ASYMMETRIC IODINATION OF THE HUMAN ERYTHROCYTE MEMBRANE*

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Summary. Exposure of erythrocyte membrane protein components on the inner and outer cell surfaces was probed using lactoperoxidase-catalyzed iodination. Resealed ghosts derived from I^{125}-labelled red cells were reiodinated with I^{131} a) on the outside by adding lactoperoxidase after the resealing process or b) on the inside by resealing with lactoperoxidase inside the ghost. SDS-polyacrylamide gel electrophoresis revealed two major radioactive components. One, ~ 90,000 daltons, was labelled on both surfaces; the other, a glycoprotein, was labelled only on the outer surface. Labelling of large molecular weight protein (> 200,000 daltons) was also observed, to a greater extent inside than outside.

Introduction. Determination of the nature and spatial disposition of membrane proteins is essential for understanding the mechanism of various membrane functions. Using the erythrocyte as a model a number of specific labelling techniques have been used to study membrane protein orientation and disposition, including labelling with non-permeant radioactive reagents (1,2), enzymic radioactive labelling (3), proteolytic digestion (4,5) and cross-linking (6,7). It has been shown that two of the major proteins are accessible at the outer surface of the erythrocyte (2,5, 8-10) and that one of these carries most of the carbohydrate of the membrane (2,5, 9-11). Evidence has also been obtained which suggests that these two components span the membrane and are thus exposed to both surfaces (2,5, 9-12).

In this study the accessibility of protein on the inner (cytoplasmic) and outer surface was probed by specific lactoperoxidase-catalyzed iodination of cells and resealed ghosts. The inner surface was labelled using ghosts resealed with lactoperoxidase (LP) inside, whereas the outer surface was labelled by adding the peroxidase after the resealing process.

Methods. Normal human blood was drawn into heparin and washed free of plasma with isotonic NaCl. Resealed ghosts were prepared by lysis of washed erythrocytes using 10 vol 5 mM phosphate, pH 7.4, at 0° for 5 min. Isotonicity was restored by addition of 5 M NaCl and the ghosts were resealed by incubating for 5 min at 0° and 30 min at 37° (13,14) and then washed twice with isotonic NaCl. Ghosts resealed

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Abbreviations: SDS, sodium dodecyl sulfate; LP, lactoperoxidase.

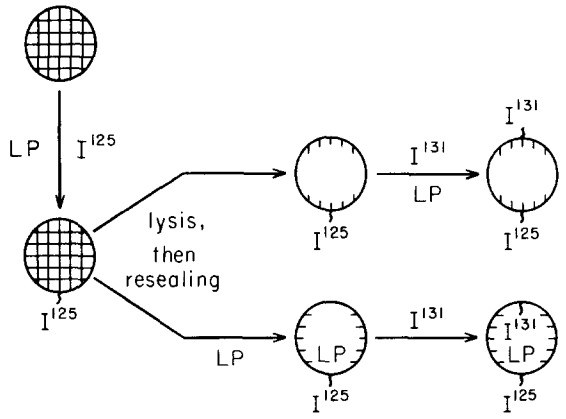

Fig. 1. Preparation of double-labelled membranes. Red cells were washed and labelled with I^{125} as described in the text. The iodinated cells were then lysed with either 5 mM sodium phosphate, pH 7.4 for relabelling the outside, or 5 mM phosphate containing lactoperoxidase (LP) for relabelling the inside. Following resealing and washing, the ghosts were relabelled with I^{131} either on the outside in a medium containing lactoperoxidase, or on the inside, in a medium lacking lactoperoxidase (see text).

with LP inside were prepared by lysis in the presence of 16 µg/ml LP.

