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ASYMMETRIC BINDING OF CYTOCHROME b_5 TO THE MEMBRANE OF HUMAN ERYTHROCYTE GHOSTS

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Summary

The intact, amphipatic form of cytochrome b_5 could bind to unsealed ghosts, but not to resealed ghosts, suggesting that the cytochrome could bind only to the inner (cytoplasmic) surface of the ghost membrane. This was further confirmed by the finding that the cytochrome could bind to closed, inside-out vesicles prepared from the ghosts. This asymmetric binding was not due to the exclusive localization of sialic acid and sugar chains on the outer surface of the ghosts membrane, because the cytochrome could not bind to ghosts even after enzymatic removal of these components. Although liposomes consisting of phosphatidylcholine or both phosphatidylcholine and sphingomyelin could effectively bind the cytochrome, this binding capacity was progressively decreased as increasing amount of cholesterol was included in the composition of phosphatidylcholine liposomes. Removal of cholesterol from resealed ghosts by incubation with egg phosphatidylcholine liposomes resulted in the binding of cytochrome b_5 to the outer surface of the treated ghosts. The possibility is discussed that the asymmetric binding is due to preferential localization of cholesterol in the outer leaflet of the lipid bilayer that constitutes the ghost membrane.

Introduction

Previous studies have shown that both cytochrome b_5 [1—3] and NADH-cytochrome b_5 reductase [4,5], purified from liver microsomes after solubilization with detergents, are amphipathic proteins, each consisting of a hydrophilic moiety carrying the prosthetic group and a hydrophobic segment. Treatment of these proteins with proteases causes the scission of the molecules at the junction between the hydrophilic and hydrophobic parts [1,3—5], and the hydro-

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philic fragments thus produced still retain their respective catalytic activities [6]. It has also been shown that the amphipatic form of these proteins, but not their hydrophilic fragments, can bind in vitro to liver microsomes, indicating that the hydrophobic segments of the proteins are responsible for their tight binding to the microsomal membrane [5,7—9]. However, their binding in vitro is not restricted to microsomes; instead, they can bind rather nonspecifically to a variety of natural and artificial lipid bilayer membranes such as those of liver mitochondria [5,7] and phosphatidylcholine liposomes [10—14]. The only reported exception to this lack of specificity is the finding of Strittmatter et al. [7] that cytochrome b_5 is unable to bind to human erythrocyte membrane although Mihara and Sato [5] have reported efficient binding of NADH-cytochrome b_5 reductase to sonicated human erythrocyte ghosts.

This paper reports that the intact, amphipathic form of cytochrome b_5 can bind only to the inner (cytoplasmic) surface of the erythrocyte ghost membrane and presents evidence suggesting that the asymmetric distribution of cholesterol in the ghost membrane is responsible for the asymmetric binding of cytochrome b_5.

Materials and Methods

Enzymes and chemicals

The intact, amphipathic form of cytochrome b_5 and its trypsin-solubilized fragment were purified from rabbit liver microsomes as described by Spatz and Strittmatter [3] and Ikeda et al. [15], respectively. Both preparations obtained were essentially homogeneous upon 15% cross-liked polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulfate and were practically free from phospholipids and detergents. Trypsin, soybean trypsin inhibitor, bovine brain sphingomyelin, and *Clostridium perfringens* neuraminidase were purchased from Sigma, and dextran T-10 and T-110 from Pharmacia. Egg-yolk phosphatidylcholine was purified by the method of Singleton et al. [16] and stored at −70°C under nitrogen gas. The other chemicals used were of the highest quality available.

