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LACTOPEROXIDASE LABELING OF ERYTHROCYTE MEMBRANES FROM THE INSIDE AND OUTSIDE*

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SUMMARY

Lactoperoxidase labeling of resealed erythrocyte ghosts has been performed as a means of determining which polypeptides of erythrocyte membranes extend through the membrane. The resealed ghost was chosen because it more nearly approximates the intact cell than does the isolated membrane. The interior surface of the membrane of the resealed ghost was labeled by sealing lactoperoxidase into the ghosts before adding $^{125}I^-$ and H_2O_2. Labeling from the outside was obtained by standard methodology. Only two polypeptides were shown to be labeled from the outside. One was the 100 000 molecular weight component (Component III) and the other was the major glycoprotein. Resealed ghosts labeled from the inside show substantial labeling of spectrin and Component III. The labeling of the glycoprotein is far less extensive, particularly when compared to the labeling of Component III. These results establish clearly that Component III spans the erythrocyte membrane in the resealed ghost, which is a close analog of the intact cell. The results for the glycoprotein are not nearly so clear. The iodinatable groups of the glycoprotein are relatively unreactive from the inside, suggesting that the protein conformation or the organization of the protein in the membrane prevents the ready accessibility of these groups to the cytoplasm.

INTRODUCTION

The structure of the erythrocyte membrane has been subjected to extensive investigation in recent years. Chemical modification and proteolytic digestion studies from a number of laboratories [1–6] have indicated the presence of two major polypeptide chains in the erythrocyte membrane which are accessible to the exterior surface of the cell. One of these is the glycoprotein which contains the bulk of the membrane sialic acid [1], and the other is a polypeptide with a mol. wt of approximately 100 000. Bretscher [7, 8] has proposed that both of these polypeptides extend through the membrane, based on chemical labeling studies of intact cells and isolated mem-

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branes, presumably labeling outside and outside plus inside in the respective species. Steck [9] has supported these proposals on the basis of proteolysis studies on inside out and rightside out vesicles, and Segrest et al. [10] have presented evidence for glycoprotein penetration of the membrane from lactoperoxidase labeling of intact cells and isolated membranes. Studies of these types have been criticized on two accounts. First, preparation of the isolated membranes may cause organizational changes in the membrane such that its structure no longer adequately represents the structure of the membrane of the intact cell [11]. In that case studies on the isolated membrane could not be directly related to the intact cell membrane structure. Evidence that such membrane alterations may occur has been presented in several studies [12–14], including some which are apparently not dependent on reagent permeability [13, 15]. Second, in permeable membranes reagents may react with components within the membrane bordering the channels through which the reagents traverse the membrane. This type of reaction would cause a misinterpretation of results obtained in comparing intact erythrocytes and isolated membranes [5]. In order to answer this question of the organization of the components in the membrane, it is clearly desirable to use a system which deviates as little as possible from the intact erythrocyte and which does not have a permeable membrane.

The resealed ghost* meets these requirements and has been used for permeability studies of erythrocyte membranes for a number of years [16]. Its permeability properties are quite similar to those of the intact erythrocyte [17, 18]. In addition proteolysis studies indicate that it has the same resistance to proteolysis as the intact erythrocyte, suggesting that there have been no major organizational changes [5]. Nonpenetrating reagents can be introduced into and maintained within the resealed ghost without significant leakage. It is clearly the best model for studies on the erythrocyte membrane and should be amenable to labeling from the inside if lactoperoxidase can be introduced into it in an active form. The present studies describe procedures for performing these experiments. Three major membrane polypeptides have been shown to be labeled from the inside to give a labeling pattern that is demonstrably different from that obtained by labeling from outside the intact cell or resealed ghost. The most noteworthy difference is the repressed labeling of the glycoprotein from the inside.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free ^{125}I was purchased as Na ^{125}I from New England Nuclear. Lactoperoxidase, isolated from bovine milk [19], was a gift from Dr K. E. Ebner. All other chemicals were of reagent grade.