Erythrocytes or resealed ghosts (approximately 50% packed cell volume) were incubated at room temperature for 30 min in a medium consisting of 1 µM KI containing 10-100 µC NaI^{125} or NaI^{131} (Frosst, carrier-free) 16 µg/ml LP (Sigma, 35-40 units/mg), 1 µg/ml glucose oxidase (Boehringer-Mannheim, 140 units/mg), 2 mg/ml glucose; these amounts of glucose and glucose oxidase generated H_2O_2 at a rate of 7 nmoles ml^{-1} min^{-1}. Erythrocytes were first labelled with NaI^{125} and then washed three times with isotonic NaCl. Resealed ghosts were similarly relabelled with NaI^{131}. Ghosts resealed with LP inside were also relabelled similarly, but with LP omitted (see Fig. 1). The labelling reactions were stopped by adding 10 vol ice-cold isotonic NaCl and then washing twice.

Membranes were prepared by the method of Dodge et al. (15) and glycoproteins were extracted by the method of Hamaguchi and Cleve (16). SDS-polyacrylamide gel electrophoresis on 3.5% or 7.5% gels was done according to the method of Lenard (17) The gels were divided into 1 mm minced segments and counted in a gamma spectrometer. Periodic acid Schiff (PAS) staining of the gels was carried out as described by Fairbanks et al. (18) and the stained gels were scanned at 560 nm. The gels were calibrated with the following molecular weight markers: ɣ-globulin, LP, bovine serum albumin, trypsin and pepsin. Proteins were determined by the method of Lowry et al. (19).

Results. When membranes are isolated from I^{131}-labelled resealed ghosts prepared from I^{125}-labelled erythrocytes and the proteins dissolved in SDS and separated by

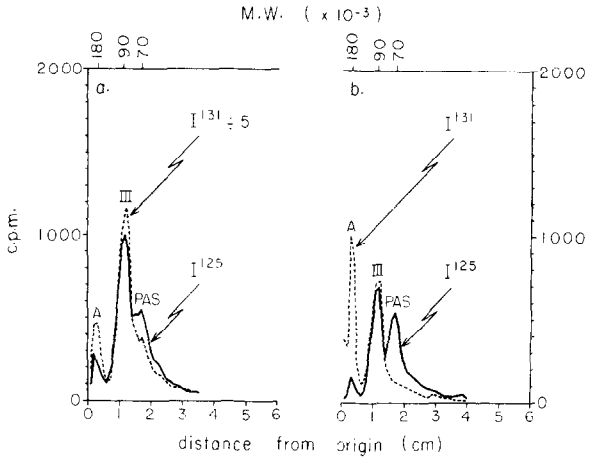

Fig. 2. Radioactivity profile of double-labelled erythrocyte membranes following electrophoresis in 7.5% SDS-polyacrylamide gels. a. I^{125}-labelled ghosts (117.2 µg protein) relabelled on the outside with I^{131}. b. I^{125}-ghosts (126.4 µg protein) relabelled on the inside with I^{131}.

polyacrylamide gel electrophoresis, two major peaks of I^{125} radioactivity are obtained. Using 7.5% gels (see Fig. 2) the one species has a molecular weight of ~90,000 and is designated as "III" in accordance with Fairbanks et al. (18). The mobility of the other corresponds to a molecular weight of 70,000 which is the same as the major PAS-positive material. (In Figs. 2 and 3, the location of the major carbohydrate-containing material determined on separate similar gels is indicated as the "PAS" peak). When the resealed ghosts are prepared from I^{125}-labelled red cells and are relabelled with I^{131} on the outside according to the scheme in Fig. 1, a similar radioactivity profile is observed (Fig. 2a). With ghosts relabelled on the inside (with lactoperoxidase inside the ghosts) the radioactivity pattern changes as seen in Fig. 2b. A peptide of ~ 90,000 daltons is relabelled but that of 70,000 daltons is not. A large molecular weight peak also appears near the top of the gel (see below). Similar results are obtained using 3.5% polyacrylamide gels (Fig. 3) except that the mobility of the major glycoprotein becomes less than that of the 90,000 M.W. protein(s), i.e. two peptides of approximately 90,000 (± 10%) and 130,000 daltons are labelled on the outside. In contrast a peptide of 90,000 daltons is labelled on the inside. In addition another broad peak of radioactivity corresponding to > 200,000 daltons is labelled, particularly on the inside, and is designated "A" on both Figures 2 and 3.

