THE ARRANGEMENT OF PROTEINS IN THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

When the iodination of intact human erythrocytes was catalyzed by the enzyme lactoperoxidase, only a single stroma protein was iodinated. This protein component thus appears to be the only protein component exposed on the exterior of the human erythrocyte surface. The apparent molecular weight of this membrane protein is 90,000, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

INTRODUCTION

An understanding of the structural arrangement of components in biological membranes is essential to a mechanistic understanding of its function. One of the most thoroughly studied biological membranes is that of the human erythrocyte. Both qualitative and quantitative data on the lipids, carbohydrates and proteins of the erythrocyte membrane have been reviewed in a number of studies (1-4). More recent work has evaluated the topography of the erythrocyte membrane with respect to the carbohydrate and lipid moieties (5-7). Determination of the protein distribution, however, has resisted thorough investigation, although recent work has defined the number of peptide units present in red cell stroma (8). An evaluation of enzyme activities associated with intact erythrocytes and stroma has suggested that certain proteins are located on the inside of the erythrocyte membrane (9-12). No technique has been devised which unequivocally establishes the spatial arrangement of the proteins within this membrane. It is the object of this preliminary report to present studies on the vectoral arrangement of membrane proteins of the red cell. With the methods employed in this work, we have been able to show that only one protein component present in the red cell stroma is in an exposed position on the exterior of the human erythrocyte membrane. This component has a molecular weight of 90,000.

METHODS

All chemical reagents used in these studies were reagent grade. Lactoperoxidase was isolated

by the method of Morrison and Hultquist (13). The concentration of lactoperoxidase was calculated from the millimolar extinction coefficient of 114 at 412 nm. Hydrogen peroxide concentrations were determined from the optical density at 230 nm, using an extinction coefficient of 72.4.

Normal A+ human erythrocytes were drawn into ACD solution. These cells were then washed free of serum protein by the method of Dodge *et al.* (4). A two hour suspension of the washed erythrocytes at 22° was included to minimize the platelet count. The packed red cells were suspended in an equal volume of distilled water and lysed by freezing and thawing. The stroma were collected by the method of Dodge *et al.* (4), and purified according to this procedure. Stroma proteins were solubilized in 2-3% sodium dodecyl sulfate. The solubilized proteins were separated by electrophoresis on a 5% polyacrylamide gel in a buffer containing 0.1% sodium dodecyl sulfate according to the procedure of Lenard (14). Molecular weight estimations were determined by calibrating the gel with standard proteins (14). Hemoglobin was partially purified from the red cell hemolysate by precipitation with 70% saturated ammonium sulfate. The crude hemoglobin precipitate was resuspended in distilled water and the precipitation repeated a second time. Finally the hemoglobin was dissolved in distilled water and passed down a Dowex 1 anion exchange resin in order to exchange free anions of solution for chloride. Concentrations of hemoglobin were determined by the use of pyridine hemochromogen method (4).

Washed platelet free erythrocytes were iodinated. These washed cells were suspended in isotonic phosphate buffer pH 7.4 to give a solution containing 3.3×10^9 cells per milliliter. The cell suspension was then made to 10^{-5}M KI and 3.3×10^{-7} M lactoperoxidase. The iodination catalyzed by lactoperoxidase was initiated by the addition of hydrogen peroxide. It was necessary to maintain low concentrations of hydrogen peroxide because of membrane oxidations, and at no time did the hydrogen peroxide concentration exceed 8 μM. Since glutathione peroxidase (15) and catalase (16) will compete with lactoperoxidase for the hydrogen peroxide, it was added in aliquots at regular time intervals to reinitiate the reaction after one aliquot of hydrogen peroxide had been utilized. At the termination of the reaction, the red cells were centrifuged, the stroma collected, and the proteins solubilized and separated by electrophoresis as indicated above. Radioactive iodide distribution in the polyacrylamide gels was determined by slicing the gels laterally to 1 mm sections, and counting the gamma emissions from each slice on a gamma spectrometer.

RESULTS

The results show that iodination of intact erythrocytes is directly proportional to the amount of hydrogen peroxide employed. As figure 1 shows, the incorporation into the stroma fraction increases linearly with hydrogen peroxide, while the hemoglobin which is on the inside of the cell is not iodinated. Membrane proteins obtained from iodinated, intact erythrocytes were

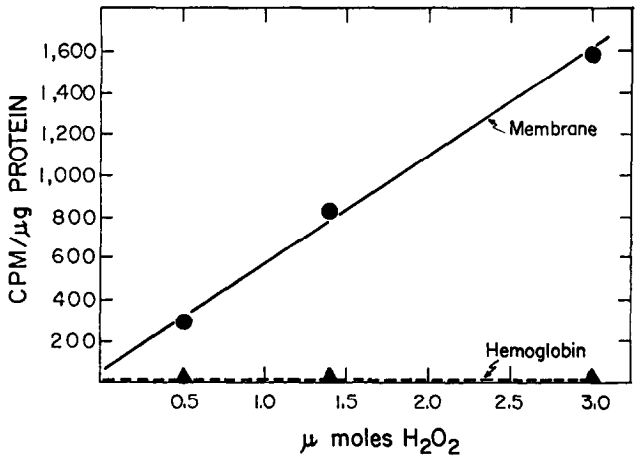

Figure 1. Iodide Incorporation Into the Stroma and Hemoglobin Fractions of Iodinated Human Erythrocytes. Erythrocytes were iodinated with increasing amounts of hydrogen peroxide as described in Methods.

