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GLUCOSE TRANSPORT INHIBITION BY PROTEOLYTIC DEGRADATION OF THE HUMAN ERYTHROCYTE MEMBRANE INNER SURFACE

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Summary

Treatment of the exterior surface of human erythrocytes with the proteolytic enzymes, trypsin or alpha-chymotrypsin (at 1 mg/ml), has no discernible effect on the carrier-mediated movement of glucose. However, the incorporation of either enzyme at much lower levels inside the erythrocyte by the method of reversible hemolysis leads to a progressive inhibition of the rate of glucose movement. Total inhibition eventually results at all tested concentrations of incorporated enzyme. These results strongly suggest that a protein susceptible to attack at the interior surface of the cell membrane is in some way involved in sugar transport. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that the spectrin band (known to be located at the inner membrane surface) gradually disappeared during the protease treatment, in close parallel with the loss in glucose transport. This was not accompanied by any appreciable modification in Band III, which has been closely identified with the glucose transport system.

Introduction

It is generally agreed that glucose traverses the human erythrocyte membrane by carrier-mediated diffusion. There have been many attempts to chemically identify and/or isolate a carrier responsible for the facilitated diffusion of glucose [1—11]. In this system, however, these attempts have been unsuccessful or subject to serious question (see refs. 12 and 13 for reviews). Recently it has been estimated in several laboratories that there are approximately 1.9—3 $\cdot 10^5$ monosaccharide binding sites per cell [7,14,15]. Assuming one glucose

Abbreviations: SDS, sodium dodecyl sulfate; N_2pH-F, 1-fluoro-2,4-dinitrobenzene.

binding site per polypeptide chain, at least nine major protein components of the erythrocyte membrane, as fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, are present in sufficient quantity ($1.8—9.4 \cdot 10^5$ peptides per cell) to account for glucose transport [16,17].

It is well established that small, non-lytic substances can be incorporated into the human erythrocyte by reversible hemolysis. In the present study, we have made use of this process to incorporate the proteolytic enzymes trypsin (EC 3.4.4.4) and alpha-cymotrypsin (EC 3.4.4.5) into depleted human erythrocytes, and have then monitored subsequent changes in the rate of glucose movement. This report correlates the effects of the slow degradation of the interior surface of the human erythrocyte membranes by these proteolytic enzymes with loss of the glucose transport system. The pattern of this degradation suggests that spectrin, a group of large molecular weight proteins located on the interior surface of the erythrocyte membrane, may in some way be involved with the glucose transport system.

A preliminary report of this work has been presented [18].

Materials and Methods

Human erythrocytes were obtained from out-dated blood in standard acid-citrate-dextrose bags from the Nassau-Suffolk (N.Y.) Inter-County Blood Services. Erythrocytes were separated by repeated centrifugation from isotonic saline (discarding the uppermost sedimented layer). The packed cells were always allowed to warm to room temperature before use. Protease loading was achieved by adding erythrocytes with stirring to 4 volumes of a 30 mM NaCl solution containing either trypsin (2×, crystalline, salt-free, Worthington Biochemical Co.) or alpha-chymotrypsin (2×, crystalline, salt-free, Worthington Biochemical Co.) at various concentrations. The lysing cells were left in the hemolytic mixture for exactly 2 min, at which time sufficient concentrated NaCl was added to make the solution isotonic. The modified cells were then separated in the centrifuge and washed twice in an isotonic sodium chloride/sucrose medium (mixture of 3 vol. isotonic NaCl and 1 vol. isotonic sucrose) containing 150 mM glucose. The entire procedure took 20 min and was done at room temperature. The modified cells were placed in a water bath at 37° C for 10 min before the first determination of the rate of glucose exit. All determinations of the rate of glucose movement were made densitrometrically as described by LeFevre [19]. In all instances, the modified and unmodified cell preparations were treated identically except for the presence of the proteolytic enzymes.

In some instances, samples of the modified cells were taken and rehemolyzed in 10 vol. of ice-cold 1 mM EDTA and 9 mM Tris · HCl buffer (pH 7.8), containing soybean trypsin inhibitor (Worthington Biochemical Co.) at 10-times the amount by wt. of the proteolytic enzyme originally used. Ghosts were prepared by the method of Masiak and Green [20]. Membrane proteins were prepared for electrophoresis by the method of Fairbanks et al. [21]. The gel system used was 0.1% SDS, 8 M urea and 5% acrylamide [22]. The gels were stained with Coomassie blue and scanned on a Beckman Acta III recording spectrophotometer with a gel-scanner attachment.

