CROSS-LINKING SIALOGLYCOPROTEINS OF HUMAN ERYTHROCYTE MEMBRANES[1]

T. H. Ji

Division of Biochemistry
University of Wyoming
Laramie, Wyoming 82070

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Summary: Human erythrocyte ghosts were treated with a bifunctional cross-linking reagent, dimethyl adipimidate dihydrochloride. On SDS-polyacrylamide electrophoresis of the cross-linked membrane proteins after solubilization, sialoglycoproteins and the proteins disappeared from the original band positions and appeared in a new band of aggregates.

Cell membranes of human erythrocytes contain a variety of proteins and glycoproteins, with a wide range of molecular weights (1-4). Most of the carbohydrates present in the erythrocytes have been found linked to membrane proteins on the outer surface of the cell (5,6). The major glycoproteins contain about 60% of the membrane sialic acid and exhibited an apparent molecular weight near 50,000 (7). Freeze-etch electron microscopy (8,9) and surface labeling of the sialoglycoproteins (10) indicated that the molecules were present in components of molecular weight near 170,000 called intramembranous particles (11). Besides the sialoglycoproteins, another species of protein has been thought to be also a component of the intramembranous particles.

However, it has recently been reported that the membrane proteins of human or bovine erythrocytes can be cross-linked but not the sialoglycoproteins (12-14), suggesting that the sialoglycoproteins are not associated with other membrane components (14).

In this communication, evidence is presented that the sialoglycoproteins of human erythrocytes are cross-linked when the membranes are treated with dimethyl adipimidate dichloride.

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Methods: Human erythrocyte ghosts, free of leucocytes, were prepared as previously (15). Human blood was collected in a heparinized vacuum bottle and kept at 5^{o} for 5-10 hours. When the erythrocytes were separated in the lower layer from the upper layer of white blood cells, the white blood cells were aspirated. Since it is very difficult to remove all of the white blood cells by aspiration, erythrocytes were collected carefully from the bottom of the erythrocyte layer using a glass syringe, and the erythrocytes near the interface between the lower and upper layer were discarded. The collected erythrocytes were washed several times in isotonic sodium phosphate buffer at pH 7.5. The procedure thus eliminates possible contamination of white blood cells and the subsequent proteolysis of the erythrocyte ghosts by the enzymes which originated from the white blood cells. For the study of erythrocyte ghost proteins, the removal of the proteolytic enzymes of white blood cells is necessary and critical (16). The washed cells were examined for contamination under a Zeiss microscope fitted with Normarski optics. The erythrocyte ghosts were prepared by the method of Dodge, Mitchell & Hanahan (17), using 10 mM of sodium phosphate buffer at pH 7.5 as the hemolyzing buffer. Ghosts were resealed in a buffer containing 0.6% sodium chloride and 100 mosM sodium phosphate buffer, pH 7.5, at 5^{o} for 15 minutes (10).

Dimethyl adipimidate dichloride (DAP) was prepared according to the method of McElvain and Schroeder (18), and was dissolved in 0.9% NaCl and 5 mM Na_2HPO_4, and the pH was adjusted to 7.5. An aliquot of the reagent was added to the erythrocyte ghost suspension and cross-linking was performed for 4 hours at room temperature. The protein concentration in the reaction mixture was always 0.15 mg/ml, but the DAP concentration was varied from 1 µg/ml to 1 mg/ml. The cross-linking reaction was stopped by washing and centrifuging the reaction mixture twice with the buffer, 0.9% NaCl in 5 mM Na_2HPO_4 at pH 7.5. The concentration of the proteins was determined by the modified Lowry method (20).

