FLUORESCENT LABELING OF THE SURFACE PROTEINS OF ERYTHROCYTE MEMBRANES USING CYCLOHEPTAAMYLOSE-FLUORESCAMINE COMPLEX

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SUMMARY

Fluorescamine was incorporated in cycloheptaamylose and the resulting complex was utilized as a new probe for fluorescent labeling of the surface proteins of erythrocytes. The complex reacted with erythrocyte membrane at 37°, pH 7 to 8 without using organic solvents. The major membrane proteins of ghosts were labeled, whereas the complex did not penetrate in the intact erythrocytes and labeled only surface proteins. Particular advantages are that the complex itself and its hydrolysis product were nonfluorescent when electrophoresed in sodium dodecyl sulfate polyacrylamide gels.

The use of water-insoluble reagents for studying membrane topology is not appropriate, since addition of the reagents in the organic solvents usually employed to dissolve the reagents is liable to destroy the membrane architecture. Schmidt-Ullrich, Knüfermann and Wallach(1) revised a dansylation method of erythrocytes membranes in which dansyl chloride was dispersed in lecithin-cholesterol micelles. Kinoshita, Iinuma and Tsuji(2) have succeeded to incorporate dansyl chloride into cycloheptaamylose and found that the reaction with proteins and a plasma membrane could be done without using organic solvents. On the other hand, fluorescamine is now widely used as a sensitive reagent for the fluometric assay of amines, amino acids and proteins(3,4) and seems to be superior to dansyl chloride in that fluorescamine itself and its hydrolysis

Abbreviation; CFC, Cycloheptaamylose-fluorescamine complex.

product are nonfluorescent. However, no one has ever used this reagent for fluorescent labeling of membranes.

In the present study, we have synthesized cycloheptaamylose-fluorescamine complex(CFC) and applied the complex to fluorescent labeling of erythrocyte membrane. The complex was soluble in water, obviated the need for use of organic solvents, and was proved to be non-penetrating in intact erythrocytes.

MATERIALS AND METHODS

Fluorescamine was purchased from Nippon Roche K. K. Cycloheptaamylose was obtained from Tokyo Kasei Kogyo Co., Ltd. Ghosts were prepared from sheep or human erythrocytes by the method of Dodge et al.(5). CFC was prepared by a modification of the method of preparation of cycloheptaamylose-dansyl chloride complex(2). To 100ml of 2% aqueous cycloheptaamylose solution(w/v), was added dropwise a solution of 125 mg of fluorescamine in 5 ml of acetone at room temperature. After 15 min, the mixture was left standing in an ice bath for 30 min. The precipitate thus formed was collected by centrifugation and dried over P_2O_5 under reduced pressure.

Proteins were incubated with CFC in 15 mM of phosphate buffer (pH 8.0) at 37° and the fluorescence of the reaction mixture was measured with a Hitachi 103 fluorescence spectrophotometer. Erythrocytes or erythrocyte ghosts were washed in 0.9% NaCl solution, resuspended in 155 mM phosphate buffer(pH 8.0) and incubated with CFC at 37°. After the reaction with CFC, erythrocytes were washed twice with 0.9% NaCl solution prior to preparing ghosts. For electrophoresis, the ghosts were dissolved in 1% sodium dodecyl sulfate and the membrane proteins were separated in 4% polyacrylamide gels, using 100 mM phosphate as buffer. Fluorescence distribution in the gels was determined with a Yamato-Asuka scanning fluorometer model SFR-21. Coomassie blue staining for protein and periodate-

Schiff staining for carbohydrate were carried out by the procedure of Fairbanks et al.(6).