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* The term resealed ghost is used to describe preparations which have been hypotonically hemolyzed at 4 °C, then brought back to isotonicity and incubated at 25 or 37 °C, without any intermediate washing procedures. Ghost or isolated membrane is used to describe preparations which have been hypotonically hemolyzed and washed with hypotonic buffers until they are essentially free of hemoglobin. Some confusion arises since the isolated membranes reseal under some conditions either in a normal or inverted condition [9, 24].

Labeling of erythrocytes from outside

Human blood was obtained and washed as previously described [20, 21]. 1 ml of washed, packed erythrocytes was suspended in 2 ml of labeling solution containing 150 mM NaCl, 10 mM Tris, 10 μM KI, 100 μCi/ml $Na^{125}I$ and 0.5 μM lactoperoxidase (pH 7.4) at room temperature. 25 aliquots of 20 μl each of 2.33 mM H_2O_2 in 0.155 M NaCl–7 mM phosphate (pH 7.4) were added at 15-s intervals to the suspension. After 5 min 400 μl of 100 mM KI and 3 ml of cold 0.155 M NaCl–7 mM phosphate (pH 7.4) were added and the suspension was centrifuged at 4 °C. The labeled erythrocytes were washed twice with 8 ml of 0.155 M NaCl–7 mM phosphate before hemolysis to obtain labeled membranes [21].

Labeling of resealed ghosts from outside

1 vol. of washed, packed erythrocytes was hemolyzed in 10 vol. of 10 mM Tris (pH 7.4) at 0 °C for 10 min, then made isotonic by adding 1 vol. of 1.42 M KCl–0.28 M NaCl–10 mM $MgCl_2$ at pH 7.4. After standing 20 min at room temperature or 37 °C, the suspension was centrifuged and resuspended in the labeling medium described above. Iodination was performed as described.

Labeling of resealed ghosts from inside

Procedure 1. 1 vol. of packed erythrocytes was hemolyzed in 10 vol. of 10 mM Tris, 10 μM KI, 70 μCi/ml $Na^{125}I$ and 0.5 μM lactoperoxidase (pH 7.4) at 0 °C, then resealed with the NaCl–KCl–$MgCl_2$ mixture described above. The resealed ghosts were washed at 4 °C, first with 20 ml of 0.155 M NaCl–7 mM phosphate, then with 8 ml of the same buffer. The washed resealed ghosts were then resuspended in 0.155 M NaCl–7 mM phosphate and iodinated by addition of H_2O_2 as described above. Membranes from the resealed ghosts were isolated as previously described [21].

Procedure 2. In order to enhance the incorporation of iodine from the inside, the washed ghosts resealed at 37 °C by Procedure 1 were resuspended in a solution containing 3.0 ml Krebs–Ringer phosphate, 0.3 ml $Na^{125}I$ (1 Ci/l) and 0.6 ml 1% bovine serum albumin. Addition of peroxide and preparation of membranes were performed as described above.

Peroxide concentration dependence studies

Membranes which had been resealed at 37 °C in the presence or absence of lactoperoxidase were suspended in 1.5 ml of labeling mixture containing 150 mM NaCl, 10 μM KI, 67 μCi/ml $Na^{125}I$ and 10 mM Tris (pH 7.4). Lactoperoxidase (0.5 μM) was also added to the samples to be labeled from outside. At room temperature 20 aliquots of 10 μl each of H_2O_2 solutions of different concentrations in 150 mM NaCl–10 mM Tris (pH 7.4) were added at 1-min intervals to the suspensions to give 1, 10, 100 and 1000 μM concentration of H_2O_2 in the reaction mixture after each addition. Subsequent steps to isolate labeled membranes were performed as above.

