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DYE-SENSITIZED PHOTOCHEMICAL LABELLING OF PROTEINS IN THE ERYTHROCYTE MEMBRANE

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SUMMARY: A new approach to the study of the molecular arrangements of proteins in membranes is described. Irradiation with visible light of native erythrocytes or washed erythrocyte membranes suspended in buffers containing a) riboflavin, fluorescein or fluorescein coupled to dextran and b) ^{3}H-labelled tryptophan resulted in incorporation of radioactivity into the membrane proteins. Polyacrylamide gel electrophoresis of solubilized membranes followed by radioactivity measurements of the separated membrane proteins revealed that in native erythrocytes the protein components known to be located at the exterior cell surface, Band 3 and the major sialoglycoproteins became specifically labelled, whereas in washed lysed cells all of the major membrane proteins were labelled.

INTRODUCTION

An important aspect of membrane structure is the location of the proteins in the membranes. The use of probes to label the surface of cells or their isolated membranes has given us considerable insight into the general arrangement of proteins in membranes. Human erythrocytes have been a convenient model system for these studies (1). Proteolytic digestion (2) or labelling of intact erythrocytes with various classes of reagents, e.g. lactoperoxidase (3) or small molecules (4-6) have revealed that some of the protein components are exposed on the outside of the cell membrane. Components exposed on the inside of the erythrocyte membrane have been demonstrated using various reagents for the labelling of leaky ghosts (6) inside-out vesicles (7) or resealed ghosts (8,9).

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Each of the various labelling reagents used has its own advantages and disadvantages. One main problem is to attain sufficiently non-specific labelling to avoid misclassifications of proteins which might lack exposed reactive residues of some particular type.

In this report the labelling of proteins in the erythrocyte membrane has been studied using a technique based on photo-sensitized coupling of labelled substances of low molecular weight (10). The biological material to be studied is mixed with a dye and a labelled low molecular weight component and the mixture is illuminated. Photochemical processes then effect coupling of the labelled component to the accessible parts of the biological structures.

MATERIALS AND METHODS

Fluorescein was obtained from G.T. Gurr, London, and was purified as described earlier (11). Fluorescein-dextran (Mw 150000) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, riboflavin from Merck, Darmstadt, W. Germany and [^{3}H] tryptophan from the Radiochemical Centre, Amersham, England.

Erythrocytes were prepared from fresh blood by washing several times with isotonic sodium phosphate buffer pH 7.4. Lysis of labelled and unlabelled cells was achieved by rapid dilution with ice-cold water. The membranes were then carefully washed with 0.01 M sodium phosphate buffer pH 7.4. Irradiation of samples was performed from a distance of 10 cm using a 500 watt tungsten lamp (Philips Argaphoto B, PF 308). The samples of erythrocytes in a glass beaker (diameter 8 cm) or lysed cells in a glass tube (diameter, 2 cm) were placed in an ice-cooled 2 M sodium nitrite solution (lower wave-length cut off about 400 nm) and the vessel was agitated vigorously during irradiation. Following irradiation the samples were carefully washed and membranes were prepared from the irradiated intact erythrocytes as described above. A portion of the membranes from each sample was then subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) according to Neville (12). After electrophoresis the gel was stained for carbohydrate and the positions of the glycoprotein bands were marked. The same gel was then stained for protein with Coomassie blue. Staining was essentially done as described by Fairbanks et al. (13). A portion of the stained wet gel representing one sample (about 50 µg of protein) was cut into pieces which were weighed and dissolved in 30% H_2O_2 at 60°C for 15 h. The radioactivity of the samples was then counted using a toluene-based scintillation mixture containing 30% Triton X-100.