Results

Before any definitive conclusions can be drawn from interior surface treatments, it must be shown that proteolytic degradation of the exterior surface has negligible (if any) effect on the rate of glucose movement, since these enzymes are most certainly available at the exterior surface during the incorporation procedures. The lack of external sensitivity to the protease is shown in Table I; this is in agreement with less extensive observations of Kahlenberg et al. [5] and Rosenberg and Guidotti [23] with trypsin. These results indicate that the exterior surface glycopeptides which are released by the proteolytic enzymes [23–25] are not essential to the glucose transport system, and that the critical proteins either are not susceptible to those enzymes or are not readily accessible from the external medium.

Fig. 1 shows the effects of the incorporation of several concentrations of trypsin into the human erythrocyte. The concentrations indicated are those in the medium at the time of incorporation; it is probable that lesser amounts were incorporated, but it is impossible to specify the actual intracellular concentration. Cells that have undergone reversible hemolysis in the absence of any proteolytic enzyme show essentially no alteration in the rate of glucose movement from that of non-hemolyzed cells. The incorporation of boiled trypsin, trypsin inhibited with soybean trypsin inhibitor, or soybean trypsin inhibitor alone at concentrations 4-times that of the active enzyme has no effect on the rate of glucose exit. Since the inhibition is progressive and dependent upon the concentration of incorporated enzyme, it is most likely due to the proteolytic action of the enzyme. Exit times of approximately 8 min are considered to represent essentially 100% inhibition of the glucose transport system. At all tested concentrations of incorporated trypsin (extending as low as 0.05 μg/ml) this total inhibition of glucose transport was eventually reached.

Fig. 2 shows similar effects of alpha-chymotrypsin on the glucose transport system. However, the membrane component involved appears to be less sensitive to alpha-chymotrypsin than to trypsin, since approximately 4-times the concentration of alpha-chymotrypsin is necessary for equivalent levels of inhib-

TABLE I

INDIFFERENCE OF ERYTHROCYTE GLUCOSE TRANSPORT TO EXTERIOR SURFACE TREATMENT WITH PROTEOLYTIC ENZYMES

Human erythrocytes (5 vol.%) were suspended in a sodium chloride/sucrose medium containing 150 mM glucose and treated with either proteolytic enzyme. The rate of glucose movement was determined densitometrically as described by LeFevre [19].

Enzyme (1 mg/ml)	Treatment time (h)	Exit time (min)
None	1	0.92–0.94
	4	0.91–0.95
Trypsin	1	0.91–0.94
	4	0.90–0.96
Alpha-chymotrypsin	1	0.90–0.96
	4	0.91–0.95

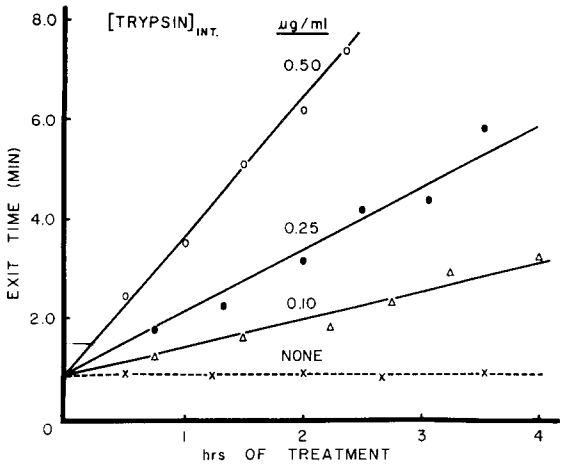

Fig. 1. Inhibition of glucose transport in the human erythrocyte by the incorporation of trypsin. The concentrations indicated were present during the hemolytic step (see text) and equilibrium across the cell membrane is assumed. The dashed line refers to modified cells containing trypsin inhibited with soybean trypsin inhibitor; equivalent data were obtained with unmodified cells, modified cells in the absence of trypsin, boiled trypsin or soybean trypsin inhibitor alone.

ition after comparable durations of exposure. Again, total inhibition was always achieved at all concentrations of incorporated enzyme.

