytic enzyme digestion. In addition, binding stoichiometry to and fractional hemoglobin release from inside-out vesicle preparations of human and rabbit membranes were compared since rabbit membranes differ significantly from human membranes only in that they lack glycophorin. Our results show that rabbit inside-out vesicles bind about 65% less human or rabbit hemoglobin under conditions of optimal and stoichiometric binding, despite being otherwise similar in composition. We suggest that this difference is either directly or indirectly due to the absence of glycophorin in rabbit membranes. Further supportive evidence includes demonstrating (a) that neuraminidase treatment of human membranes did not affect hemoglobin binding and (b) that reconstitution of isolated glycophorin into phospholipid vesicles increased the hemoglobin binding capacity in a manner proportional to the fraction of glycophorin molecules oriented with their cytoplasmic sides exposed to the exterior of the vesicle. Proteolysis of human inside-out vesicles either before or after addition of hemoglobin reduced the binding capacity by about 25%. This is consistent with the known proportion of total hemoglobin binding sites involving band 3 protein and the selective lability of the cytoplasmic aspect of band 3 protein to proteolysis. Phospholipid involvement in hemoglobin binding was determined using various phospholipase C preparations which differ in their reactivity profiles. Approximately 38% of the bound hemoglobin was released upon cleavage of phospholipid headgroups. These results suggest that the predominant sites of binding for hemoglobin on the inner surface of the red cell membrane are the two major integral membrane glycoproteins.

Introduction

There are several conditions where increased membrane-bound hemoglobin has been observed. They include sickle cell anemia [1–4]; the thalassemias [5,6]; and chemically induced and hereditary Heinz body anemia [7]. The association of hemoglobin with the red cell membrane may be a normal occurrence as the cell ages [8,9]. The significance of hemoglobin binding to the survival of the cell in the circulation is unknown. However, there has been some suggestion that hemoglobin-membrane interactions may lead to selective increases in K^+ permeability [10]. The first evidence that hemoglobin could bind to the erythrocyte

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Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonate; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

membrane came from studies designed to prepare isolated ghosts [11]. Subsequent work began to define the membrane binding capacity, the dependence of binding on conditions of pH and ionic strength and the preferential binding of certain hemoglobin variants [12–14]. Hemoglobin has been shown to bind to the integral components of the membrane [15]. The total hemoglobin binding capacity of the membrane appears to involve cytoplasmic sites. Removal of sialic acids with neuraminidase or digestion of extracellular proteins with chymotrypsin has no significant effect on binding [16]. Hemoglobin does not bind reversibly to extracellular phospholipids under non-denaturing conditions [17]. Furthermore, myoglobin binds to the membrane under the same conditions with an affinity which is 1 to 2 orders of magnitude lower than hemoglobin, suggesting a significant degree of specificity even under the non-physiologic conditions necessary to study binding [18]. There is recent evidence for binding to unsealed ghosts at near physiologic pH but low ionic strength [19]. More recently, Eisinger et al. [20] have presented evidence that hemoglobin may bind or closely approach the vicinity of band 3 protein within intact cells.

Several lines of evidence have suggested that there are multiple sites of association for hemoglobin on the inner surface [16,21,22]. The high affinity site has been suggested to be band 3 protein. The evidence supporting this position includes (a) competition studies with glyceraldehyde-3-phosphate dehydrogenase [22,23] which is known to bind specifically to band 3 [15]; (b) alterations in hemoglobin's affinity for cytoplasmic sites in a manner which correlated with the specific binding of DIDS to band 3 [16]; (c) sensitivity of the cytoplasmic sites to proteolytic enzymes known to cleave band 3 at that surface [16] and (d) demonstration of hemoglobin cross-linking to band 3 protein under stoichiometric binding conditions [24,25]. Finally, we have reported that isolated cytoplasmic segments of band 3 did alter hemoglobin function in solution [16] in a manner similar to that seen for membrane-bound hemoglobin [23].