RESULTS

A suspension (1.3 ml) of native erythrocytes (corresponding to 0.5 ml of packed cells) in 0.12 M sodium phosphate buffer pH 7.4 containing 0.8 mM riboflavin and 0.25 M ^{3}H-labelled tryptophan was illuminated for 10 min. Only very slight hemolysis occurred during the illumination. After centrifugation, washing and preparation of the membrane fraction, the latter was subjected to SDS-polyacrylamide gel electrophoresis. The staining pattern was the same as that observed with a normal membrane preparation. The radioactive labelling pattern was determined by counting measurements on slices of the stained gel as shown in Fig. 1. The main labelled component corresponds in migration velocity to PAS-1, the main sialoglycoprotein band. The peak on

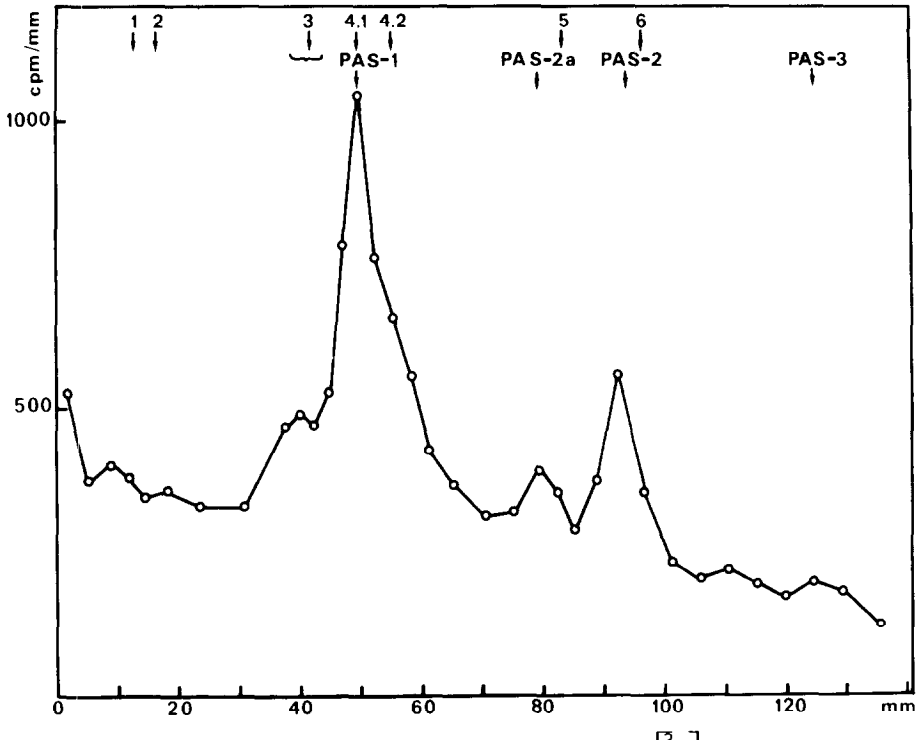

FIG. 1. Labelling of intact erythrocytes with [^{3}H] tryptophan. Radioactivity profile from a sliced polyacrylamide gel. The count rate per mm of gel was calculated and plotted against the migration distance. The positions of the main protein components as determined from the stained gel are indicated.