Trypsin and chymotrypsin treatments of labeled erythrocytes and resealed ghosts

Erythrocytes labeled from outside or resealed ghosts labeled from inside (approximately $3 \cdot 10^9$ cells) were treated with trypsin or chymotrypsin (0.5 mg enzyme per ml packed erythrocytes) in a volume of 2.0 ml of 0.155 M NaCl–7 mM phosphate (pH 7.4) for 60 min at room temperature. The enzymes were inhibited with phenyl-

methanesulfonylfluoride added in dimethylsulfoxide [5] and washed with incubation buffer before hemolysis to prepare membranes.

Analytical procedures

Membrane samples were solubilized and subjected to polyacrylamide gel electrophoresis on 5% gels as previously described [21] or subjected to electrophoresis on 7.5% gels (20 : 1, acrylamide : methylenebisacrylamide) for 18 h at 8 mA/gel. Appropriate quantities of washed membranes, hemolysates (after dialysis to remove iodide) or gel slices [22] were digested with NCS solubilizer (Amersham/Searle) in counting vials at 50 °C for 3 h. After addition of Bray's solution [23], the samples were counted on a Packard Tri-Carb Model 3320. Glyceraldehyde-3-phosphate dehydrogenase was assayed in the presence and absence of dodecylsulfate as previously described [24]. Hemolysate protein was determined as hemoglobin [25]. Membrane protein was determined by the method of Lowry et al.[26].

RESULTS

Labeling of human erythrocytes, resealed ghosts and ghosts with lactoperoxidase

Iodination with lactoperoxidase and ^{125}I has been used successfully to label surface proteins of intact erythrocytes [6, 27]. In order to label the erythrocyte from the inside it would be necessary to seal the lactoperoxidase into the membranes. It is desirable to perform the resealing immediately without any intermediate washing procedures in order to prevent membrane inversion or vesicularization. Procedures for resealing and for incorporating impermeable substrates into the resealed ghosts have been well documented [16, 18]. About 10% of the hemoglobin of the original erythrocyte remains within the resealed ghost, which shows permeability properties similar to those of the intact erythrocyte. We routinely use the activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase, measured in the presence and absence of sodium dodecylsulfate (which destroys membrane structure), to determine the extent of resealing in both resealed ghost and isolated membrane. A similar procedure has also been used by Steck [9] to determine sealing of isolated membranes.

In our initial experiments using Procedure I both lactoperoxidase and $^{125}I^-$ were sealed into the resealed ghosts at 25 °C, and the resealed ghosts were washed twice in the cold to remove membrane-external lactoperoxidase. Since the leakage of iodide at 4 °C was slow, both lactoperoxidase and most of the iodide were maintained within the membrane. The resealed ghosts were then treated with H_2O_2 at 25 °C to initiate labeling. The results of this type of experiment were compared to experiments in which erythrocytes and resealed ghosts were labeled from the outside. The resealed ghosts with lactoperoxidase inside were labeled and the distribution of the label into hemoglobin and the membranes was indicative of labeling from the inside (Table I). The membranes isolated from labeled intact erythrocytes and resealed ghosts were subjected to acrylamide gel electrophoresis in dodecylsulfate. Radioactivity profiles were obtained by slicing and counting gels, protein patterns by Coomassie blue staining and glycoprotein patterns by periodate–Schiff staining. Protein bands were identified by their position in the electrophoretic pattern, the distinctive shapes of certain bands and their calculated molecular weights. The designation of the bands by number is according to the scheme outlined in previous publications

TABLE I

LACTOPEROXIDASE LABELING OF CELLS, RESEALED GHOSTS AND MEMBRANES

Iodine incorporation into hemoglobin and membranes of erythrocytes, resealed ghosts labeled from inside (Procedure 1) and isolated membranes. Ghosts were resealed at 25 °C.