Although hemoglobin binds to band 3, the total binding capacity under the conditions used exceeds the number of available copies of band 3 protein by a factor of about 4 to 5 [16]. The purpose of this study was to identify the other major binding site(s) on the inner surface of the membrane using inside-out vesicles. Our results suggest that the predominant second binding site involves the cytoplasmic aspect of glycophorin and that binding to phospholipid, although extant, constitutes a small fraction of the total binding capacity.

A preliminary account of this work has been published in abstract form [26].

Materials and Methods

Recently out-dated human whole blood or packed cells were obtained from the Omaha chapter of the American Red Cross. Fresh human blood was obtained from healthy volunteers. Fresh rabbit blood was obtained from 2.5–3.5-kg New Zealand white rabbits according to the method of Burke [27]. Most of the materials used here have been described in previous publications from this laboratory [16,28]. Phospholipases from *Clostridium welchii (perfringens)* and from *Bacillus cereus* were obtained from Sigma Chemical Co. (St. Louis, MO). Inositol specific phospholipase C from *Staphylococcus aureus* was the generous gift of Professor Martin G. Low. Cellulose, concanavalin A, chymotrypsin, neuraminidase, lithium diiodosalicylate and dipalmitoyl phosphatidylcholine were from Sigma.

Preparative methods

Unsealed ghosts from human or rabbit red blood cells were prepared as previously described [28]. Inside-out vesicles were prepared according to the method given by Steck [29]. The vesicles were routinely salt stripped in 5 mM Bistris-HCl (pH 8) +200 mM NaCl.

Isolation of inside-out vesicles was performed using the concanavalin A-cellulose method of Kondo et al. [30] and is based on the principle that red cell membrane sugars are exclusively located on the outer surface. After isolation the inside-out vesicles were washed in 5 mM phosphate (pH 6).

Isolation of glycophorin from human red cell membranes was performed essentially as described by Marchesi and Andrews [31] with the following modifications. After the extraction of glycophorin

from the membranes with lithium diiodosalicylate, 9 vol. of ethanol were added to 1 vol. of the glycophorin-containing water phase. Following this, 0.1 vol. of a 1 M Tris, 10 mM EDTA solution (pH 7.4) was added which resulted in precipitation of glycophorin in agreement with the findings of Van Zoelen et al. [32]. The glycophorin precipitate was centrifuged at 4000 rpm in the CRU 5000 for 10 min and redissolved in water. The material was filtered over a coarse glass filter. The sample was dialyzed against 10 mM Tris-HCl (pH 9) for 8 h at 4°C followed by a second dialysis against distilled water for 8 h. The protein was lyophilized and then resuspended in water. Confirmation of the final product was made by SDS gel electrophoresis as previously described [28], staining with periodic acid Schiff's base stain. Samples of unsealed ghosts were electrophoresed for comparison. Our final sample contained 2.4 mg per ml of glycophorin based on protein determinations by the method of Lowry et al. [33] using bovine serum albumin as a standard.

PC vesicles lacking and containing glycophorin were prepared exactly as described by MacDonald and MacDonald [34]. Both samples were evaporated to dryness, followed by a wash in 5 mM phosphate (pH 6). The amount of glycophorin present in the one sample was calculated as above.

Hemoglobin was prepared essentially as described by Salhany et al. [35] with the exception that cells were washed in phosphate-buffered saline, pH 8, lysed in 5 mM phosphate (pH 8) and dialyzed, after chromatography, in 5 mM Bistris-HCl (pH 6) for 72 h at 4°C.

Analytical procedures and data analysis

Hemoglobin concentrations were determined spectrophotometrically at 577 nm using 14.6 $mM^{-1} \cdot cm^{-1}$ as the extinction coefficient [36]. In addition, each sample analyzed was reduced by addition of dithionite and concentration determined at 555 nm using 12.5 $mM^{-1} \cdot cm^{-1}$ [36].