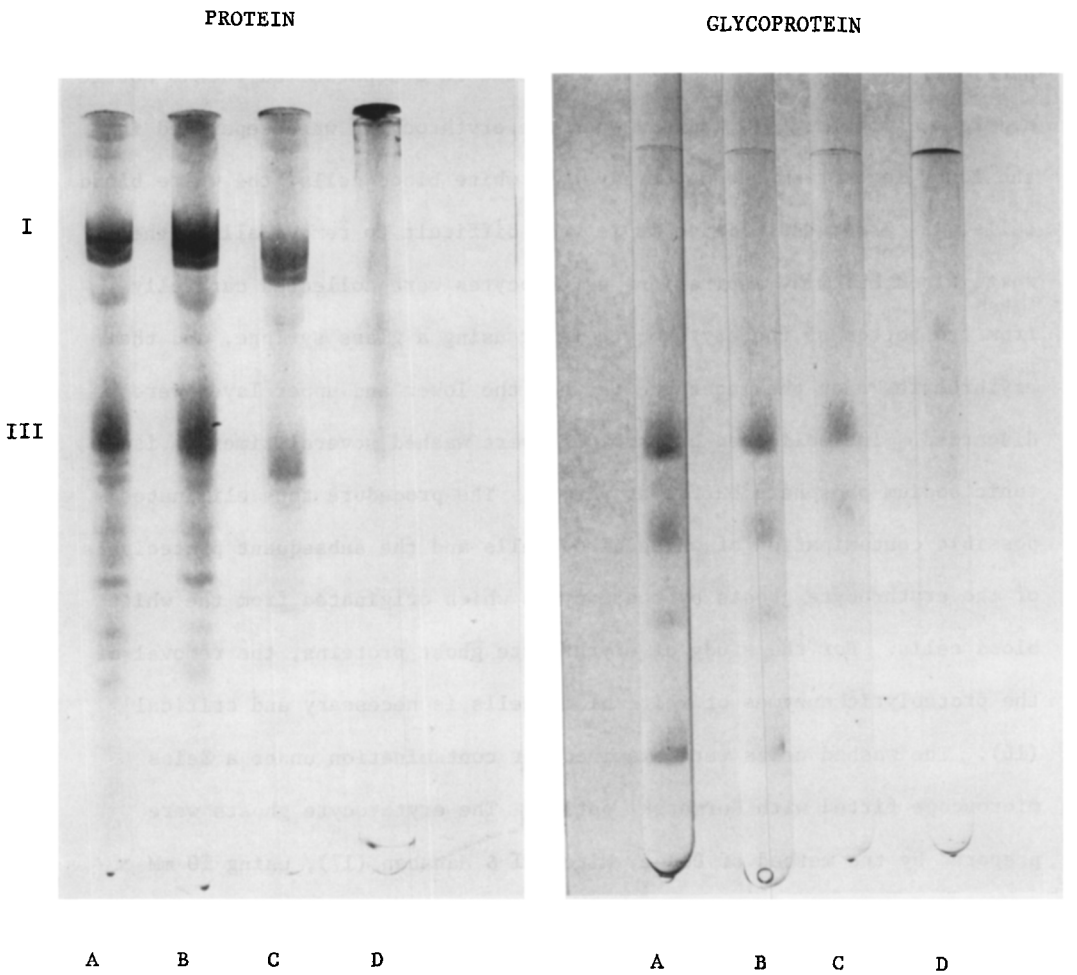

Figure 1: The erythrocyte ghost proteins (0.15 mg/ml) were cross-linked with DAP of different concentrations for 4 hours at room temperature. The ghosts were washed and sedimented twice to stop the cross-linking reaction. The microscopic examination showed no ghost aggregation. The ghosts were solubilized in 1% SDS at room temperature for acrylamide gel electrophoresis. The gels were stained either for protein or for glycoprotein. The DAP concentration was 1 μg/ml for gel A, 10 μg/ml for gel B, 100 μg/ml for gel C, and 1 mg/ml for gel D.

