EXTERIOR PROTEINS ON THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

Membranes from pronase treated human erythrocytes were isolated and the membrane proteins separated by disc gel electrophoresis in sodium dodecyl sulfate. A comparison of pronase treated cells and untreated cells show that pronase treatment of intact human erythrocytes results in the alteration of three membrane proteins with molecular weights of 90,000, 95,000, 105,000 and reduces their molecular size. All of the proteins with molecular weights of 90,000 and 105,000 were altered indicating that all proteins with these molecular weights must be exposed on the outside of the membrane. Protein fragments formed from proteolytic digestion remain with the membrane, and have an apparent molecular weight of 65,000. The residual fragments are insensitive to further hydrolysis, and appear to be portions of the hydrolyzed proteins which is buried within the membrane structure.

INTRODUCTION

Evidence from a number of investigators has suggested that the human erythrocyte membrane is asymmetric. For example, most of the carbohydrate of the membrane is exposed on the exterior surface. All the sialic acid can be removed by treatment of erythrocytes with neuraminidase (1), while carbohydrate containing antigenic determinants can be removed by proteolytic enzymes (2-4). From studies of the localization of enzyme activities present in the erythrocyte membrane, data have accumulated suggesting an asymmetric distribution of functional membrane proteins. Most of the enzyme activities, including ATPase (5), 2,3-diphosphoglycerate phosphatase (6) as well as the glycolytic enzymes (7) are oriented towards the inside of the red cell. One notable exception is the acetylcholine esterase activity which can be removed by proteolytic enzymes and appears to be on the exterior surface of the red cell membrane (8,9). Chemical reagents which react with protein functional groups, although not definitive, have also provided evidence that the proteins are asymmetrically assembled in the erythrocyte membrane (10,11).

Most convincing with regard to asymmetric distribution of proteins in the red cell membrane is the recent finding that the proteins in the molecular weight class of 90,000 are exposed to the exterior surface and can be catalytically iodinated by lactoperoxidase (12). The

remaining membrane proteins, however, can only be iodinated when the cell was lysed prior to iodination (13). These data indicate that only a few membrane proteins are exposed to the outside of the cell, while most membrane proteins are exposed to the hemoglobin containing cell interior (13).

Pronase is a non-specific protease which will hydrolyze proteins almost completely to their amino acids (14). This enzyme has been shown to remove much of the carbohydrate from the erythrocyte membrane surface (2). In this report, those membrane proteins modified by pronase are examined in order to further characterize the proteins on the outside of the erythrocyte membrane.

MATERIALS AND METHODS

Human erythrocytes were washed free of serum proteins as previously described (13). The cells were then suspended in 0.106 M phosphate buffer, pH 7.4, to a hematocrit of 20% containing 25 μg/ml pronase (Calbiochem B grade) and incubated at 37°C with constant shaking. After varying periods of time, the cells were cooled to 4°C, and centrifuged at 1,500 X *g* for 15 minutes. The cells were then suspended in 10 volumes of the 0.106 M phosphate buffer and again centrifuged. This procedure was repeated a second time to free the cells of pronase and the hydrolysis products. The membranes were isolated as previously described (13). The membrane proteins were solubilized in sodium dodecyl sulfate and electrophoresed on 5% polyacrylamide gels by standard procedures (13).

RESULTS AND DISCUSSION

The effect of pronase treatment of intact human erythrocytes on the membrane protein composition is shown in Fig. 1. Gel A represents the membrane proteins isolated from untreated erythrocytes. The pattern of separated proteins is similar to that published previously (13,15). Identical protein patterns were obtained from cells incubated in the 0.106 M phosphate buffer for 60 minutes at 37°C, indicating that these conditions do not remove proteins from the membrane.

Pronase treatment of the cells, however, does alter the molecular weights of selected membrane proteins. Gels B and C in Fig. 1 represent the membrane protein composition from erythrocytes treated with 25 μg/ml pronase at 37°C for 15 and 60 minutes, respectively. After 15 minutes incubation, the concentrations of proteins with molecular weights between 90,000 and 105,000 is decreased. Band III, the 90,000 molecular weight protein is completely removed. Bands I and II, with molecular weights of 105,000 and 95,000, respectively, become identifiable as two separate components. Concomitant with the decrease in the 90,000-105,000 molecular weight proteins is an increase of proteins with a molecular weight of 65,000, Band IV. After treatment of

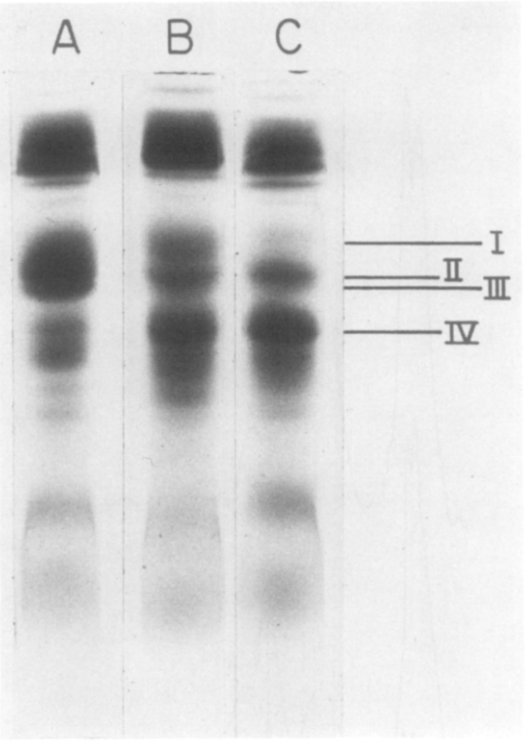

Figure 1 - Acrylamide gels of membrane proteins from normal and pronase treated erythrocytes. Stroma were isolated from untreated erythrocytes, gel A, and from erythrocytes treated at a 20% hematocrit with 25 μg/ml pronase at 37°C for 15 and 60 minutes, gels B and C respectively. The stroma were solubilized in 3% SDS, electrophoresed on 5% polyacrylamide gels containing 0.1% SDS and stained for protein with coomassie brilliant blue. Protein molecular weight bands labeled correspond to molecular weights of 105,000, 95,000, and 90,000, and 65,000, numbers I, II, III and IV respectively.