RESULTS AND DISCUSSION

Fluorescamine readily formed a complex with cycloheptaamylose and the resulting CFC was moderately soluble in water. The reaction of CFC with proteins gave fluorophors with excitation and emission maxima at 390 nm and 480 nm, respectively. This fluorescence is of the same type as the one generated from the reaction of fluorescamine in acetone with amino acids. Curve A in Fig. 1 shows the reaction time of CFC with bovine serum albumin at 37° and pH 8.0. It takes about 30 min for the completion of the reaction. Since fluorescamine dissolved in acetone reacted with the protein within 10 min(curve B in Fig. 1), it is evident that the fluorescamine was stabilized by the complex formation with cycloheptaamylose. A preliminary experiment revealed that fluorescence intensity of amino acids and peptides labeled with fluorescamine was enhanced about 3-fold by the addition of cycloheptaamylose(Kinoshita, manuscript in preparation). Kinoshita, Iinuma and Tsuji have previously found that cycloheptaamylose en-

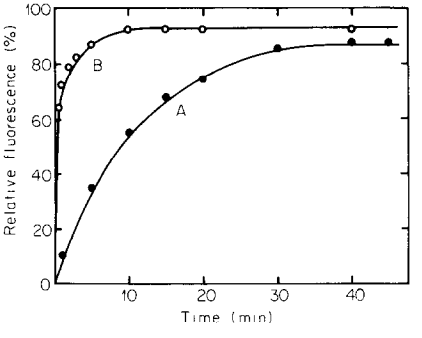

Fig. 1. Variation of fluorescence with time. Bovine serum albumin (3.4×10^{-7} M) was incubated with exess concentration of CFC(curve A) or fluorescamine in acetone(curve B) at 37° in 10 mM phosphate buffer at pH 8.0. Excitation was at 390 nm and emission was measured at 480 nm.

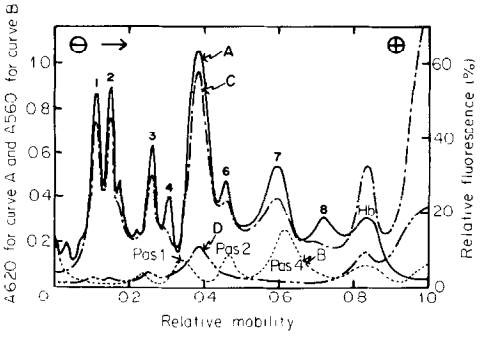

Fig. 2. Gel patterns from intact sheep erythrocytes and their ghosts treated with CFC(0.5 mg/mg protein) at 37° in 155 mM phosphate buffer at pH 8.0 for 30 min. Coomassie blue(curve A) and periodate-Schiff staining(curve B) of ghosts. Curves C and D shows fluorescence distribution of ghosts and intact erythrocytes, respectively. The ordinate gives absorbance at 620 nm for Coomassie blue and at 560 nm for periodate-Schiff staining, respectively, and arbitrary linear fluorescence units. The numbering of the proteins is adopted from that of Schmidt-Ullrich et al.(1).

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hanced the fluorescence of dansyl amino acids(7) and solubilized dansyl chloride(2). They attributed these phenomena to the incorporation of dansyl derivatives in the hydrophobic cavity of cycloheptaamylose molecule(7). Cycloheptaamylose may presumably exert a similar effect on fluorescamine and its derivatives.

In order to examine whether CFC reacts with proteins or erythrocyte ghosts, sheep erythrocyte ghosts were treated with CFC in 155 mM phosphate buffer(pH 8.0) at 37° and their components were analyzed on sodium dodecyl sulfate gels(Fig. 2). Comparison of the membrane protein bands recognizable by Coomassie blue(curve A in Fig. 2) or periodate-Schiff staining(curve B) with the fluorescent bands(curve C) demonstrates that the major membrane proteins in ghosts are accessible to CFC. A broad and strong fluorescent zone with two peaks just behind the tracking dye may arise from the reaction of CFC with hemoglobin and membrane lipids. Similar fluorescent band produced by the reaction between dansyl chloride and lipids was reported by Schmidt-Ullrich et al.(1).