Centrifugation measurements of hemoglobin binding were performed at 4°C using a Sorvall centrifuge with rotor SS-34 essentially as described in a previous report [16]. Human or rabbit inside-out vesicles prepared as described above, were brought to a total protein concentration of 5.75 mg/ml determined by the method of Lowry et al. [33]. The vesicles in 5 mM Bistris-HCl (pH 6), were mixed 1:1 with known concentrations of oxyhemoglobin under conditions of stoichiometric binding [16]. Each sample was incubated for 10 min at 25°C and then centrifuged at 15000 rpm for 20 min. Each supernatant fraction was removed and assayed to determine the hemoglobin concentration. The amount of hemoglobin bound was then calculated.

Hemoglobin binding to reconstituted PC vesicles was performed in a similar manner. The amount of hemoglobin added was roughly equivalent to the amount of glycophorin present assuming a 1:1 stoichiometry with the hemoglobin tetramer.

Proteolytic and lipolytic enzyme-induced hemoglobin release experiments were performed as follows. Inside-out vesicles (5.75 mg/ml) were mixed 50:50 with less than saturating concentrations of hemoglobin again under stoichiometric binding conditions and allowed to incubate at 25°C for 10 min. The hemoglobin-bound vesicles were then divided into six groups of equal volume (4 ml/sample) and centrifuged for 20 min as described. The supernatant fraction was removed to determine the fraction of hemoglobin bound. The first sample served as a control to which enough 5 mM Bistris-HCl (pH 6) was added to bring the volume back to 4 ml. The remaining samples had 2 ml of the following added and reactions proceeded for respectively: (a) phospholipase C from *C. welchii* (0.34 mg/ml in 5 mM Bistris-HCl (pH 6) at 25°C for 1 h); (b) phospholipase C from *B. cereus* (0.072 mg/ml in 5 mM Bistris-HCl (pH 6) at 25°C for 1 h); (c) inositol-specific phospholipase C from *S. Aureus* (5 μg/ml in 5 mM Bistris-HCl (pH 6) at 25°C for 1 h); (d) chymotrypsin (200 μg/ml in 5 mM Bistris-HCl (pH 6) at 37°C for 1 h) or (e) Tris base (20 mM). Each sample was then brought to a final volume of 4 ml by addition of 5 mM Bistris-HCl (pH 6). The final pH for the Tris-containing sample was 8.95 and this caused all the hemoglobin to be released and was used to determine a 100% release value. All samples, except the chymotrypsin containing sample were incubated at 25°C for 60 min. The chymotrypsin sample and an appropriate control were incubated at 37°C. Each sample was then centrifuged and

the percentage of hemoglobin released determined as described above.

In order to check for the activity of the phospholipase C preparations, the supernatant fraction from large batches of vesicles treated with the respective enzymes was analyzed using ^{31}P-NMR. The instrument and data collection procedures were the same as previously described [37]. Peaks were identified using standard spectra of the expected products of the enzyme digestion collected under identical conditions.

Finally, titration of intact rabbit erythrocytes with DIDS was performed as described previously [16] except that the cells were washed in 90 mM phosphate buffer (pH 7.4) instead of sulfate.

Results

The fraction of chymotrypsin-sensitive hemoglobin binding sites on inside-out vesicles

Our previous work on hemoglobin binding to the erythrocyte membrane used light scattering to measure the stoichiometry of binding to unsealed ghosts [16]. It was shown that band 3 protein accounted for about 25 to 30% of the total binding capacity based on stoichiometric titration experiments with glyceraldehyde-3-phosphate dehydrogenase, an enzyme known to bind exclusively to the cytoplasmic portion of band 3 protein [38]. This part of band 3 protein is also quite sensitive to proteolysis by chymotrypsin [39]. The cytoplasmic portion of glycophorin, the other predominant integral membrane glycoprotein, is known to be insensitive to chymotrypsin proteolysis when membrane bound [40,41]. The sensitivity of the cytoplasmic aspect of band 3 protein to proteolysis was previously used to show a diminution in hemoglobin binding capacity when unsealed ghosts were digested with chymotrypsin [16].