Unsealed and resealed human erythrocyte ghosts

Outdated human blood in acid citrium dextrose solution was supplied by Midori Juji Company Ltd., Osaka. The erythrocytes obtained from the blood were washed three times with 5 mM sodium phosphate buffer, pH 8.0, containing 0.15 M NaCl and then subjected to hemolysis in the buffer containing no NaCl. The resultant ghosts were washed repeatedly with 5 mM sodium phosphate buffer, pH 8.0, and finally suspended in 5 mM sodium phosphate buffer, pH 7.0. This suspension was used as the unsealed ghost preparation. Resealed ghosts were prepared essentially as described by Bodeman and Passow [17]. 10 ml of freshly prepared unsealed ghosts in 5 mM sodium phosphate buffer, pH 8.0 (about 4 mg protein per ml), was slowly added with gentle stirring to 200 ml 5 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.1 mM $MgSO_4$ (referred to as "isotonic buffer"). The mixture was incubated at 37°C for 60 min and centrifuged at 20 000 × *g* for 10 min. The precipitate was resuspended in the isotonic buffer to about 0.8 mg of protein per ml, and 15

ml of the suspension was layered over 15 ml of a dextran T-10 solution (1.6 g of dextran was dissolved in 50 ml of the isotonic buffer). This was then centrifuged at 22 500 rev./min for 60 min in a Beckman SW 25.1 rotor. The resealed ghosts floating at the surface of the dextran layer were collected washed with 10 vol. of the isotonic buffer, and suspended in the same buffer.

Inside-out vesicles derived from erythrocyte ghosts

Inside-out vesicles were prepared from erythrocyte ghosts essentially as described by Steck and Kant [18]. The pelleted unsealed ghosts were diluted with 40 vol. of 0.5 mM sodium phosphate buffer, pH 8.0, and the suspension was incubated at 0°C for 90 min. The ghosts recovered from the incubation mixture by centrifugation were converted to small vesicles by forcing them to pass through a hypodermic needle of gauge 27 at least 3 times. The homogenized ghosts were mixed with 2 vol. of 0.5 mM sodium phosphate buffer, pH 8.0. This suspension (5 ml) was layered over 5 ml of a dextran T-110 solution (8 g of dextran was dissolved in 100 ml of 0.5 mM sodium phosphate buffer, pH 8.0) and centrifuged at 37 000 rev./min for 60 min in a Beckman SW 41 rotor. The inside-out vesicles accumulating at the interface of the two layers were collected, washed with 0.5 mM sodium phosphate buffer, pH 8.0, and suspended in an appropriate buffer.

Phosphatidylcholine liposomes containing cholesterol or sphingomyelin

A desired amount of cholesterol or sphingomyelin was dissolved in a chloroform/methanol (2 : 1, v/v) solution containing a known amount of egg-yolk phosphatidylcholine. After removal of the solvent by evaporation, 50 mM Tris · HCl buffer, pH 8.0, containing 1 mM EDTA was added to the residue to make the phosphatidylcholine concentration 10.6 mM, and the suspension was mixed vigourously in a Thermo-Mixer (Thermonics, Ltd., Tokyo). The preparation thus obtained contained large, multilamellar phosphatidylcholine liposomes containing a known amount of cholesterol of sphingomyelin. These liposomes could be sedimented from the suspension by centrifugation at 105 000 × *g* for 30 min.

Trypsin and neuraminidase treatment of resealed ghosts

3 ml of resealed ghosts in the isotonic buffer (about 5 mg of protein per ml) was mixed with 3 ml of the buffer containing 136 μg of trypsin per ml, and the mixture was incubated at 37°C for 60 min. The ghosts were then precipitated and washed once with the isotonic buffer. The washed ghosts were resuspended in 5 ml of the isotonic buffer containing 124 μg of trypsin inhibitor per ml and the suspension was incubated at 37°C for 15 min. The trypsin-treated ghosts were again sedimented, washed with the isotonic buffer, and finally suspended in the same buffer. For neuraminidase treatment, resealed ghosts (about 15 mg of protein) suspended in 3 ml of the isotonic buffer were mixed with 30 ml of 20 mM sodium phosphate buffer, pH 6.5, and then 3 ml of the isotonic buffer containing 0.36 unit of neuraminidase. The mixture was incubated at 37°C for 60 min and then centrifuged. The precipitate was washed once with the isotonic buffer and suspended in the same buffer.