Since it is of interest to know what protein components are being degraded by these enzymes, samples were taken at various time intervals after the initiation of the treatment, concurrently with the determinations of the rate of glucose movement, and prepared for gel electrophoresis. Fig. 3 shows the results for trypsin at 0.25 µg/ml. It is apparent that the spectrin (Bands I and II, as designated in ref. 21) progressively disappears and is totally degraded at about the same time the glucose transport is fully inhibited (compare with Fig. 1). Similar results were obtained for other concentrations of incorporated trypsin.

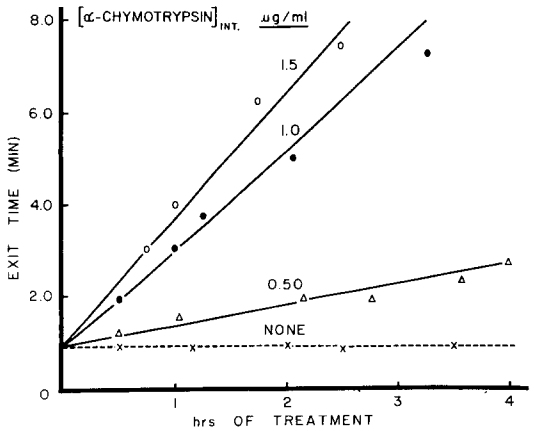

Fig. 2. Inhibition of glucose transport in the human erythrocyte by the incorporation of alpha-chymotrypsin. Conditions are similar to Fig. 1.

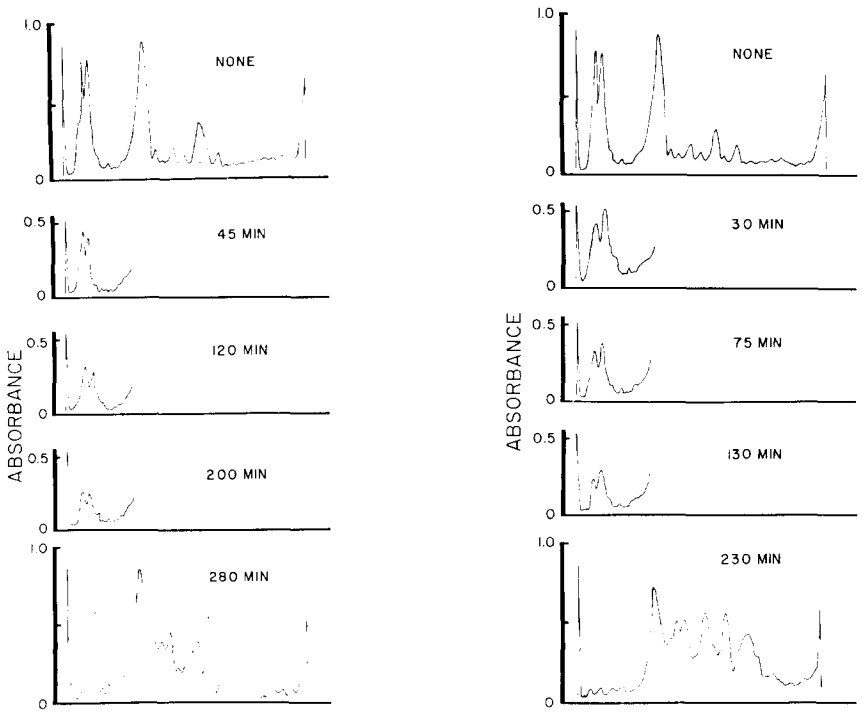

Fig. 3. SDS-polyacrylamide gel electrophoresis of trypsin-treated erythrocyte membranes. The times indicated are the length of treatment with trypsin (0.25 μg/ml). The gels were run until the tracking dye (Bromphenol blue) was at the bottom of the gel (right side of the scan). Only the spectrin portions of the scans are shown at the intermediate times since no specific interpretation of the record to the right is possible because of the appearance of the degradation products of spectrin as illustrated in the final pattern.

Fig. 4. SDS-polyacrylamide gel electrophoresis of alpha-chymotrypsin-treated erythrocyte membranes. The times indicates are the length of treatment with alpha-chymotrypsin (1.0 μg/ml). Other conditions are similar to Fig. 3.

It should be emphasized that band III is not noticeably altered during the treatment period. Unfortunately, the degradation products of spectrin are not released during the ghosting procedure and completely mask any degradation that may have occurred in the proteins below 90 000 daltons.