In order to assess the fraction of chymotrypsin-sensitive binding sites on the inner surface of the membrane, we treated both hemoglobin-bound and free human inside-out vesicles with chymotrypsin. The present approach is a more quantitative one than that used previously, where unsealed ghosts were the starting material. The chymotrypsin-induced decrease in hemoglobin binding capacity in that case may have partially resulted from the formation of some sealed right-side-out vesicles [29]. Analysis of the digested membranes using SDS-gel electrophoresis and staining with both Coomassie blue and periodic acid Schiff's base gave identical results to those reported by Steck and co-workers [39,40]. Table I shows the fraction of hemoglobin released from inside-out vesicles at less than and greater than half saturation with hemoglobin (see below) due to chymotrypsin proteolysis. The same results were obtained when we investigated the fraction of hemoglobin remaining in the supernatant fraction when vesicles were first treated with chymotrypsin and then added to the hemoglobin solutions under

TABLE I

PERCENT HEMOGLOBIN RELEASED FROM INSIDE-OUT MEMBRANE VESICLES BY VARIOUS ENZYMES

Each column is for a given percentage of hemoglobin saturation. The concentration of inside-out vesicles was 2.87 mg of protein per ml. The experiments were conducted in 5 mM Bistris-HCl (pH 6).

Treatment	Human 35% satd.	Human 78% satd.	Rabbit 51% satd.
Control	0.9±0.5	3.2±1.8	1.2±0.04
Chymotrypsin	30.0±5.5	23.1±2.3	38.6±5.0
Phospholipase C *B. cereus*	15.1±2.1	38.5±0.1	62.5±0.6
Phospholipase C *C. welchii*	3.9±0.5	22.7±1.4	18.6±3.4

otherwise the same conditions. It is worth noting that increasing the fractional saturation of the membrane does not affect the fraction of chymotrypsin-sensitive sites which are present. The fraction of hemoglobin released remains constant at 23–30%. This percentage agrees very well with the titration measurements reported earlier [16].

Fraction of phospholipase C-sensitive hemoglobin binding sites on inside-out vesicles. Studies with phospholipase C preparations from three bacterial species

There is considerable evidence that hemoglobin can interact with phospholipids under artificial conditions such as liposome preparations [42–44]. Studies aimed at assessing the extent to which hemoglobin binds to phospholipids on the intact red cell membrane are virtually nonexistent. However, Szundi et al. [17] and Shaklai and co-workers [44], have shown that hemoglobin does interact more strongly with phospholipids known to exist on the inner surface of the membrane, especially PS.

In order to study the interaction of hemoglobin with the inner surface phospholipids of intact red cell membrane bilayers, we studied the fraction of hemoglobin released after addition of specific phospholipase C preparations which are known to selectively cleave certain phospholipids and not others.

The acidic phospholipids, PS, PE, and PI are known to lie on the cytoplasmic side of the bilayer [45] and are the most likely sites for hemoglobin binding [16]. The possibility that hemoglobin might bind to PI is particularly interesting since inositol phosphates are known to bind tightly to hemoglobin [46]. When PI specific phospholipase C from *S. aureus* was added to hemoglobin-bound vesicles, no significant amount of hemoglobin was released despite ^{31}P-NMR evidence for full enzyme activity.