Treatment of resealed ghosts with egg phosphatidylcholine liposomes

About 0.55 g of egg phosphatidylcholine dispersed in 10 ml of the isotonic buffer was placed in an ice bath and sonicated for 30 min in a Branson sonifier under a stream of nitrogen gas. The sonicated suspension was then centrifuged at 105 000 × *g* for 60 min and the supernatant was used as liposomes. Incubation of ghosts with the liposomes was conducted essentially as described by Bruckdorfer et al. [19]. A suspension containing resealed ghosts (21.8 mg of protein) and 415 mg of egg phosphatidylcholine liposomes in 12.9 ml of the isotonic buffer was incubated for 5 h at 37°C. The ghosts were then collected by centrifugation after 11-fold dilution with the isotonic buffer. The ghosts whose membrane had become permeable to macromolecules during the incubation were separated from resealed ghosts by centrifugation on the dextran solution as described above and only the resealed ghosts were used for the binding experiments.

Binding of cytochrome b_5 to ghosts and inside-out vesicles

Binding experiments with unsealed ghosts were conducted in 5 mM sodium phosphate buffer, pH 7.2, to prevent resealing during incubation, whereas the isotonic buffer was used for both resealed and inside-out vesicles. A mixture containing 60 μM cytochrome b_5 (either the intact form of the hydrophilic fragment) and ghosts (1.45 mg of protein, either unsealed or resealed) in 0.5 ml of the buffer was incubated at 37°C for 20 min. After the incubation, the mixture was diluted with 10 ml of the ice-cold buffer. The ghosts were then sedimented by centrifugation, suspended in 5 ml of the buffer, and analyzed for protein and cytochrome b_5. Binding of cytochrome b_5 to inside-out vesicles was similarly studied, except that the incubation mixture contained 55 μM cytochrome b_5 and inside-out vesicles (0.55 mg of protein) in a final volume of 0.5 ml.

Binding of cytochrome b_5 to liposomes

The incubation mixture contained, in a final volume of 0.5 ml, 50 mM Tris · HCl buffer, pH 8.0, 1 mM EDTA, 30 μM cytochrome b_5 (intact form), and multilamellar phosphatidylcholine liposomes containing a varying amount of cholesterol or sphingomyelin (9.54 mM with respect to phosphatidylcholine). After incubation at 37°C for 30 min, the mixture was diluted with 4.5 ml of 50 mM Tris · HCl buffer, pH 8.0, containing 1 mM EDTA and centrifuged at 105 000 × *g* for 30 min. The precipitated liposomes and the supernatant were then separately analyzed for cytochrome b_5.

Analytical and assay methods

Protein was determined by the Lowry et al. method [20]. Phospholipid phosphorus was estimated after digestion by heating in concentrated H_2SO_4 according to the method of Bartlett [21]. Phospholipid phosphorus in ghost preparations was assayed after precipitation of ghost membrane with 10% trichloroacetic acid followed by extraction of phospholipid with ethanol/ethyl ether (3 : 1, v/v). Cholesterol was determined as described by Zak [22]. Membrane-bound and neuraminidase-released sialic acid was measured by the method of Warren [23]. Cytochrome b_5 in transparent sample was determined from

the intensity of the oxidized Soret absorption peak at 413 nm, assuming a millimolar extinction coefficient of 117 [24]. The cytochrome in membrane preparations was estimated, after addition of 1% Triton X-100, from the dithionite-reduced minus oxidized difference spectrum, assuming that the increment of millimolar extinction coefficient between 424 and 409 nm is 185 [25]. Acetylcholine esterase and glyceraldehyde 3-phosphate dehydrogenase were assayed as described by Steck and Kant [18] in the absence and presence of 0.1% Triton X-100.