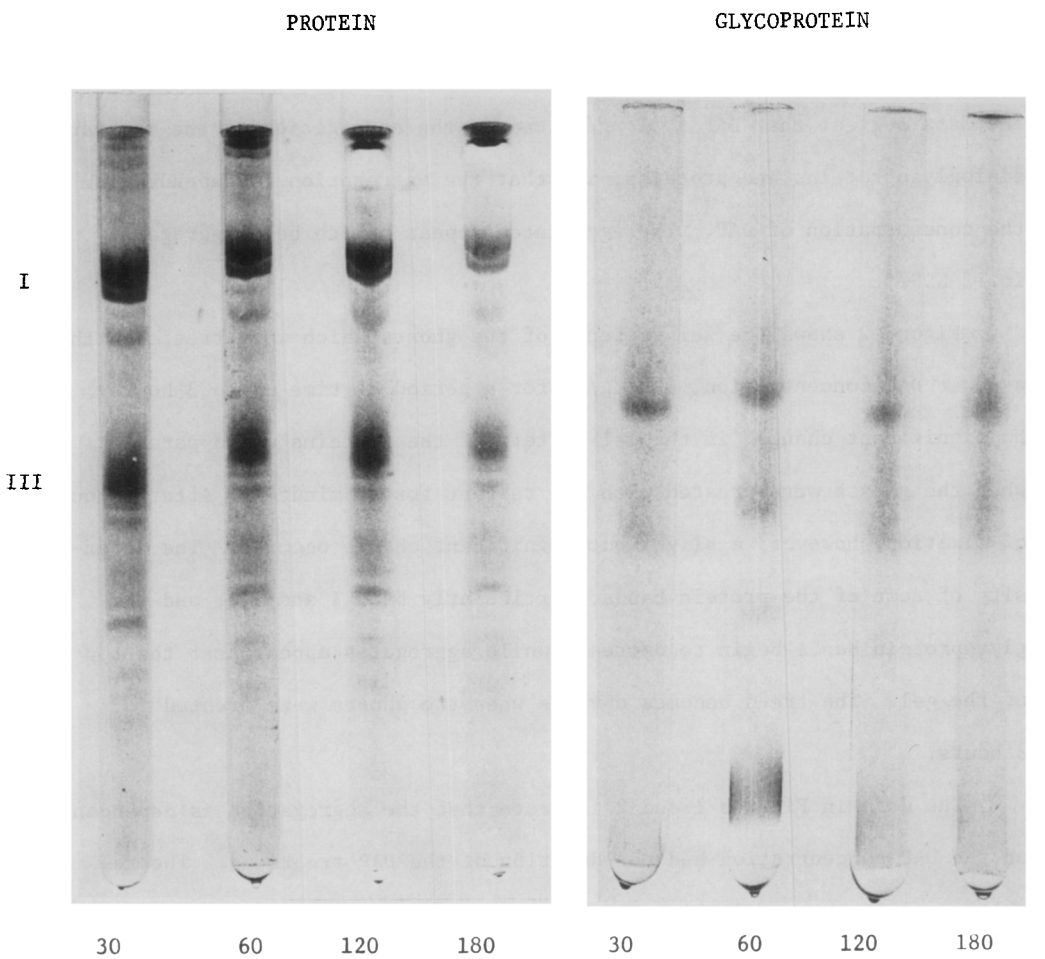

Figure 2: The erythrocyte ghost proteins (0.15 mg/ml) were cross-linked with 0.3 mg/ml of DAP for the following periods of time: 30 min., 60 min., 120 min., and 180 min. Refer to the legend for Figure 1.

SDS-gel electrophoresis was performed as described by Fairbanks *et al.* (19), and stained for proteins or glycoproteins.

Results and Discussion: Figure 1 shows the SDS-acrylamide gel electrophoregram of the erythrocyte ghosts which were fixed with DAP of different concentrations for 4 hrs. at room temperature. At the concentration below 0.1 mg/ml of DAP the gel patterns did not change. However, at 1 mg/ml

of DAP, most of the protein bands and glycoprotein bands disappeared from their original positions and a new band appeared near the top of the gel. The data suggest that DAP at 1 mg/ml caused the aggregation of the membrane sialoglycoproteins and proteins, and that the aggregation is dependent on the concentration of DAP. The aggregates appear not to be deaggregated in 1% SDS.

Figure 2 shows the gel patterns of the ghosts which were treated with a lower DAP concentration, 0.3 mg/ml for a period of time up to 3 hours. No significant change in the gel pattern of the proteins was apparent when the ghosts were treated with the reagent for 30 minutes. After 1 hour of fixation, however, a slight but significant change occured. The intensity of some of the protein bands, particularly band I and III, and the glycoprotein bands begin to decrease while aggregates appear near the top of the gel. The trend becomes obvious when the ghosts were treated for 3 hours.

The data in Figures 1 and 2 indicate that the aggregation is dependent on the DAP concentration and the duration of the DAP treatment. The results exclude the possibility that the aggregation was simply due to the presence of the reagent and that the detergency of SDS became less efficient due to the reagent. Therefore, it is reasonable to consider that the sialoglycoproteins and the proteins were cross-linked to form the aggregates by DAP. The cross-linking could be between any combination of the membrane constituents, sialoglycoproteins, proteins, and lipids. However, it seems unlikely that the membrane lipids were involved extensively in the aggregation, since DAP reacts mainly with free amino groups (21) and the molecular weight of the membrane lipids is too low to cause the glycoproteins to appear near the top of the gels. After the DAP treatment, 0.3 mg/ml for 3 hours, the ghosts were diluted 10 times with 0.9% NaCl in 5 mM Na_2HPO_4 and examined under a Zeiss microscope equipped with Normalski differential interference optics. Most of the ghosts appeared

to be separated from one another. Any ghost aggregates could be dispersed by adding the buffer. It is improbable that there were any cross-linkings between the ghosts.

It is not easy to correlate accurately the intensity of the bands to the concentration of protein and glycoprotein. The concomitant appearance of the aggregates and disappearance of the protein bands and glycoprotein bands, while some protein bands were left unchanged, suggest the possibility that a part of the aggregates could originate from the cross-linking between sialoglycoproteins. It is also possible that the proteins and the glycoproteins may be cross-linked and in a close proximity for cross-linking. As apparent in Figure 2, the cross-linking is time-dependent. The result may represent the reaction of the cross-linking or it may be due to a constant movement of and the change of the distance between the proteins and glycoproteins. It is interesting to note that the three sialoglycoprotein bands disappeared as they aggregated.

It has been reported in the literature that the membrane proteins of human or bovine erythrocytes can be cross-linked but not the sialoglycoproteins (12-14). There are several possible causes for the discrepancy between the previously reported data and the present data. The most probable explanation is that the reactivity of the previously used bifunctional reagents may be different from DAP which was used in this study. It may be also possible that the maximum distance of cross-linking of DAP, 8.6 A^{o}, may be significantly different from that of monomeric glutaraldehyde and others. According to the present data and the previous report (13), any failure to cross-link molecules, however, should not be interpreted as evidence that the molecules are not associated with each other.

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