Table I shows the effect of phospholipase C preparations from *B. cereus* and *C. welchii* on the fraction of hemoglobin released at two fractional saturations. In both cases phospholipase C from *B. cereus* released significantly more hemoglobin than did phospholipase C from *C. welchii*. ^{31}P-NMR analysis of the supernatant fractions showed that the enzymes were fully active as compared with their activity in the absence of hemoglobin. It has been well established that these two phospholipase C preparations differ in that the enzyme from *B. cereus* does hydrolyze PS while the enzyme from *C. welchii* does not [47]. Although phospholipase C from *C. welchii* does hydrolyze PE at a slower rate than the *B. cereus*, our reaction times were long enough to hydrolyze this phospholipid [47]. The *C. welchii* enzyme also does not hydrolyze PI [47]. Since phospholipase C from *B. cereus* cleaves all of the inner surface phospholipids except PI, which we have shown is not involved in hemoglobin binding, the results in Table I suggest that about 38% of the total hemoglobin binding capacity of the red cell membrane involves binding to the inner surface phospholipids. Furthermore, since the *B. cereus* enzyme cleaves PS while the *C. welchii* enzyme does not, about 10% of the bound hemoglobin is sensitive to specific cleavage of PS (Table I).

Comparison of the hemoglobin binding capacity of human and rabbit inside-out vesicles. Evidence for binding to the cytoplasmic aspect of glycophorin

At this point we have accounted for about 50 to 70% of the total hemoglobin binding capacity of the inner surface of the red cell membrane by identifying the chymotrypsin sensitive and phospholipase C-sensitive components. The other predominant cytoplasmic facing component of the membrane not directly sensitive to these treatments, which could potentially bind hemoglobin, is the major sialoglycoprotein known as glycophorin [48]. To our knowledge, the best estimate of the number of glycophorin molecules per cell is $1 \cdot 10^6$ [49]. Furthermore, the cytoplasmic aspect of this protein has a cluster of highly acidic amino acids which would favor hemoglobin binding [50].

In order to determine if hemoglobin binds to the cytoplasmic aspect of glycophorin in intact membranes, we have used a convenient 'experiment of nature'. It is known that rabbit erythrocyte membranes differ significantly from human membranes only in that they lack glycophorin [51,52]. We have compiled a table which compares the composition of human and rabbit membranes (Table II). The similarities in qualitative and quantitative composition of these two membranes are striking.

TABLE II

COMPOSITION OF HUMAN VERSUS RABBIT ERYTHROCYTE MEMBRANES

G3PD, glyceraldehyde-3-phosphate dehydrogenase.

Component	Human	Ref.	Rabbit	Ref.
Protein				
spectrin	+	58	+	51
band 3	+		+	
bands 4.1–4.2	+		+	
band 5 (actin)	+		+	
band 6 (G3PD)	+		+	
band 7	+		+	
Glycophorin	+		–	
Total lipid	$5.09 \cdot 10^{13}$	61	$4.15 \cdot 10^{13}$	62
Total cholesterol	$1.23 \cdot 10^{13}$		$1.19 \cdot 10^{13}$	
Total phospholipid	$3.11 \cdot 10^{13}$		$2.73 \cdot 10^{13}$	
All in (gm/cell)				
Phospholipid (%)		61		63
Lysophosphatidylcholine	1.62		0.3	
Phosphatidylcholine	30.56		33.90	
Phosphatidylethanolamine	27.18		31.90	
Phosphatidylserine	12.96		12.20	
Phosphatidylinositol	1.83		1.60	
Phosphatidic acid	1.78		1.60	
Sphingomyelin	25.75		19.00	
Others	1.50		–	
Fatty acid (%)		64		64
Saturated	42		40	
Mono-saturated	18		15	
Poly-saturated	40		45	