Experiment	Sample	Specific activity (cpm/μg protein)		Iodine distribution (% of total)	
		Membrane	Hemolysate protein	Membrane	Hemolysate protein
1	Erythrocytes	403	1.6	88	12
	Membranes	36 200	—	—	—
2	Erythrocytes	122	0.3	91.3	8.7
	Ghost labeled from inside	14	5.1	41.4	58.6

[5, 20, 21, 24]. The results of labeling intact erythrocytes were consistent with previous studies. Only Band III and the glycoprotein were labeled significantly. The labeling of resealed ghosts from the inside showed three peaks, corresponding to Bands I, III and IV. There was no detectable labeling of the glycoprotein. Membranes from three preparations of the resealed ghosts labeled from the inside were subjected to electrophoresis and stained. Bands I, III and IV were sliced from the gels and shown to contain 20, 30 and 25% of the radioactivity from the gel, respectively.

The initial experiments suffered from the fact that very low levels of iodine were incorporated into the membranes (Table I) because of leakage of iodide during washing and labeling. As a result the radioactivity peaks after electrophoresis were only about 5 times background and there was some variability in the relative amounts of iodine incorporated into Bands III and IV. In order to circumvent this problem the membranes were resealed at 37 °C and additional iodide was added from outside the membranes immediately before adding peroxide (Procedure 2). This resulted in substantially higher specific activities of the membrane proteins (compare Table II

TABLE II

SPECIFIC ACTIVITIES OF CELLS AND RESEALED GHOSTS LABELED AT 10 μM PEROXIDE ALIQUOT CONCENTRATION

Specific activities of membranes from lactoperoxidase labeling of intact cells, resealed ghosts from outside and resealed ghosts from inside. Ghosts were resealed at 37 °C. Labeling from the inside was performed by Procedure 2.

Sample	Specific activity (cpm/μg protein)	
	Membrane	Hemolysate protein
Erythrocyte	537	2
Resealed ghost (outside)	37 600	82
Resealed ghost (inside)	980	291

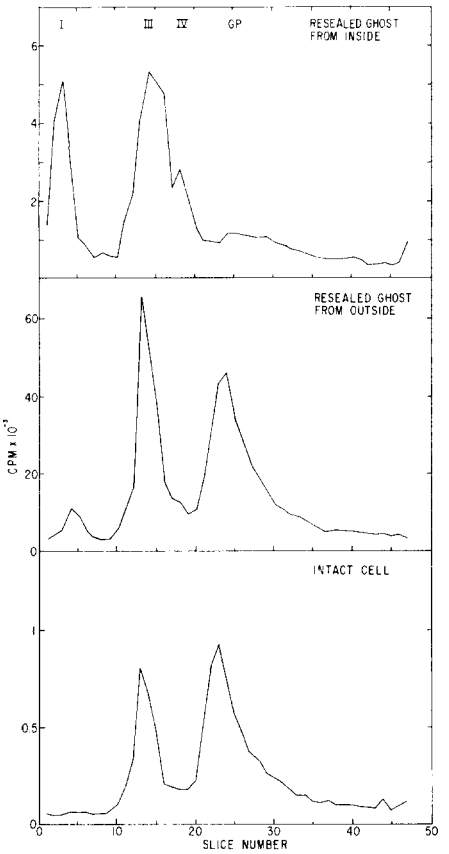

Fig. 1. Lactoperoxidase labeling of intact human erythrocytes from outside, resealed ghosts from outside and resealed ghosts from inside using Procedure 2. Cells and resealed ghosts were labeled as described under Experimental Procedures at 10 μM peroxide aliquot concentrations. Membranes were isolated and subjected to electrophoresis on 7.5 % acrylamide gels in dodecylsulfate for 18 h. Gels were cut into 2-mm slices for counting as previously described [21]. Components are labeled as reported previously [20]. GP, glycoprotein.