Results

Sealing status and membrane sidedness of ghosts and vesicles

As a first step of this study, it was necessary to check the sealing status and membrane sidedness of the erythrocyte ghosts preparations prepared as described above. For this purpose, glyceraldehyde 3-phosphate dehydrogenase [26] and acetylcholine esterase [27] were used as markers of the inner and outer surfaces of the ghost membrane, respectively. It was assumed that the intact ghost membrane is impermeable to the substrates of these enzymes and that this impermeability barrier is abolished completely by addition of 0.1% Triton X-100.

As shown in Table I, glyceraldehyde 3-phosphate dehydrogenase activity of the resealed ghost preparation was mostly cryptic in the absence of Triton X-100, but became accessible to the exogenously added substrates on addition of the detergent. Acetylcholine esterase, on the other hand, was fully active regardless of the presence and absence of Triton X-100. These results indicated that most of the ghosts in this preparation were completely sealed. In the case of unsealed ghost preparation, both marker enzymes were fully accessible to their substrates even in the absence of the detergent. Thus, it was evident that the ghosts in this preparation were actually unsealed, i.e. their membrane had

TABLE I

SEALING STATUS OF UNSEALED AND RESEALED HUMAN ERYTHROCYTE GHOST PREPARATIONS

Glyceraldehyde 3-phosphate dehydrogenase (inner surface marker) and acetylcholine esterase (outer surface marker) activities of unsealed and resealed ghost preparations were determined in the absence and presence of 0.1% Triton X-100. It was assumed that the membrane barrier against the substrates of the marker enzymes was completely abolished by the detergent. Accessibility represents: (activity in the absence of Triton X-100)/(activity in the presence of Triton X-100).

Marker enzyme	Ghost preparation	Triton X-100	Specific activity (unit/mg protein)	Accessibility (%)
Glyceraldehyde 3-phosphate dehydrogenase	Unsealed	+	1.98	98
		—	1.94	
	Resealed	+	0.134	14
		—	0.019	
Acetylcholine esterase	Unsealed	+	1.51	103
		—	1.55	
	Resealed	+	1.42	106
		—	1.50	

holes permitting free entry of exogenously added substances. It is to be noted that glyceraldehyde 3-phosphate dehydrogenase activity of the resealed preparation was much lower than that of the unsealed preparation even after full activation by Triton X-100. This was due to inactivation of the enzyme caused by exposure to 37°C for 60 min during the resealing process.

In contrast to the case of resealed ghosts, glyceraldehyde 3-phosphate dehydrogenase activity of the inside-out vesicles prepared from the ghosts was fully active even in the absence of Triton X-100, whereas acetylcholine esterase became fully accessible to the substrate only after the destruction of the membrane by the detergent (Table II). These findings indicated that most of the vesicles in the preparation actually had reversed membrane sidedness and were completely sealed. This conclusion could be further confirmed by measuring the neuraminidase-induced release of sialic acid, which is exclusively located on the outer surface of the ghost membrane [28]. Thus, in the absence of Triton X-100 only a small amount of sialic acid was releasable from the vesicles by neuraminidase, but most of the sialic acid could be released in the presence of the detergent.

Selective binding of cytochrome b_5 to inner surface of ghost membrane

Table III shows that both intact cytochrome b_5 and its hydrophilic fragment could not bind to the resealed ghosts to significant extents, indicating that the outer surface of the ghost membrane was devoid of the cytochrome b_5-binding capacity. It was, however, found that a significant amount of intact cytochrome b_5 was bound by the unsealed ghosts in which the inner surface of the membrane was available for the added cytochrome, suggesting that the binding took place at the inner surface. The possibility could, however, not be ruled out that the apparent binding was due to artifacts such as simple trapping of the cytochrome in the internal space of the ghost caused by resealing during the incubation. To avoid confusions arising from such artifacts, the binding of

TABLE II

MEMBRANE SIDEDNESS OF INSIDE-OUT VESICLES PREPARATION DERIVED FROM HUMAN ERYTHROCYTE GHOSTS

Glyceraldehyde 3-phosphate dehydrogenase and acetylcholine esterase activities of the inside-out vesicle preparation were determined in the absence and presence of 0.1% Triton X-100 as described in Table I. The vesicles (2.12 mg protein/ml in 50 mM Tris/maleate buffer, pH 5.7) were treated with neuraminidase (0.2 unit/ml), also both in the absence and presence of 0.1% Triton X-100, at 30°C for 40 min. The mixture was then directly analysed for released sialic acid. It was assumed that the intact membrane was impermeable to neuraminidase.