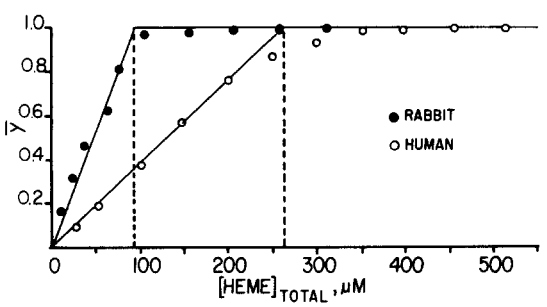

Fig. 1. Centrifugation measurements of the hemoglobin binding to human and rabbit inside-out vesicles under stoichiometric binding conditions. Fractional saturation is defined as the [heme] bound divided by the [heme] at saturation. The hemoglobin binding sites in rabbit inside-out vesicles are saturated with 88 μM hemoglobin [heme basis]. Human inside-out vesicles are saturated with 262.5 μM hemoglobin [heme basis]. Experiments were 5BT6. Both rabbit and human inside-out vesicles were matched to 2.87 mg of total protein per ml. Temperature prior to centrifugation was 25°C.

We have found that when matching concentrations of rabbit and human inside-out vesicles are studied for their hemoglobin binding capacity (i.e. stoichiometry), rabbit vesicles bind approximately 65% less hemoglobin (Fig. 1). This result was obtained in three separate experiments using blood from three different rabbits. The difference in binding capacity can roughly account for the remainder of those hemoglobin binding sites at the cytoplasmic surface not sensitive to the proteolytic or lipolytic enzymes used here. The difference in binding capacity was also observed when rabbit hemoglobin was used.

Although Table II indicates that both human and rabbit red cells contain band 3 protein, it was necessary to show that these two membranes have about the same number of band 3 sites. Since it is well established that DIDS binds almost exclu-

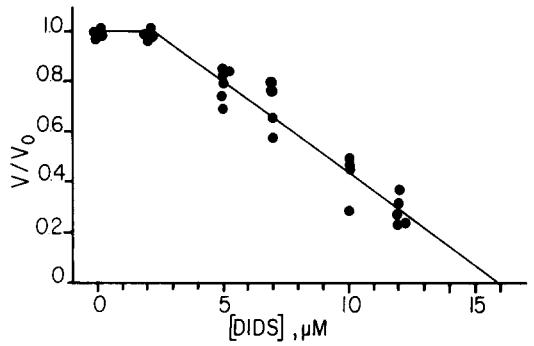

Fig. 2. DIDS inhibition of dithionite-phosphate exchange in intact rabbit erythrocytes. The results are from four different rabbits. The best linear least-square line was determined from the data after the 2 μM level owing to the obvious non-linear nature of this inhibition curve under stoichiometric conditions. The value of the intercept was 15.9 μM DIDS. Similar experiments with human cells gave a value of about 21 μM.

sively to band 3 under the reaction conditions employed, we studied DIDS inhibition of anion transport and compared the extrapolated concentration of DIDS necessary to cause 100% inhibition for a 50% rabbit erythrocyte suspension, with the same number previously published for intact human cells [16]. The results are shown in Fig. 2. The extrapolated intercept was about 16 μM while that reported previously for human was about 21 μM.

It will be noted that we always observe non-linear inhibition of transport by DIDS both for human and rabbit cells when the cell concentrations are high and stoichiometric binding conditions prevail. This has been thought to be a consequence of secondary binding of DIDS to glycophorin at the outer surface [53,54]. However, the fact that the same result is seen with rabbit cells which lack glycophorin suggests that other explanations be sought. Although the two intercepts do differ by 25% this small difference clearly can not account for 65% less hemoglobin binding capacity observed for rabbit vesicles vs. human (Fig. 1).

The cytoplasmic aspect of glycophorin was implicated in binding since treatment of unsealed human ghosts with neuraminidase had very little effect on the hemoglobin binding capacity as compared with control (approx. 5% less binding at the 78% saturation level). The absence of a significant effect of neuraminidase is consistent with our previous result which showed that selective digestion of membrane proteins at the outer surface, which also releases sialic acids [53,54], had no significant effect on hemoglobin binding [16].