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the erythrocytes for 60 minutes, this pattern is further accentuated with a decrease in the concentration of protein in Band I, and a further increase in the intensity of Band IV. The concentration of the other protein molecular weight classes are not affected by the pronase treatment.

These protein alterations are more easily visualized by the densitometer tracings of the stained gels shown in Fig. 2. No alterations in protein composition appear in the higher molecular weight regions, $>$ 200,000. However, in the regions of Bands I, II and III there is a marked decrease in protein which coincides with the increase in the amount of protein in Band IV. Band II, is greatly reduced in intensity by 15 minutes pronase treatment, however, no further changes in this band occur after an additional 45 minutes incubation. Band I, however, is completely removed during this time period.

The present results show that pronase reduces the molecular weight of all proteins with molecular weights of 90,000 and 105,000. This means that all membrane proteins with these molecular weights must be on the outside of the membrane. The 95,000 protein molecular weight

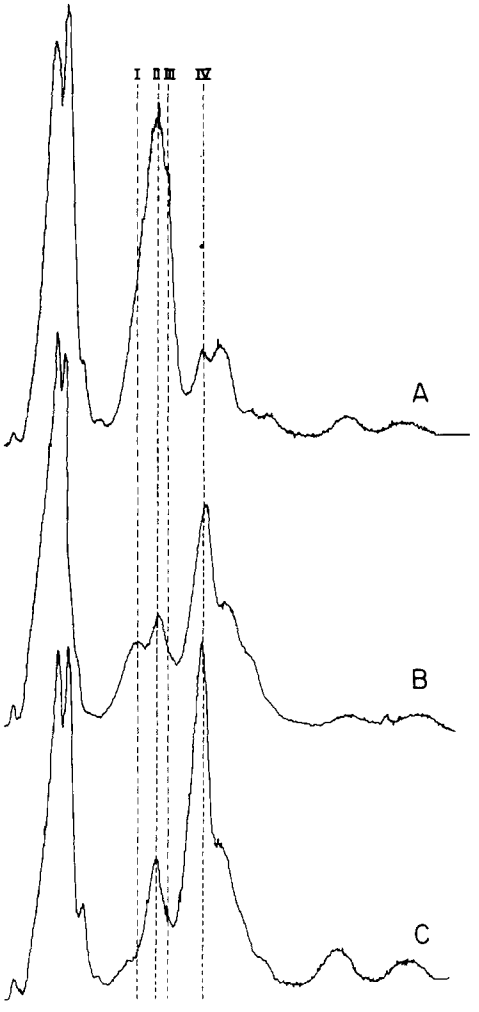

Figure 2 - Densitometer tracings of the acrylamide gels shown in Fig. 1. The letters correspond to the gels and the numbers to the protein molecular weight classes in Fig. 1.

class is only partially removed by pronase and appears to be composed of two types of proteins. One of these is digested and one is unaffected by this enzyme. It is possible that these two proteins are identical with different orientation in the membrane or alternatively they may be entirely different proteins: one located on the outside of the membrane and thus digestable and the other buried in the membrane and inaccessible to the enzyme.

Band IV of Figs. 1 and 2 is not present in the stroma from untreated erythrocytes and is a hydrolytic product resulting from pronase treatment. Since this protein material increases in concentration with time of pronase treatment it appears that this peptide fragment is protected from further pronase hydrolysis by non protein membrane components, and that this lack of

hydrolysis is a function of the membrane organization, and not of the protein structure.

The new peptide formed in the present study could represent a portion of proteins in the 90,000 to 105,000 molecular weight region which are buried in the membrane and are covered by lipid. That this 65,000 fragment is not covered by protein seems evident due to the lack of susceptibility of the peptide fragment to further hydrolysis. Indeed, a twenty-fold increase of pronase concentration used to incubate intact erythrocytes has no effect on this peptide molecular weight.

It is not clear if all the proteins altered by pronase treatment represent protein components on the membrane surface. Lactoperoxidase catalyzed iodination of intact erythrocytes results in the labeling of the 90,000 molecular weight class of membrane proteins as estimated by 5% polyacrylamide disc gel electrophoresis (12). Recent evidence indicates that two different proteins in this 90,000 molecular weight class are labeled (16). These 90,000 molecular weight proteins are also very susceptible to pronase giving further evidence that this molecular weight class of proteins are indeed on the sruface of the membrane. The other proteins on the membrane surface or more probably protein that becomes exposed to the solution when the proteolytic enzyme removes protein and carbohydrate from the membrane surface. Thus, these latter proteins are inaccessible to lactoperoxidase iodination either because of the lack of exposed tyrosines or because they are covered by carbohydrate or protein.

Since this manuscript was completed, two publications (17,18) have appeared whose results are in essential agreement with the data presented in this paper.

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