Marker	Triton X-100	Activity (unit/mg protein)	Accessibility (%)
Glyceraldehyde 3-phosphate dehydrogenase	+	2.37	95
	—	2.25	
Acetylcholine esterase	+	1.85	24
	—	0.44	
Sialic acid released	+	118 *	14
	—	16.8 *	

* nmol of sialic acid released per mg of vesicle protein.

the cytochrome to the inside-out vesicles was examined. As is also shown in Table III, these vesicles, though mostly constituting a closed system as discussed above, could effectively bind the intact cytochrome, providing firm evidence that the inner surface of the ghost membrane possessed the cytochrome-binding capacity. The hydrophilic fragment of cytochrome b_5 was also apparently bound by the vesicles, but to a much lesser extent than the intact cytochrome. It was likely that this "binding" of the fragment was due to some artifacts.

Effect of removal of sialic acid and sugar chains from ghosts

It is known that sugar chains associated with erythrocyte glycoproteins are located on the outer surface of the membrane [29,30] and the major glycoprotein of erythrocyte membrane contains most of the cell surface sialic acid at the terminal position of its sugar chains [28,30]. It was, therefore, likely that the strongly negative charge of sialic acid on erythrocyte membrane surface [31] is a factor preventing the binding of cytochrome b_5, an acidic protein [3], to the outer surface of the ghost membrane. To test this possibility, about 80% of the membrane-bound sialic acid was removed from the resealed ghosts by neuraminidase treatment. As shown in Table IV, however, this treatment could not endow the ghosts with the cytochrome b_5-binding capacity. Another possibility is that the densely distributed sugar chains on the outer surface exert steric hindrance against the cytochrome binding. An attempt was, therefore, made to remove the sugar chains from the resealed ghosts by trypsin treatment. It was hoped that this treatment would cleave a part of the glycoprotein molecule together with attached sugar chains from the outer surface of the membrane. As seen in Table IV, this treatment decreased the sialic acid content of the ghosts from 31.3 to 12.7 μg per mg of protein. This result indicated that the treatment had removed considerably more than 60% of the sugar chains, when the fact that trypsin also removed a significant portion of membrane protein was taken into consideration. At any rate, practically no binding of cytochrome b_5 could be observed to the trypsin-treated ghosts. It was, therefore, unlikely that the densely distributed sugar chains act as a barrier against the cytochrome binding. These results also suggested that trypsin did not affect the sealed nature of the resealed ghosts.

TABLE III

BINDING OF CYTOCHROME b_5 TO VARIOUS PREPARATIONS OF HUMAN ERYTHROCYTE GHOSTS

Binding reactions and determination of bound cytochrome b_5 were carried out as described in Materials and Methods.

Membrane preparation	Cytochrome b_5 added	Cytochrome b_5 bound (nmol/mg protein)
Resealed ghosts	Intact	0.16
	Hydrophilic fragment	0.10
Unsealed ghosts	Intact	1.35
	Hydrophilic fragment	0.17
Inside-out vesicles	Intact	1.98
	Hydrophilic fragment	0.52

TABLE IV

EFFECTS OF NEURAMINIDASE AND TRYPSIN TREATMENTS ON THE CYTOCHROME b_5-BINDING CAPACITY AND SIALIC ACID CONTENT OF RESEALED ERYTHROCYTE GHOSTS

Treatments of resealed ghosts with neuraminidase and trypsin were carried out as described in Materials and Methods. The ghosts thus treated were analyzed for sialic acid and also subjected to cytochrome b_5-binding experiments under the same conditions as employed for intact resealed ghosts. For comparison, the data obtained for untreated resealed ghosts are also included.