with Table I). The radioactivity profiles for labeled intact cells and resealed ghosts (labeled from outside and inside) are shown in Fig. 1. As expected, the intact cells show two peaks, corresponding to Components III and the glycoprotein. The labeling of resealed ghosts from the outside also shows primarily labeling of these two components, but there is additionally a small amount of the iodine label in Components I (spectrin) and IV, probably resulting from the small population of unsealed membranes. The resealed ghost labeled from the inside shows three peaks, corresponding to Components I, III and IV, with very slight labeling in the region of the glycoprotein. For comparison protein and glycoprotein profiles for the membranes are shown in Fig. 2. There were no significant differences in these profiles for membranes prepared from any of the labeled preparations or untreated control preparations. In order to achieve good resolution of the glycoprotein from Components III and IV,

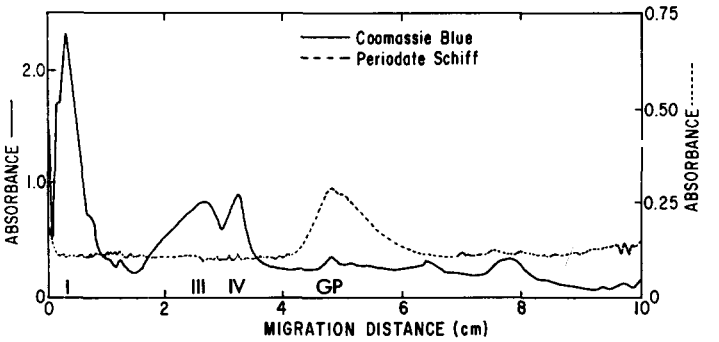

Fig. 2. Protein and glycoprotein patterns of companion gels from experiment described in Fig. 1. No differences were noted between patterns from labeled vs unlabeled or resealed ghost vs intact cell membranes. The gels were run for 18 h on 7.5 % gels to achieve satisfactory resolution of Components III and IV from the glycoprotein. As a result Component III has been distorted to show even greater asymmetry than usual and Component IV shows only one band instead of the usual two [5]. That the positions of the radioactivity are coincident with stained bands was shown by slicing out the stained sections for counting as well as by comparison of the profiles. About 60–70 % of the radioactivity on the gels could be recovered as the summation of the major peaks for each experiment.

the electrophoreses were run on 7.5% gels for long periods of time. As a result Band III shows even greater asymmetry than usual [20, 28] and the lower molecular weight polypeptides have been run off the gel.

One possible explanation for the low degree of labeling of glycoprotein is that the glycoprotein is less reactive than the other proteins and does not compete well for the iodine label. Therefore, the dependence of iodination on H_2O_2 concentration was studied in an attempt to saturate the labeling sites. Fig. 3 shows the incorporation of the iodine into the membrane and hemolysate as a function of aliquot concentration of H_2O_2 in the reaction mixture. Incorporation from the outside of resealed ghosts shows a maximum at 100 μM and a lower value at 1000 μM. Labeling of the membrane from the inside is higher at 1000 μM but does show indications of leveling off. Samples at concentrations higher than 100 μM showed considerable formation of methemoglobin and aggregates of membrane protein. Because of the possibility of membrane structural changes under these conditions, membrane labeling patterns were not examined at H_2O_2 concentrations above 100 μM.

Fig. 4 shows the radioactivity profiles of resealed ghosts labeled from the inside and outside using 100 μM peroxide. Under these conditions again only Component III and the glycoprotein are labeled from the outside. Spectrin and Component III show extensive labeling at higher peroxide concentrations with lactoperoxidase incorporated inside, but the relative labeling of Component(s) IV appears decreased. In addition, a small peak of label corresponding to the glycoprotein is demonstrated under these conditions.