Hemoglobin binding to glycophorin in reconstituted PC vesicles

Although the results comparing the hemoglobin binding capacities of human and rabbit vesicles offer strong evidence for the involvement of the cytoplasmic aspect of glycophorin in binding, they do not offer evidence for direct binding. We have attempted to obtain such evidence by studying hemoglobin binding to PC vesicles under stoichiometric binding conditions with and without isolated glycophorin prepared as described above. The vesicles which contained glycophorin had about 44 μM present per unit sample volume in the final suspension with hemoglobin. Assuming 1 : 1 binding with hemoglobin tetramer, we added 174 μM (heme basis) to both samples. After an incubation at room temperature for 10 min, the vesicles were centrifuged and the fraction of hemoglobin bound was measured as described above. PC vesicles bound about 17.8 ± 3.9% of the hemoglobin while PC vesicles containing glycophorin bound 38.3 ± 1.8% of the hemoglobin. The difference in binding capacity between the two samples is attributed to binding to glycophorin and is about 20% or about 35 μM in heme. If all of the glycophorin molecules had their cytoplasmic portions facing the external surface of the vesicle, we should have seen about 100% binding. However, when the vesicle preparations were incubated with neuraminidase (1 h 37°C with 50 U/mg of glycophorin) 80% of all sialic acid could be removed, in agreement with other findings [32]. This number suggests that only 20% of the glycophorin molecules have their cytoplasmic sides facing out in very good agreement with our findings.

Proteolytic- and lipolytic-induced hemoglobin release from rabbit inside-out vesicles

If the diminution in binding capacity of rabbit vesicles is due to the absence of glycophorin, then that hemoglobin which does bind to the rabbit membrane must predominantly bind to the cytoplasmic aspect of band 3 protein and to phos-

pholipids. As noted above, we have titrated intact rabbit erythrocytes with DIDS and found that they contain about the same number of band 3 sites as human cells. Digestion with chymotrypsin and phospholipase C from *B. cereus* should release virtually all of the bound hemoglobin. Table I compares these enzyme digestion experiments for human and rabbit inside-out vesicles. The combination of the fractional hemoglobin released from chymotrypsin plus phospholipase C from *B. cereus* was about a 100% for rabbit vesicles but only about 60% for human as noted previously. It will be noted that although the measurements shown in Table I were made at somewhat different fractional saturations, the ratio of chymotrypsin-sensitive binding sites to those which are sensitive to phospholipase C from *B. cereus* is approximately the same for the two membranes. This supports the view established in Table II and Fig. 2 and stated above that the phospholipid composition and number of band 3 protein molecules are the same for both membranes.

Discussion

Hemoglobin binds to the cytoplasmic side of the erythrocyte membrane in a reversible manner which depends on pH, ionic strength and hemoglobin concentration. Under conditions of optimal binding, multiple sites of association appear to exist [15]. The results of this paper suggest that the predominant sites of association are the cytoplasmic portions of the two integral membrane proteins, band 3 and glycophorin. Binding to phospholipid, although extant, appears to be a smaller fraction of the total binding capacity.

The total binding capacity of our human inside-out vesicle preparations (2.87 mg of protein per ml) was about 260 μM in heme or 65 μM on a tetrameric basis (Fig. 1). About 25% of the bound hemoglobin molecules were released upon digestion with chymotrypsin, which under our conditions, selectively cleaves the cytoplasmic portion of band 3 protein [40,41]. This fractional release seems reasonable when the concentration of band 3 protein in the sample volume is calculated. Since these vesicles are depleted of cytoskeletal proteins and glyceraldehyde-3-phosphate dehydrogenase, it may be assumed, for the sake of discussion, that the predominant mass of protein comes from band 3. With a molecular weight of 95 kDa and 2.87 mg/ml of protein present, we have about 30 μM of band 3 monomers present per unit sample volume. With a 1 : 1 stoichiometry of band 3 protein per Hb, we should expect about 46% of the binding capacity to involve band 3 protein which agrees with our findings within the uncertainties of the calculations.