Glycoprotein was extracted from I^{131}(I^{125})-labelled ghosts, dissolved in 2% SDS containing 40 mM dithiothreitol and electrophoresed on 7.5% gels. As shown in Fig. 4 (typical experiment, the same preparation as the experiment shown in Fig. 3), glycoprotein from ghosts relabelled on the outside (Fig. 4b) is labelled with both

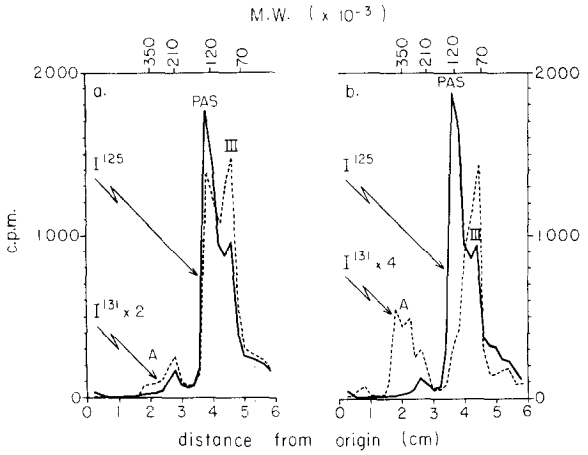

Fig. 3. Radioactivity profile of double-labelled erythrocyte membranes following electrophoresis in 3.5% SDS-polyacrylamide gels. a. I^{125}-ghosts (91.8 µg protein) relabelled on the outside with I^{131}. b. I^{125}-ghosts (90.5 µg) relabelled on the inside with I^{131}.

isotopes, whereas glycoprotein from ghosts relabelled on the inside is labelled almost exclusively with I^{125} (Fig. 4b). Staining of the extracted glycoprotein with PAS revealed one major and one minor component (Fig. 5); the mobility of the former in 7.5% gels coincides with that of the radioactivity peak in Figs. 4a and 4b.

In order to compare relabelling of glycoprotein on the inside with relabelling outside the ghost, the scale of the ordinate for I^{131} in Fig. 4b is twice that for the I^{131} in Fig. 4a, respectively. This adjustment corresponds to the difference in total I^{131} radioactivity (sum of all fractions) inside (2.2×10^4 cpm/mg) compared to total I^{131} outside (5.67×10^4 cpm/mg) as determined from the experiment in Fig. 3, i.e. if all protein components of both sides of the ghosts were relabelled equally, the relative areas under the I^{131} peaks shown in Figs. 4a and 4b would indicate the relative labelling of glycoprotein on the outside compared to the inside, respectively. This type of comparison may, in fact, maximize the I^{131} inside:I^{131} outside.

Discussion. Studies from other laboratories using enzymic iodination (3,8) and specific labelling with non-permeant reagents (1,2) have shown that two major protein species are exposed on the outer surface of the erythrocyte membrane. One of these proteins has a molecular weight of 90,000 daltons, migrates with ~25% of the membrane protein on polyacrylamide gels and spans the membrane (5,9), i.e. it is exposed on both the internal (cytoplasmic) and external cell surfaces. The present study using resealed ghosts with lactoperoxidase inside in the one case and outside, in the other, confirms this observation although the possibility that