The critical question in these experiments is whether labeling actually occurs from inside the membrane. The following observations support our contention that labeling does occur at the interior surface of the membrane. (1) The membranes are virtually all resealed. This was shown by glyceraldehyde-3-phosphate dehydrogenase assays in the presence and absence of dodecylsulfate (Table III). From these

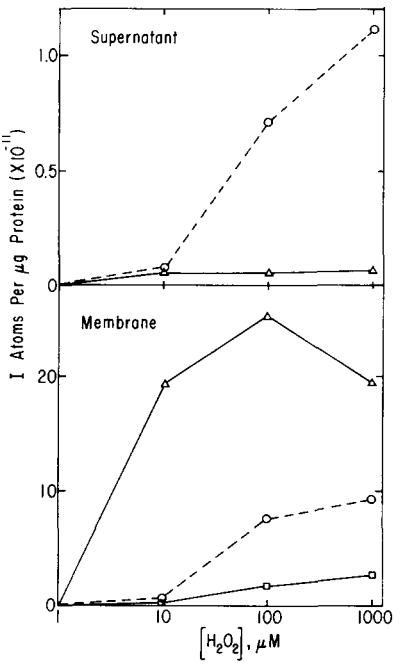

Fig. 3. Peroxide concentration dependence of labeling of membrane (bottom) and hemolysate proteins (top) of intact cell (□–□), resealed ghost labeled from inside (○---○) and resealed ghost labeled from outside (△–△).

TABLE III

DETERMINATION OF MEMBRANE RESEALING

Glyceraldehyde-3-phosphate dehydrogenase activity as a measure of resealing of ghost. Activity was measured in presence and absence of dodecylsulfate as described previously [24]. Activity is expressed as μmoles/min per ml erythrocytes.

Dodecylsulfate treatment	Glyceraldehyde-3-*P* dehydrogenase activity	% activity
None	0.51	3.6
1 mM	13.5	100

data it appears that about 95% of the membranes are resealed. This eliminates the possibility that labeling occurs within the membrane structure to a significant extent, since there is no movement of the lactoperoxidase across the membrane. (2) The ratios of the specific activities of membrane protein to hemolysate protein were 3.5 for resealed ghosts labeled from the inside and 460 for the resealed ghost labeled from the outside. The ratios of the total label incorporated into hemolysate protein to that of membrane protein were 2.3 for resealed ghosts labeled from the inside and 0.02 for resealed ghosts labeled from the outside. Both of these indicate that hemolysate labeling (primarily of intracellular hemoglobin) is about 100 times greater when

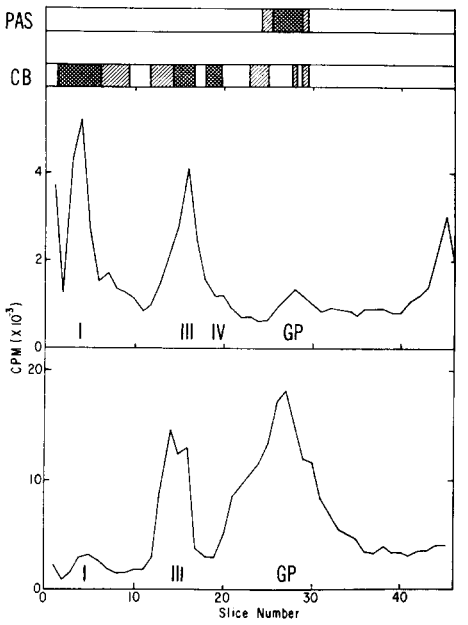

Fig. 4. Labeling of resealed ghosts from inside (top) and outside (bottom) at higher peroxide concentration as in Fig. 3. Aliquot addition gave 100 μM H_2O_2 in reaction mixture compared to 10 μM in Fig. 1. The specific activities of the iodide were not the same for the two experiments, so direct comparison of the iodide incorporated in each peak is not possible. PAS, periodate–Schiff reagent; CB, Coomassie blue reagent.