Table I shows that the fraction of hemoglobin released by digestion with phospholipase C from *B. cereus* was about 38% at 78% total saturation for human inside-out vesicle preparations. Assuming this fractional release is maintained to 100% saturation, only about 63% of the total hemoglobin binding capacity of human membranes is attributable to band 3 protein and inner surface phospholipids. The results comparing human and rabbit membranes suggest that roughly one-half of the binding capacity of human membranes may be attributed to binding to the cytoplasmic side of glycophorin. This conclusion may be supported as follows. Firstly, we have shown in Table II, that these two membranes differ significantly in composition only in that rabbits lack glycophorin. Secondly, it is known that there are about the same number of copies of glycophorin per membrane ($1 \cdot 10^6$ [49]) as band 3 protein [55–58] and that these two proteins may form a 1 : 1 complex in the membrane [59]. Thus, we would expect our inside-out vesicle preparations to also contain about 30 μM of glycophorin per unit of sample volume. This number would account for the circa 50% of the total hemoglobin binding capacity not accounted for by hemoglobin binding to band 3 protein and phospholipids. Thirdly, the hemoglobin binding capacity of a matched suspension of rabbit inside-out vesicles is reasonably comparable to the amount of hemoglobin released by chymotrypsin and *B. cereus* phospholipase C digestion of human membranes, while the same enzymes can release virtually all of the hemoglobin from rabbit inside-out vesicles (Table I). Fourthly, neuraminidase treatment of ghosts does not significantly change the hemoglobin binding capacity. Finally, reconstitution of glycophorin into PC vesicles showed enhanced hemoglobin binding to a degree proportional to the fraction of

cytoplasmic-facing glycophorin molecules in the reconstituted system.

It should be noted that we purposely selected conditions so that stoichiometric and not affinity differences would be detected. Therefore, although the absence of glycophorin may be indirectly affecting the affinity of hemoglobin for the membrane, the more likely interpretation is that the difference reflects a diminution in the number of sites present. Similarly, the basic assumptions implicit in our experimental approach was that enzymatic cleavage directly releases hemoglobin from specific sites and that indirect effects are insignificant. We have shown that predigestion of the vesicles reduces the binding capacity to an extent comparable to the amount of hemoglobin release when, for example, chymotrypsin is added to human inside-out vesicles with hemoglobin bound. Despite these checks, it is true that the total amount of hemoglobin binding sites attributed to glycophorin (65%), to band 3 protein (25%), and to phospholipid (38%) exceeds 100% by about 28%. This difference certainly could be due to indirect effects. Considering the relatively high reproducibility of the experiments reflected by the small standard deviations, random experimental error is probably not a factor.

The most likely contribution to indirect effects in our experiments very probably are the effects seen with the phospholipases. Cleavage of phospholipid headgroups is probably not as specific as the action of chymotrypsin on band 3 protein in inside-out vesicle preparations or the absence of glycophorin in rabbit membranes. The removal of phospholipid head-groups might effect the structure of band 3 protein and glycophorin to a significant enough extent to cause some hemoglobin to be released. Whether or not the effect of phospholipases is considered direct or indirect, it is clear that PI is not involved in hemoglobin binding and that PS is, since more hemoglobin was released with the *B. cereus* enzyme than with the *C. welchii* enzyme.

The interaction of hemoglobin with glycophorin under the present conditions, raises the possibility that the interaction may occur in the intact cell under physiologic conditions. The study by Eisinger et al. [20] referred to above, used fluorescent energy transfer to study the interaction of hemoglobin binding with the membrane in intact cells; using DIDS-labeled band 3 protein and anthroyl stearate (n-AS) labels inserted into the lipid bilayer as donor molecules. Although there are several lines of evidence suggesting that hemoglobin can bind to band 3 protein [15], the binding observed in the intact cell might also involve glycophorin owing to the possibility that these proteins may form a 1:1 complex in the membrane [59,60]. We are currently planning experiments to measure the relative affinities of hemoglobin for isolated band 3 protein and glycophorin.

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