Ghost preparation	Sialic acid (μg/mg protein)	Cytochrome b_5 added	Cytochrome b_5 bound (nmol/mg protein)
Untreated	31.3	Intact	0.16
		Fragment	0.10
Neuraminidase-treated	5.9	Intact	0.33
		Fragment	0.49
Trypsin-treated	12.7	Intact	0.23
		Fragment	0.46

Effects of cholesterol and sphingomyelin on cytochrome b_5 binding to phosphatidylcholine liposomes

In contrast to intracellular membranes such as microsomes, plasma membranes of animal cells including human erythrocytes are characterized by high contents of cholesterol. It has also been reported that sphingomyelin is present in erythrocyte membrane in a significant amount [32] and that this phospholipid and phosphatidylcholine are both concentrated in the outer leaflet of the erythrocyte membrane bilayer [33—35]. It is, therefore, likely that cholesterol and sphingomyelin have something to do with the asymmetric binding of cytochrome b_5 to the ghost membrane. To obtain a clue to the possible roles of these lipid components in regulating the cytochrome binding, we took advantage of the fact that phosphatidylcholine liposomes can bind intact cytochrome b_5 effectively [10—14]. Thus, multilamellar liposomes composed of egg-yolk phosphatidylcholine and either cholesterol or sphingomyelin at various ratios were prepared, and the binding of cytochrome b_5 to these liposomes were examined. As shown in Fig. 1, the capacity of phosphatidylcholine liposomes to bind the intact cytochrome decreased progressively as the content of cholesterol in the liposomes was increased. Inclusion of sphingomyelin to phosphatidylcholine liposomes, on the other hand, did not reduce, but considerably increased, the amount of cytochrome b_5 incorporated into the liposomes. It was thus clear that the presence of cholesterol, but not sphingomyelin, inhibits the binding of cytochrome b_5 to phospholipid bilayer membranes.

Cytochrome b_5 binding to cholesterol-depleted resealed ghosts

Since addition of cholesterol to phosphatidylcholine liposomes inhibited the binding of cytochrome b_5 to the liposomal membrane as described above, it seemed likely that cholesterol is exerting a similar effect in the ghost membrane, especially in view of the fact that the molar ratio of cholesterol to total phospholipids in human erythrocyte membrane is nearly 1 : 1 [38]. To confirm this possibility, we examined whether or not cytochrome b_5 could bind to resealed ghosts from which cholesterol had been partly removed. Depletion of cholesterol was effected by incubation of resealed ghosts with an excess of egg

