EFFECTS OF OXYGEN TENSION ON CHEMICAL SUSCEPTIBILITIES OF ERYTHROCYTE MEMBRANES AS EXAMINED WITH 1-DIMETHYLAMINONAPHTHALENE-5-SULFONYL CHLORIDE

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Received May 24,1974

Chemical susceptibilities of erythrocyte membranes toward 1-dimethyl-amino-naphthalene-5-sulfonyl chloride were examined under aerobic and anaerobic conditions to see the effects of oxygen equilibrium of hemoglobin on the structure of erythrocyte membranes. Gel filtration on Sepharose 4 B equilibriated with 1% sodium dodecylsulfate revealed that membrane proteins and glycolipids of deoxygenated erythrocytes react with the reagent to a greater extent than do those of oxygenated erythrocytes.

Molecular organization of proteins of erythrocyte membranes have been studied from various approaches. Lenard(1,2), Fairbanks et al.(3), Kobylka et al.(4) and Triplett et al.(5) analyzed proteins and glycoproteins of human erythrocyte membranes and those of other various animals by polyacrylamide disc gel electrophoresis in the presence of sodium dodecylsulfate(SDS). Clarke(6) and Capaldi (7,8) extracted water soluble membrane proteins of high molecular weight from bovine erythrocyte ghosts. Chemical modification using membrane impermeable reagents was applied to intact erythrocytes, cell ghosts and resealed ghosts(9-14).

There exist two distinct states in erythrocytes in blood, oxygenated and deoxygenated states. Hamasaki et al.(15) and Hamasaki and Minakami(16) reported that glycolysis in human erythrocytes is enhanced by deoxygenation with concomitant accumulation of 2,3-diphosphoglycerate. They discussed these phenomena in relation to uptake of proton and/or of 2,3-diphosphoglycerate by hemoglobin upon deoxygenation.

In our previous studies, we applied chemical modification reagents or denaturation reagents to proteins such as hemoglobin(17,18) and serum albumin(19) to

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elucidate the tertiary structure and its changes upon binding with various ligands. In the present paper, we describe changes of chemical susceptibilities of erythrocyte membranes toward DANSCl by oxygen equilibrium of hemoglobin.

Bovine blood was defibrinized and then filtrated through 3 sheets of gauze. Erythrocytes in serum were centrifuged at 4000 x g for 20 min. Serum and buffy coat were sucked off and erythrocytes were washed once with 0.9% NaCl and two times with Krebs-Ringer phosphate buffer(pH 7.4). DANSCl, DANS-alanine and Sepharose 4 B were purchased from Seikagaku Kogyo Co.

Washed erythrocytes were suspended in Krebs-Ringer phosphate buffer to make the concentration of hemoglbin 15%. A cell suspension(70 ml) was oxygenated by a gentle flow of oxygen and another(70 ml) was deoxygenated by evacuation and filling with pure nitrogen in a closed vessel. Oxygen and nitrogen gases were washed by passing through water. Oxygen concentration was followed by a Clark oxygen electrode and oxygen partial pressure during modification was less than 28 mmHg for deoxygenated erythrocytes and was 600 mmHg for oxygenated erythrocytes. Modification was initiated by dropwise addition of 1.0 ml of DANSCl in acetone and continued for 1 hr at 37^{o}C with gentle stirring by a magnetic stirrer at about 50 rpm. The concentration of DANSCl in the sample mixture was 2.0 mM.

The modified cells were centrifuged at 9000 x g for 15 min, and the extent of hemolysis during modification was calculated from the concentration of hemoglobin in the supernatant fluid. The extent of lysis was 2.0% for both samples. Modified erythrocytes were washed with 4 volume of 0.9% NaCl for 3 times. Centrifugation was carried out at 270 x g for 5 min. Cell ghosts were obtained according to Dodge et al.(20) and were solubilized with SDS(1). Gel filtration was carried out on Sepharose 4 B column(2.5 x 90 cm) equilibriated with 1% SDS. Each fraction(5.3 ml) from the column was analyzed by SDS-polyacrylamide gel electrophoresis(acrylamide, 7.5%)(ref. 21) and densitometric measurements were carried out with a Gilford 2400-S spectrophotometer. Protein concentration was determined according to Lowry et al.(22) using bovine serum albumin as a standard, and carbohydrate concentration was determined by the method of Dubois et al.(23).

Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer MPF 2 A and the sensitivity of photomultiplier was controled using DANS-alanine in 1 % SDS as a standard. Excitation was at 350 nm with 10 nm bandwidth and emission was measured at 510 nm with the same bandwidth.

Gel filtration of modified erythrocyte membranes. Fluorescence of the modified ghosts arises from the DANS groups linked covalently to membranes and from those adsorbed by membranes. Therefore, membrane proteins were separated by gel filtration to remove the effects of the adsorbed DANS groups and also to avoid the quenching of fluorescence by residual hemoglobin.

Fig. 1 shows the results of gel filtration of ghost proteins obtained from

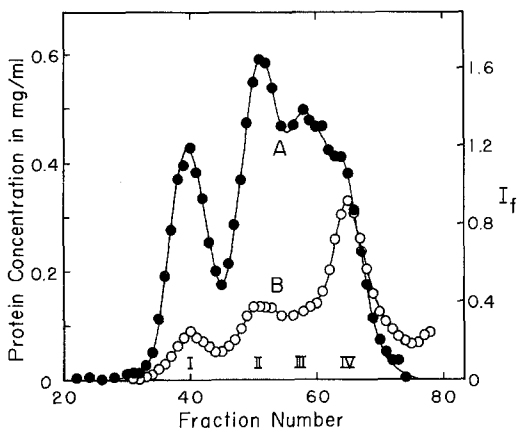

Fig. 1. Sepharose 4 B gel filtration(2.5 x 90 cm column) of DANS-modified oxygenated erythrocyte membranes(Ca. 74 mg). Fraction(5.3 ml) were collected. Curve A, protein concentration and Curve B, fluorescent intensity at 510 nm (arbitrary unit).

oxygenated DANS-modified erythrocytes on Sepharose 4 B. Curves A and B are the elution curves measured by protein concentration and by fluorescent intensity, respectively. Membrane proteins are separated roughly into 4 components, I, II, III and IV. This elution profile is quite similar to that for bovine erythrocyte ghosts reported by Kobylka et al.(4).

Membrane proteins obtained from deoxygenated erythrocytes are also separated into 4 components(Curve A in Fig. 2) and the elution pattern agrees well with

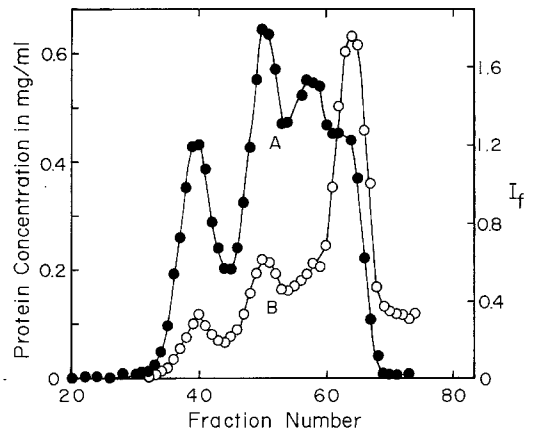

Fig. 2. Sepharose 4 B gel filtration(2.5 x 90 cm column) of DANS-modified deoxygenated erythrocyte membranes(Ca. 79 mg). Fractions(5.3 ml) were collected. Curve A, protein concentration and Curve B, fluorescent intensity at 510 nm(arbitrary unit).

that obtained for oxygenated erythrocytes. The fluorescent profile obtained for membrane proteins of deoxygenated erythrocytes(Curve B in Fig. 2) is similar to that for oxygenated erythrocytes. However, fluorescent intensity of each fraction of deoxygenated erythrocytes is much higher than that of oxygenated erythrocytes.

The relative fluorescent intensity per unit protein concentration was calculated for each fraction. The relative values of components I, II, III and IV of deoxygenated erythrocytes are about 1.3, 1.5, 1.5 and 2.0 times, respectively, those obtained from oxygenated erythrocytes. The results suggested that the structure of erythrocyte membranes changed upon deoxygenation and that the structural changes made the membrane proteins or glycolipids more susceptible to the reagent.