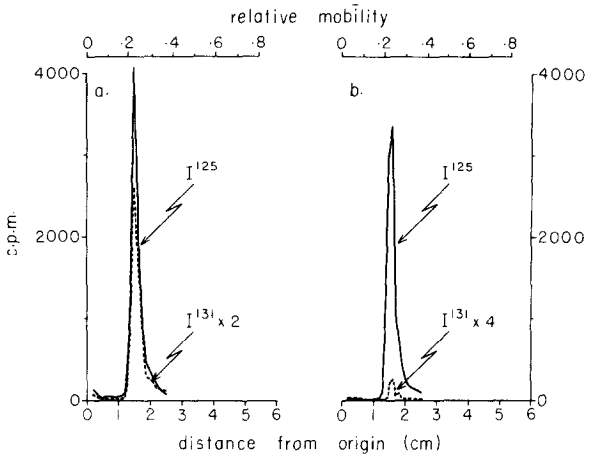

Fig. 4. SDS-polyacrylamide gel electrophoresis on 7.5% gels of glycoprotein extracted from double-labelled erythrocyte membranes. a. Radioactivity profile of glycoprotein extracted from ghosts relabelled on the outside with I^{131} Approximately 15,000 cpm I^{125} were applied to the gel. b. Radioactivity profile of glycoprotein extracted from ghosts relabelled on the inside with I^{131}. Approximately 12,500 cpm I^{125} were applied to the gel.

different proteins of similar molecular weight are labelled on either surface cannot be ruled out in the present study. A second major protein carried all of the sialic acid and most of the carbohydrate of the membrane (2,5, 9-11) and its apparent molecular weight on SDS polyacrylamide gels varies with the percentage gel as described previously (20) and confirmed in this study.

Previous studies have indicated that the major glycoprotein also spans the membrane; the evidence was based on studies using lactoperoxidase added extracellularly and showed that the major glycoprotein ('glycophorin') of intact cells and of resealed ghosts was labelled only at the N-terminal end of the polypeptide chain where all the sialic acid residues are located; however, "leaky" ghosts were labelled at both the N- and C-terminal ends of glycophorin (11,12). Assuming that glycophorin is a major fraction of the glycoprotein extracted in the present study and that the order of cyanogen bromide peptide fragments elucidated by Segrest et al. (12) is correct, then there should be sufficient tyrosine residues on the C-terminal fragment to be iodinated if this fragment does indeed penetrate the membrane and is exposed to the inside of the cell. Our study raises doubts that the glycoprotein can penetrate beyond the hydrophobic interior of the membrane, since I^{131}-radioactivity of glycoprotein extracted from ghosts labelled on the inside was almost negligible. The difference between our results and those from previous studies (11,12) is probably due to the difference in ghost preparations used; in the present study ghosts were resealed by isotonic reconstitution and labelled from the one side or the other. In other studies labelling of the outside

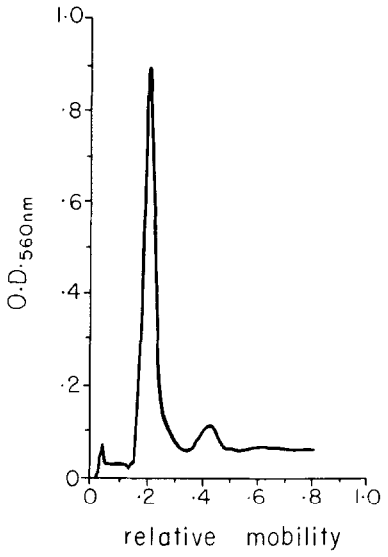

Fig. 5. 560 nm absorption profile of extracted glycoprotein.

of intact cells or resealed ghosts was compared to the labelling of "leaky" or fragmented ghosts.

The present observation that the membrane glycoprotein(s) has limited, if any, accessibility to the cell interior has important implications regarding its conformation within the hydrophobic domain of the membrane and its possible interactions with other membrane components. Recently observed cis- and trans-membrane perturbations of the surface distribution of acidic anionic residues (mainly N-acetyl neuraminic acid) suggest that glycoprotein molecules interact with components of both surfaces (21); our experiments suggest that interaction with components exposed to the cell interior would occur within rather than at the inner surface of the membrane. The inaccessibility of glycoprotein to the interior of resealed ghosts (c.f. reference 22) and its accessibility in "leaky" ghosts may, in fact, be evidence for glycoprotein mobility within the membrane under different conditions.

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