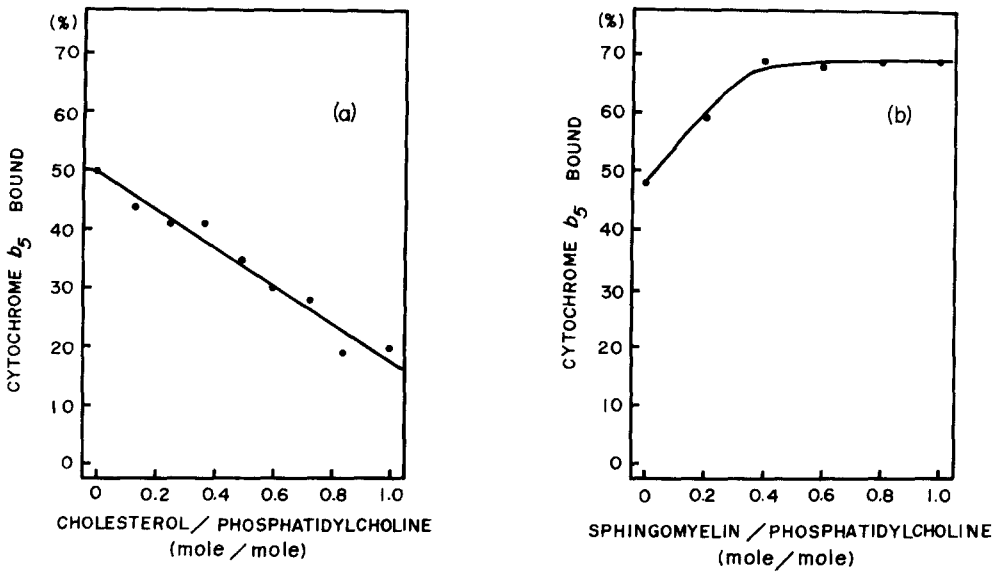

Fig. 1. Effects of cholesterol and sphingomyelin on the capacity of egg-yolk phosphatidylcholine liposomes to bind the intact form of cytochrome b_5. Preparation of phosphatidylcholine liposomes containing an indicating amount of cholesterol or sphingomyelin and cytochrome b_5 binding experiments were conducted as described in Materials and Methods. Under the conditions employed about 50% of the cytochrome b_5 added was bound by phosphatidylcholine liposomes. (a) Effect of cholesterol. (b) Effect of sphingomyelin.

phosphatidylcholine liposomes essentially as described by Bruckdorfer et al. [19]. The ghosts thus incubated were subjected to centrifugation on the dextran solution to remove unsealed ghosts that had been formed during the incubation. As shown in Table V, this treatment actually decreased the cholesterol content in the ghost membrane by 43%, though the ratio of phospholipids to protein in the ghosts remained essentially unchanged. The data in Table VI indicate further that the cholesterol-depleted ghosts thus prepared could bind a much more amount of intact cytochrome b_5 than the untreated ghosts. The actual amount of cytochrome b_5 bound to the depleted ghosts was, however, not so high as that observed with unsealed ghosts and inside-out vesicles (cf. Table III); this was probably due to the considerable amount of cholesterol

TABLE V

EFFECTS OF EGG PHOSPHATIDYLCHOLINE TREATMENT ON CHOLESTEROL CONTENT OF RESEALED ERYTHROCYTE GHOSTS

Treatments of resealed ghosts with egg phosphatidylcholine liposomes was conducted as described in Materials and Methods. The content of total phospholipid in ghost membrane was determined by organic phosphorus assay assuming the average molecular weight of 770 for phospholipid in ghosts.

Ghost preparation	Cholesterol/phospholipid (mg/mg)	Phospholipid/protein (mg/mg)
Resealed	0.427 (100%)	0.454
Phosphatidylcholine-treated	0.245 (57%)	0.429

TABLE VI

EFFECTS OF EGG PHOSPHATIDYLCHOLINE TREATMENT ON THE CYTOCHROME b_5-BINDING CAPACITY OF RESEALED ERYTHROCYTE GHOSTS

The conditions employed for cytochrome b_5-binding experiments were the same as for intact resealed ghosts.

Ghost preparation	Cytochrome b_5 added	Cytochrome b_5 bound (nmol/mg protein)
Resealed	Intact	0.16
	Fragment	0.10
Phosphatidylcholine-treated	Intact	0.54
	Fragment	0.08

remaining in the ghost membrane even after prolonged incubation with phosphatidylcholine liposomes. Although these results appeared to support the view that high concentrations of cholesterol in the membrane prevents the cytochrome binding, the possibility still existed that the observed binding of cytochrome b_5 to the treated ghosts was a result of unsealing of the membrane during the cytochrome-binding assay. When the ghosts after incubation with cytochrome b_5 were examined by centrifugation on the dextran solution, it was in fact found that a considerable quantity of unsealed ghosts has been formed during the incubation, probably because of the fragility of the cholesterol-depleted membrane upon exposure to a high temperature. It was, however, found that high contents of cytochrome b_5 were present not only in the unsealed ghosts but also in the sealed ones (data not shown), indicating that the cytochrome had actually bound to the outer surface of the cholesterol-depleted ghost membrane.