resealed ghosts are labeled from the inside than from the outside. This is consistent with the presumed interior localization of the lactoperoxidase. (3) Component I (spectrin) is heavily labeled from the inside but not the outside of resealed ghosts. Spectrin has previously been localized at the interior surface of the ghost by electron microscopy using ferritin-labeled anti-spectrin antibody [29]. (4) The glycoprotein is not appreciably labeled from the inside, except at high peroxide concentrations. Even this labeling is suspect, since some membrane structural alterations may be occurring under these conditions. This result does show that there is no lactoperoxidase outside the cells, or the glycoprotein would be labeled as it is with intact cells. The fact that there was negligible leakage of hemoglobin during the experiments also indicates that the membrane was not being traversed by proteins. (5) Limited proteolysis of labeled intact erythrocytes with trypsin or chymotrypsin releases 15–20% of the membrane-bound iodine, but no radioactivity is released from resealed ghosts labeled from the inside. The low amount of radioactivity released from the intact cells is indicative of the failure of the glycopeptides to release from the cell surface under the conditions used and the fact that the membrane sites were not completely labeled in this experiment. Hubbard and Cohn [6] noted about 40% of label released by a trypsinization of more heavily labeled cells.

All of the above criteria indicate that labeling of the erythrocyte membrane has been performed from the inside when lactoperoxidase was incorporated into the resealed ghosts.

DISCUSSION

The main focus of this work has been to determine whether the two major polypeptides accessible to the exterior surface of the erythrocyte membrane are also accesible to the interior surface, as was proposed originally by Bretscher [7, 8]. To do this we have designed a procedure for labeling the membranes from the inside by sealing lactoperoxidase into the membranes by techniques that have been used previously for incorporating metabolic substrates or ions for transport studies [16, 30]. This technique should be a general one that can be used for any type of membranes which can be sealed to give a reasonably impermeable vesicle. It is not even necessary that the entire population be resealed, as long as lactoperoxidase does not bind to the membranes significantly. Any membranes that are not sealed will simply lose their lactoperoxidase during washing and will not contribute to the labeling pattern. We have noted the same basic labeling patterns for partially resealed erythrocyte ghosts (with lactoperoxidase incorporation) as with those populations which were essentially completely resealed.

The resealed ghost was chosen for these studies as the closest model to the intact cells which could be experimentally managed. The available evidence strongly supports the conclusion that the interior surface of the membrane was labeled by lactoperoxidase incorporated into the resealed ghosts. The results of the labeling from the inside and outside indicate that Component III is accessible from both sides of the membrane. The situation with the major glycoprotein is not as clearly defined. Calculations of the amount of iodine incorporated from the outside of the resealed ghost indicate that approximately 10^6 atoms are incorporated per cell, using conditions of maximum incorporation. This would correspond roughly to one atom of iodide per molecule of glycoprotein and Component III, using Bretscher's estimations for the number of molecules of each component per cell [8]. Labeling from the inside under the same conditions attains only one-half to one-third that value per cell. This would correspond to somewhat less than one atom of iodide per molecule of labeled spectrin plus Component III based on the number of chains computed by Bretscher [8] or by Fairbanks et al. [28]. Certainly the amount of label incorporated into the glycoprotein from the inside would correspond to considerably less than one atom per glycoprotein molecule. These values are clearly only estimates because of the uncertainty in the number of polypeptide chains, but they do yield some idea of the amount of labeling which is possible under the stated conditions.

The major question which arises from this work concerns the limited susceptibility of the glycoprotein to labeling from the inside. One possibility is that the portion of the glycoprotein which is not accessible from the outside is buried within the membrane bilayer. However, Segrest et al. [10] have shown that both the C-terminal region, which has two tyrosine residues, and the N-terminal region, which has three, can be labeled with lactoperoxidase in the isolated leaky membrane. These results were taken to infer that the glycoprotein extends into the cell interior. Unfortunately, no indication was given of the quantitative distribution of iodine in these preparations, so it is not possible to determine what fraction of the glycoproteins may be labeled. If the C-terminal is buried within the membrane in the intact cell, it would have to undergo a structural rearrangement during hemolysis and washing of the ghosts to make it accessible in the isolated membrane. A schematic for this behavior is shown