Fractions from gel column showed fluorescent maxima around 510 nm, while DANS-alanine in 1% SDS showed a maximum at 535 nm. This implied that the environmental state, e. g., hydrophobicity, of DANS groups linked to membrane proteins is different from that of DANS-alanine in 1% SDS or that the groups reacting with DANSCl are not α-amino groups. However, the concentration of DANS groups of components I, II, III and IV were calculated assuming that the

fluorescent quantum yield of the DANS group is equal to that of DANS-alanine. Components I, II, III and IV of oxygenated erythrocytes contain 7.9, 8.5, 9.4 and 30 nmoles of DANS groups per g of protein, respectively, and those of deoxygenated erythrocytes 10, 13, 14 and 61 nmoles per g of protein, respectively.

Three experiments were carried out to see the reproducibility of the experimental data, since some of DANSCl tend to precipitate under our experimental conditions. The fluorescence intensity of DANS groups introduced to membrane proteins of deoxygenated erythrocytes were always higher than that of oxygenated ones. For example, with 4 mM DANSCl, fluorescence intensities of components I, II, III and IV of deoxygenated erythrocytes were 1.2, 1.3, 1.2 and 1.5 times, respectively, those of oxygenated erythrocytes.

SDS-disc electrophoresis. Fig. 3 shows densitometric traces of gels obtained

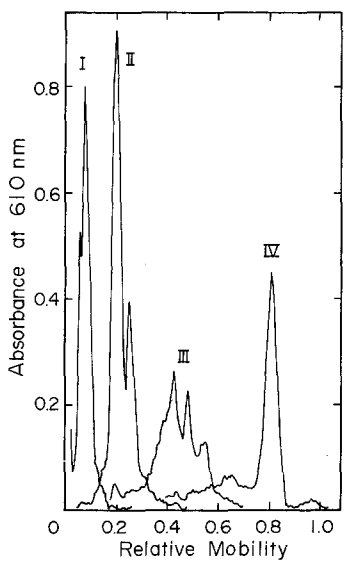

Fig. 3. Densitometric traces of Amino black 10 B stained polyacrylamide gels of single fractions taken at I, II, III and IV in Fig. 1.

for single fractions at components I, II, III and IV in Fig. 1. Each component from Sepharose 4 B gel chromatogram contains several proteins. Capaldi(7) reported that there are at least twelve proteins in bovine erythrocyte membranes and those were numbered from 1 to 12. The proteins in component

I may be the protein 1 and 2 of Capaldi's designation. Component II contains two proteins and their apparent molecular weight was 110,000 and 97,000, respectively. Component III contains three proteins of apparent molecular weight of 56,000, 47,000 and 39,000.

Gel of component IV shows an intense protein band and its apparent molecular weight was 17,000. The protein of component IV is, therefore, assumed to be the subunits of hemoglobin. Component IV was analyzed by polyacrylamide gel electrophoresis and the results are shown in Fig. 4. Gels 1 and 2 are the

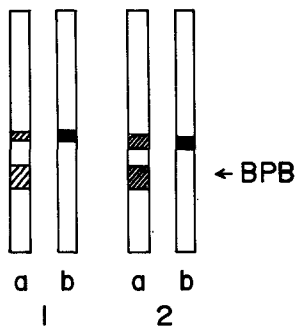

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns of single fractions taken at IV of oxygenated erythrocytes(Gel 1) and deoxygenated erythrocytes(Gel 2). Fluorescent bands in Gels 1a and 2a are detected under 365 nm light and protein band in Gels 1b and 2b was stained with Amino black 10 B.

electrophoretic patterns of the component IV obtained from oxygenated and from deoxygenated erythrocytes, respectively. As shown by Gels 1a and 2a, two fluorescent bands were seen under 365 nm light. On the other hand, after Amino black 10 B staining, only one band was detected(Gels 1b and 2b together with Fig. 3). The fluorescent band other than globin may arise from glycolipid as reported by Kobylka et al.(4), since component IV was found to contain carbohydrate of high concentration. Fluorescent intensity of these two bands obtained from deoxygenated erythrocytes were much more intense than the corresponding bands obtained from oxygenated erythrocytes.

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