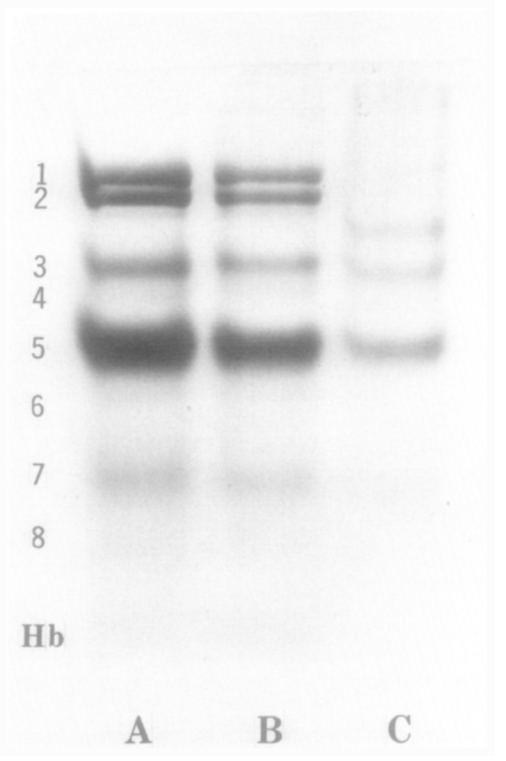

Fig. 3. Coomassie blue stained slab gel patterns from (A) untreated ghosts; (B) ghosts treated with CFC; (C) ghosts treated with cycloheptaamylose-dansyl chloride complex.

It is advantageous that CFC treatment did not alter the mobilities of the protein bands in the sodium dodecyl sulfate gels. Untreated ghosts which were electrophoresed and stained by Coomassie blue gave sodium dodecyl sulfate gel patterns identical with those obtained from CFC treated ghosts(Fig. 3). Another advantage of CFC is that neither unreacted CFC nor its hydrolysis product gave any fluorescent band when electrophoresed in the sodium dodecyl sulfate gels. To illustrate these advantages of CFC, similar experiments were conducted using cycloheptaamylose-dansyl chloride complex as a reagent. Dansylation of ghosts changed their protein distribution pattern, causing disappearance of bands 1 and 2 and appearance of a new band between band 2 and 3 by an unknown reason(Fig. 3). The same phenome-

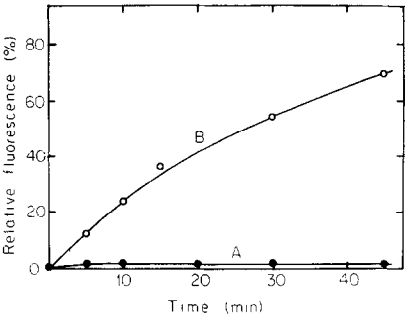

Fig. 4. Time course of the reaction of CFC with hemoglobin in intact(curve A) and broken erythrocytes(curve B) at 37° and pH 8.0. Fluorescence was measured after extraction of heme from hemoglobin as described in the text.

non was observed by Schmidt-Ullrich et al.(1) using dansyl chloride in lecithin-cholesterol micelles as a reagent. In addition, the hydrolytic product of cycloheptaamylose-dansyl chloride complex gave the strong fluorescent band in the middle of the sodium dodecyl sulfate gels and interferes with the measurement of dansylated protein bands.

Evidence for the impermeability of the membrane to CFC can be obtained by measuring the fluorescence of the globin part of the hemoglobin in the erythrocytes externally treated with CFC. Heme was extracted from hemoglobin with acetone-HCl(8), because it quenches the fluorescence. As shown by curve A in Fig. 4, the globin isolated from erythrocytes labeled from the exterior was practically free from fluorescence. In contrast, when hemoglobin from an equivalent number of erythrocytes were treated identically, the globin part was remarkably labeled(curve B in Fig. 4). The result indicates that CFC does not penetrate into erythrocytes. The size and hydrophilic hydroxyl groups of cycloheptaamylose probably prevent the complex from passing through the lipid bilayer.

When intact erythrocytes were treated with CFC and analyzed on sodium dodecyl sulfate gels, only a few bands were labeled with

fluorochrome. Lysis during the reaction was negligible(less than 0.3%). Curve D in Fig. 2 shows fluorescent distribution from intact sheep erythrocytes treated with CFC. Bands 3 and 5 were labeled significantly and traces of fluorochrome were observed for bands 1 and 2. This pattern is very similar to that obtained by the treatment with dansyl chloride in lecithin-cholesterol micelles(1). It seems that glycoproteins in intact erythrocytes were not labeled. The simplest explanation for this is that CFC could not be accessible to glycoproteins probably by virtue of the steric hindrance of carbohydrate part of the proteins. In the case of intact human erythrocytes, only band 3 was labeled and no label could be detected in bands 1 and 2, as has been found with most other procedures.

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