Fig. 4 shows a similar treatment using alpha-chymotrypsin at 1 μg/ml. Again, the spectrin band is totally degraded at about the same time that 100% inhibition of glucose transport is reached. Band III is also partially degraded by alpha-chymotrypsin; but this is probably not related to the response in the glucose transport system, since band III was not similarly affected by trypsin. These results are quantitatively in agreement with Triplett and Carraway [26], who indicated that spectrin is less susceptible to the action of alpha-chymotrypsin than of trypsin and that band III is more susceptible to alpha-chymotrypsin.

Discussion

The present study shows that the incorporation of the proteolytic enzymes, trypsin or alpha-chymotrypsin, into the human erythrocyte causes a progressive inhibition of glucose transport which is dependent upon the amount of enzyme initially incorporated. Furthermore, it is shown that the time-course of the degradation of spectrin, a major protein of the erythrocyte membrane, corresponds reasonably well with the progressive inhibition of the transport system. These observations raise the question whether spectrin may in some manner be involved in glucose transport. But since the spectrin fragments do not dissociate from the membrane during the ghosting process and thereby obscure the electrophoretic resolution of smaller membrane proteins (less than 90 000 daltons), one cannot conclude that these smaller components are not also modified by the proteolytic digestion.

The present suggestion of a possible spectrin involvement in glucose transport further exacerbates the current conflict of evidence concerning which membrane proteins may actually be involved in this transport system. Carter et al. [3] have shown that glucose transport by membrane vesicles formed upon limited digestion of ghosts with trypsin was unaffected by prior extraction of four major membrane protein bands, one of which was spectrin. Taverna and Langdon [27], using D-glucosylisothiocyanate as an "active-site-directed" irreversible inhibitor, reported that the specific incorporation occurred in the proteins of band III and IV, but not spectrin. However, the single figure they present in support of this claim fails to demonstrate the specific localization. Studies with cytochalasin B, a potent competitive inhibitor of glucose transport, have indicated that the high-affinity binding sites of cytochalasin B are located on band III and that spectrin is not involved [15]. Also, Kahlenberg [28], using a combination of extraction and protein cross-linking, indicates that band III is the only membrane protein involved in glucose transport.

However, Eady and Widdas [29], using a differential labeling of the glucose carrier of human erythrocytes which was based on the fact that 1-fluoro-2,4-dinitrobenzene (N_2ph-F) inactivation of this system is enhanced by 2-deoxy-D-glucose but retarded by 4,6-*O*-ethylidene-D-glucose, indicated that a protein of high molecular weight was appropriately labeled. Similarly, Jung and Carlton [30] applied a double-isotopic, differential labeling technique, observing the effect of the presence and absence of D-glucose on dinitrophenylation of membrane proteins of human erythrocyte ghosts by N_2ph-F, and found a high molecular weight protein (180 000) appropriately labeled. Also, LeFevre et al. [8] and Shanahan and Jacquez [10] developed double-labeling techniques which made use of the fact that cytochalasin B provides significant protection against inhibition of glucose transport by *N*-ethylmaleimide or by N_2ph-F, respectively, and both found the appropriate labeling to occur only in the gel region near the spectrin band or preceding it (at even higher apparent molecular weight). The present study tends to support these latter findings [8,10,29,30] in showing that internal surface treatment with low levels of trypsin does not demonstrably alter band III at all and that alpha-chymotrypsin has only a small effect, while both enzymes completely inhibit glucose transport.

Currently we have no satisfactory explanation for the mutually inconsistent

results obtained from the several laboratories attempting to identify the glucose carrier proteins. It appears likely that the specific chemical treatments of the membranes involved in the several studies may result in variable inter-protein associations or cross-linkages, resulting in shifts in the localizations of the various "Labels" on the gels. Recently, Kasahara and Hinkle [9] have obtained a protein fraction from human erythrocyte ghosts (by solubilization with Triton X-100 or octylglucoside) that catalyzed D-glucose uptake when reconstituted in sonicated liposomes. However, gel electrophoresis of this protein fraction indicated at least partial retention of the majority of the protein bands of the erythrocyte membrane. Resolution of the above inconsistencies will presumably have to await development of more specific extraction or differential labeling techniques.

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Since the acceptance of this manuscript, M.F. Shanahan and P.R. Hinkle, in independent public presentations, have indicated that neither spectrin nor band III are involved in glucose transport. Their data indicate that a low molecular weight protein (in the region of band 4.5, as designated in ref. 21) is associated with glucose transport.

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