Discussion

The results reported here indicate that the intact form of cytochrome b_5 can bind almost exclusively to the inner, but not outer, surface of the membrane of human erythrocyte ghosts. It is evident that this asymmetric binding is a manifestation of the asymmetry of the organization of the ghost membrane. Based on the results obtained by several different techniques such as freeze-fracture electron microscopy [36] and enzymatic and chemical labeling of proteins in situ [29,37], it has been reported that proteins of the erythrocyte membrane are more densely located in the inner layer and the outer surface is not saturated with proteins. If this is so, it is unlikely that the presence of proteins on the outer surface is the reason for the lack of cytochrome b_5 binding. As already discussed, it is also inconceivable that the negative charge of sialic acid and the presence of sugar chains of glycoproteins act as barriers against the cytochrome binding to the outer surface of the ghost membrane. Although there is the possibility that the sugar chains of membrane glycolipids, which cannot removed by trypsin treatment, is responsible for the lack of binding capacity, it does not appear likely that sugar of glycolipids behave so differently from those of glycoproteins.

It seems most likely that the asymmetric binding of cytochrome b_5 is mainly

caused by asymmetric distribution or organization of lipid components within the erythrocyte membrane. Several investigators have actually presented evidence that phosphatidylcholine and sphingomyelin are concentrated in the outer layer of the erythrocyte membrane [33—35]. However, the preferential localization is phosphatidylcholine and sphingomyelin in the outer layer does not seem to be responsible for the phenomenon in question, because liposomes composed of phosphatidylcholine alone or both phosphatidylcholine and sphingomyelin can bind cytochrome b_5 as reported in this study and by other workers [10—14].

One of the characteristic features of plasma membranes of animal cells including human erythrocytes is their high contents of cholesterol. This lipid component has, therefore, to be considered as a candidate causing the asymmetric binding of cytochrome b_5. The present study has shown that the cytochrome binding to egg-yolk phosphatidylcholine liposomes is inhibited by inclusion of cholesterol in the composition of the liposomes. It has further been found that the outer surface of ghost membrane becomes available for the cytochrome binding when cholesterol is depleted partially. Thus it is highly likely that the high content of cholesterol is mainly responsible for the inability of the outer surface of erythrocyte ghost membrane. As an inevitable consequence of these considerations, one has to postulate that the cholesterol content in the outer layer of the ghost membrane must be much higher than that in the inner layer. As a matter of fact, direct evidence for this asymmetric distribution of cholesterol in the erythrocyte membrane has recently been provided by Fisher [39] who developed a method to obtain the outer and inner halves of the membrane separately based on the freeze-fracture technique. Although further work is still needed to reach a decisive conclusion, it may be tentatively concluded that the preferential localization of cholesterol in the outer leaflet of the lipid bilayer of the erythrocyte membrane is mainly responsible for the asymmetric binding of cytochrome b_5.

At present, nothing is known of the mechanism by which cholesterol inhibits the binding of cytochrome b_5 to phospholipid membranes. Recent NMR studies have, however, provided evidence that cholesterol in phosphatidylcholine-cholesterol liposomes forms a complex with phosphatidylcholine at a molar ratio of 1 : 1 [40]. If this is so, then it is likely that cholesterol inhibits the binding by competing with cytochrome b_5 for phosphatidylcholine which also acts as the cytochrome binding site [10—14]. Cholesterol has also been shown to decrease the fluidity of various membranes [41]. This effect may also be involved in the cholesterol-induced inhibition of the cytochrome binding.

It is very likely that the property of preferential binding to the inner surface of the ghost membrane is not restricted to cytochrome b_5, but is shared by many other amphipathic membrane proteins such as NADH-cytochrome b_5 reductase. In this connection, the observation by Mihara and Sato [5] that the amphipathic form of the reductase could bind effectively to sonicated human erythrocyte ghosts deserves comments, since we have also observed efficient binding of intact cytochrome b_5 to the same preparation. It seems that sonication of the ghosts produces unsealed vesicles or membrane fragment, as reported by Mason and Lee [42], and in these preparations the inner surface of the membrane is freely available to the proteins added exogenously.

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