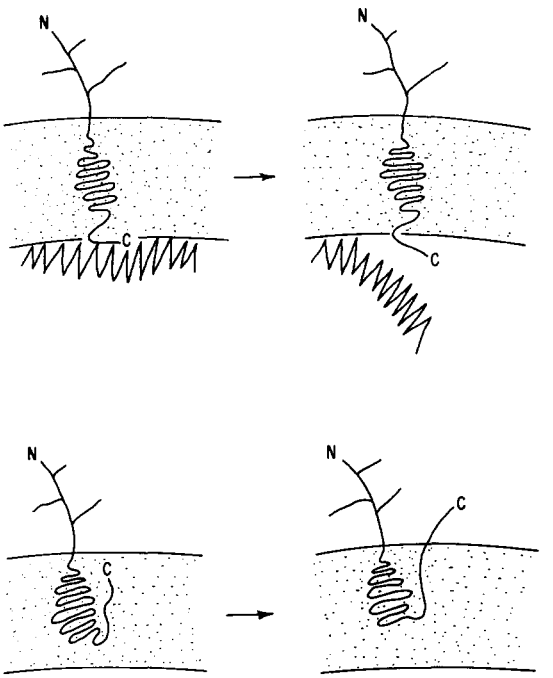

Fig. 5. Membrane models of the possible organizational or conformational changes of the glycoprotein during membrane preparation. These might account for the accessibility of the C-terminal peptide region of the glycoprotein in isolated leaky membranes [10] and its relative inaccessibility from either the inside or outside of the membrane in the resealed ghost. The bottom drawing shows how the glycoprotein might undergo a conformational change to render its C-terminal accessible to the outside surface of the membrane. The top drawing shows how hypotonic washing might dissociate spectrin from the membrane to some extent and expose the C-terminal of the glycoprotein which was previously buried.

as the bottom part of Fig. 5. A similar possibility is that the glycoprotein C-terminal extends into the cell interior in the intact cell, but is in a conformation that prevents iodination. It would then have to undergo a conformational change during ghost preparation to make it iodinatable to accommodate the results of Segrest et al. [10]. The final and most attractive possibility is that the glycoprotein extends through the bilayer but is blocked from accessibility to the cell interior by some component(s) at the inside of the membrane. If these were partially or wholly desorbed during the preparation of the leaky ghost, the glycoprotein would then become accessible from the inside. An interesting candidate for this masking would be spectrin, the fibrous protein which is present at the interior surface of the erythrocyte membrane. A model for this hypothesis is presented in Fig. 5. According to this proposal, spectrin is oriented along the interior face of the membrane and blocks access to the glycoprotein. During hypotonic hemolysis and washing the spectrin interaction with the membrane is broken (either partially or wholly), and spectrin may be partially depolymerized. At this stage the glycoprotein becomes accessible. Spectrin remains within the ghost either because it is still partially polymerized and too large to pass through the leaky membrane, or because it is still loosely attached to the membrane. Addi-

tional washing at low ionic strengths or with EDTA has been shown to further depolymerize spectrin, fragment the membranes and release soluble spectrin. The proposal is consistent with known properties of spectrin and with the behavior of the membrane during washing [31, 32]. Evidence that such an interaction is feasible has been presented by Nicolson [33], who showed that the distribution of cell surface sialic acid of the glycoprotein is altered after the perturbation of spectrin with anti-spectrin antibody.

The possibility of a direct interaction between spectrin and the glycoprotein introduces the possibility of considering a glycoprotein–spectrin complex as a transducing system to transmit information across the membrane and around its inner surface in a cooperative fashion. For example, a substance acting on the glycoprotein at the cell surface could trigger a conformational change in the glycoprotein which would cause concomitant changes in spectrin. The system could act in the reverse sense from the inside. This type of system is clearly of interest with regard to hormone action and other examples of trans-membrane phenomena.

After this work was completed a report appeared by Reichstein and Blostein [34], detailing similar studies on iodination of the inside of the erythrocyte membrane. They conclude that the glycoprotein interacts with components within the membrane rather than at its inner surface, but do not discuss alternative explanations for the lack of iodination of the glycoprotein from the inside of the cell.

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