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separated by polyacrylamide gel electrophoresis as is shown in figure 2. This gel system separates proteins on the basis of their molecular weight and confirms the protein pattern for human erythrocyte stroma recently presented by Lenard (14). Identical results were obtained from

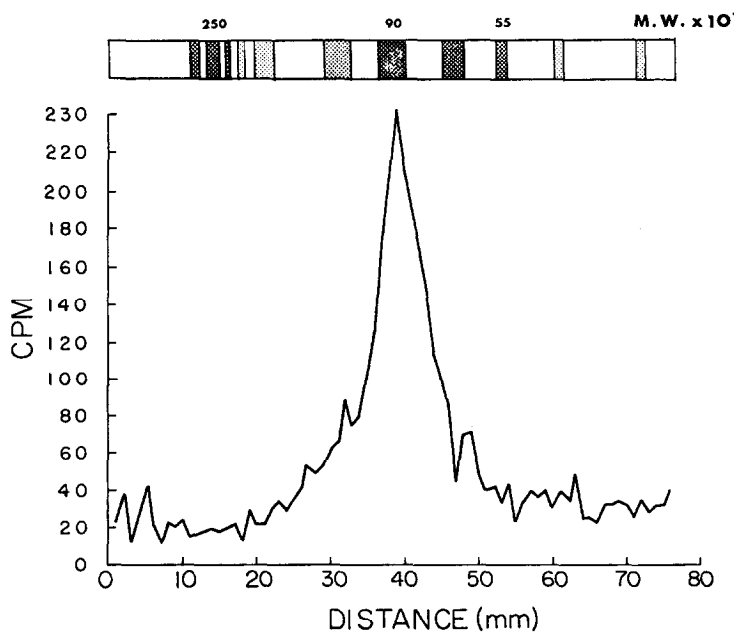

Figure 2. Separation of stroma protein components by SDS polyacrylamide gel electrophoresis. The iodine distribution into these components from iodinated intact erythrocytes is shown on the graph below.

untreated stroma, indicating that iodination has no effect on the resolution of the membrane proteins. The fractionated stroma shown in figure 2 were obtained from a preparation in which all available sites on the intact erythrocyte had been iodinated. The radioactive iodide distribution among the membrane proteins shown in figure 2 indicates that a single protein component with a molecular weight of 90,000 contains radioactive iodine.

DISCUSSION

In the present study iodide is employed as a membrane specific reagent to determine the vectoral arrangement of proteins in a direction perpendicular to the surface of the membrane. Lactoperoxidase catalyzes iodide incorporation into the exposed tyrosine and histidine groups on proteins according to the following equation.

$$\text{Protein} + H_2O_2 + {}^{125}I \xrightarrow{\text{Lactoperoxidase}} \text{Protein}\text{—}{}^{125}I$$

The predominant reaction occurs with tyrosine iodinating the aromatic ring ortho to the hydroxyl group (17). At the pH studied, 7.4, no detectable I_2 is formed by lactoperoxidase, and the iodination reaction has been shown to occur via an enzyme substrate complex between the protein substrate and lactoperoxidase (17). In the iodination of intact erythocytes, only those proteins on the exposed, exterior surface of the membrane are iodinated, since the high molecular weight of lactoperoxidase (M.W. = 78,000) (18) prevents its diffusion into the cell. This is clearly shown in figure 1 where hemoglobin is not significantly labeled in the iodination of intact erythrocytes. Hemoglobin can be iodinated when exposed to lactoperoxidase (19).

Other techniques have been used with varying degrees of success to determine distributions of erythrocyte membrane components. Protein reagents have been employed; however, these reagents can produce erroneous results. They pass into the membrane and thus do not give an exclusive vectoral label (20,21) and they disrupt the membrane which also yields equivocal results (20). Enzymatic digestion of membranes removes groups from the surface of the membrane (22); however, this approach has provided little reliable data on the protein distribution except when antigenic determinants have been altered (23). Antigen-antibody reactions clearly indicate the location of antigenic determinants on the surface of the membrane; however, use of this technique to determine protein distribution is limited since most of the red cell antigens are not proteins (24). Finally, the activities of certain enzymes have suggested their location in the membrane (9-12); however, diffusion of substrates and products of these enzymes have made interpretations difficult. In contrast, the present experiments with lactoperoxidase catalyzed iodination gave no evidence of membrane penetration or membrane damage. Iodinated erythrocytes maintain normal potassium

ion concentrations and resistance to lysis upon standing 18 hours at 4°.

When intact erythrocytes are iodinated, only one protein in the isolated stroma contains radioactivity (figure 2). All membrane proteins, however, can be iodinated by lactoperoxidase once solubilized by lipid extraction (19). The 90,000 molecular weight protein appears to be the only protein in the isolated stroma that is exposed to the supporting media when the membrane exists as the intact erythrocyte. It is unlikely that proteins exist on the membrane surface with all the hydrophylic tyrosine groups buried (25).

Many reports have indicated the abundance of nonproteinaceous material on the outer surface of the erythrocyte membrane. Eylar *et al.* (5) have demonstrated that all the sialic acid in the membrane is on the exterior of the erythrocyte. Other antigenic determinants which are predominantly glycopeptides and glycolipids (24) are also positioned on the cellular surface, although some have been shown to be partially buried (26). Phospholipase C reacts readily with erythrocyte membrane indicating that phospholipids are in close association with the surface of the cell (25). Cholesterol which is 20% by weight of the membrane, has also been shown to exist on the exterior of the erythrocyte membrane (7). These studies and the present data suggest that the exterior of the human erythrocyte membrane is composed primarily of glycoproteins, glycolipids, and lipids. Therefore, it would appear that most proteins are either buried within the membrane or are on its interior surface while only one stroma protein component is exposed to the supporting media on the intact erythrocyte.

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