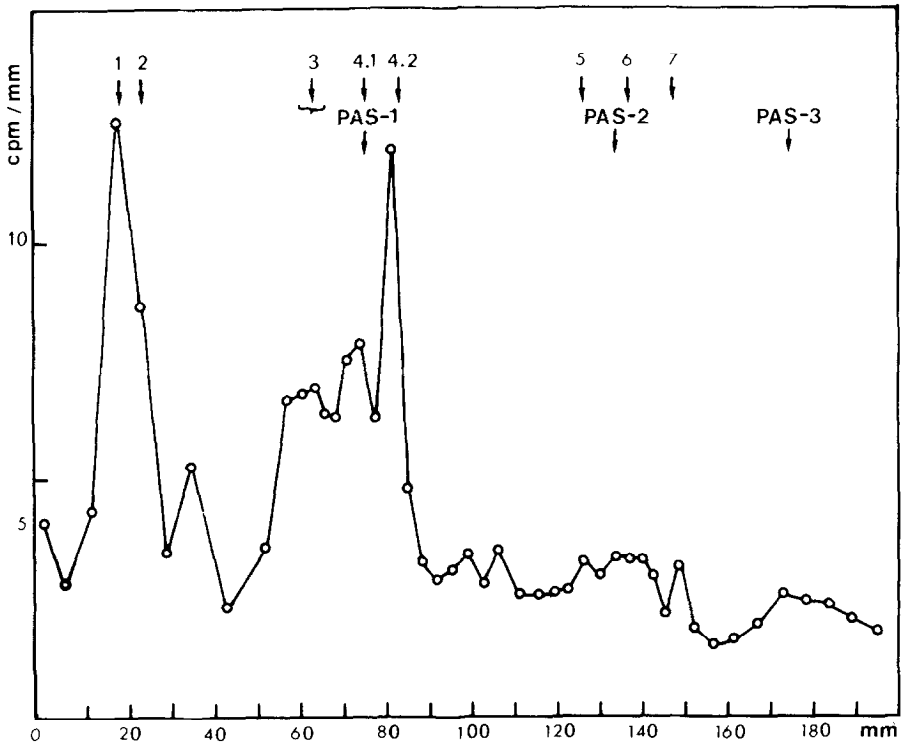

FIG. 2. Labelling of erythrocyte membranes with [^{3}H] tryptophan. Radioactivity profile of the sliced gel (cf. Fig. 1).

the left side corresponds to components in the Band 3 region. The minor labelled components on the right side correspond in migration velocity to the sialoglycoproteins PAS-2a, PAS-2 and PAS-3 (14). The same experiment was also performed using 1 mM fluorescein instead of riboflavin as the exciting dye. Similar results were obtained but the degree of labelling was much lower than was obtained with riboflavin.

Suspensions of 0.4 ml washed erythrocyte membranes from 0.15 ml packed cells containing 0.25 mM ^{3}H-labelled tryptophan and 0.8 mM riboflavin or 1 mM fluorescein were illuminated for different periods from 10 sec to 1 min. Analysis by SDS-polyacrylamide gel electrophoresis showed that considerable labelling had occurred, but also that most of the membrane proteins had formed

aggregates too large to penetrate the gel surface even with the shortest illumination times used. To avoid such aggregation, fluorescein coupled to dextran was used as the exciting dye. A suspension of 0.4 ml washed erythrocyte membranes in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.25% fluorescein-dextran corresponding to 0.3 mM fluorescein was illuminated for 1 min. Analysis by SDS-polyacrylamide gel electrophoresis showed that very little aggregation had occurred. The labelling pattern shown in Fig. 2 is quite different from that obtained with intact erythrocytes. All of the major protein components of the erythrocyte membrane seem to have been labelled, including bands 1 and 2.

DISCUSSION

Spikes and MacKnight reported earlier that only solvent exposed amino acid residues in proteins were accessible for dye-sensitized photo-oxidative reactions (15). Their finding seems to apply also for the coupling reaction described in this study. Labelling of native erythrocytes results mainly in labelling of the sialoglycoproteins and Band 3, all which are known to be on the outside of the erythrocyte membrane. Upon similar treatment of washed, unsealed membranes protein components which are known to be on the inside of the membrane (e.g., Bands 1 and 2) (1) become labelled as well.

The heavy labelling of the sialoglycoproteins as compared to Band 3 of native erythrocytes in this study indicates that the carbohydrate part has been labelled. The results obtained are consistent with the assumption that labelling occurs both with exposed proteins and the carbohydrate portion of the sialoglycoproteins, which has been shown to be highly exposed at

the exterior of the cell surface (1). The data thus suggests that the dye-sensitized photolabelling technique (10) is well suited for surface labelling of components located at the surfaces of biological membranes.

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