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EFFECT OF PROTEOLYTIC DIGESTION ON GLUCOSE TRANSPORT CARRIER OF HUMAN ERYTHROCYTE GHOSTS

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SUMMARY

Practically hemoglobin-free resealed human erythrocyte ghosts which retain the glucose transport carrier function intact were treated with pronase, and resulting proteolytic digestions and their effects on the carrier activity were directly compared. The ghosts, after a loss of more than 50% of their original membrane protein by the pronase treatments, were found to retain the glucose carrier activity intact. The diffusion barrier to D-mannitol was also found to be left grossly intact after the same digestions. Sodium dodecyl sulfate-gel electrophoresis of these glucose carrier-intact, protein-depleted, pronase-treated ghosts revealed that the majority of the membrane proteins which were retained in the ghosts after the digestion, were grossly degraded into small molecular entities.

INTRODUCTION

Transport of many biologically important metabolites across cell membranes utilizes a so called "carrier mediation" mechanism, where, it appears, a specific membrane component selectively mediates movement of solutes across an otherwise virtually impermeable membrane diffusion barrier[1]. In an effort to chemically identify and isolate the carrier responsible for the facilitated diffusion of glucose across the human erythrocyte membrane[2], we previously attempted to selectively remove various protein components from the membrane and correlate the release of a particular protein with concomitant loss, or preservation, of the carrier activity in the residual ghosts[3,4]. It was found that the carrier activity is extremely resistant to the attempted extraction by changes in ionic strength, pH, and divalent cation contents. These findings suggested that the glucose carrier, as distinguished from many "binding proteins" of bacterial systems[1], is a component of the fundamental structural core of the membrane, presumably located in a hydrophobic area[3]. In the present communication, we report that the ghosts, after treatment with pronase which released more than 50% of the membrane proteins and degraded 70% of the remaining proteins, still performed the glucose carrier function af full capacity.

MATERIALS AND METHODS

Fresh human blood drawn from healthy donors was used within 3 days. Two different kinds of ghost preparations were used, namely, pink ghosts prepared in 1/10 isotonic balanced salt solution (1/10 balanced salt solution) and white resealed ghosts prepared in 1/15 isotonic phosphate buffer solution (1/15 phosphate buffer) and resealed in 1/10 balanced salt solution. These preparations were detailed elsewhere[3,5].

The pronase (*Streptomyces griseus* protease, Calbiochem, Los Angeles, Calif.) treatments were carried out by incubating the ghosts (5 mg non-hemoglobin membrane protein per ml reaction mixture) suspended in 1/10 balanced salt solution, pH 7.4. After designated intervals of the incubation, the ghosts were washed by adding 20 vol. of prechilled 1/10 balanced salt solution for three times at 4 °C to remove residual enzyme and digested membrane proteins. Usually the same batch of ghosts treated in this manner was used for both the flux measurements and the chemical analysis of proteolytic digestions. For these ghost samples which were used for the sodium dodecyl sulfate gel electrophoresis, the pronase reactions were terminated by heating the digestion mixture at 100 °C for 10 min prior to the repeated wash described above. The insertion of this heating step did not appreciably affect the protein release.

The glucose transport carrier activity of ghosts was assayed as previously described[4]. Total protein was assayed by the Lowry modification of the Folin assay[6]. The phospholipid-phosphorus was measured by the method of Bartlett[7]. The hemoglobin content was determined as described by Dodge *et al.*[8]. The membrane proteins were solubilized for the gel electrophoresis by adding the following reagents into 2 ml of ghost suspension containing 10 mg of the membrane protein: 0.25 ml of 20% sodium dodecyl sulfate, 0.5 ml of 400 mM dithiothreitol, 2.5 ml of 20% sucrose, 0.05 ml of 1 M Tris–HCl, pH 8.0, 0.025 ml of 0.2 M EDTA, pH 8.0, 0.1 ml of 0.5 mg/ml of pyronin Y and water to make up the total 5 ml. If required, molecular markers were added at this step. The resulting clear solutions were incubated at 37 °C for 30 min.

Electrophoresis with 5.6% acrylamide gels containing 1% sodium dodecyl sulfate was carried out essentially by the method of Fairbanks *et al.*[9]. With the voltage gradient at about 9 V/cm and the current at 8 mA/tube, the running time was usually about 55 min to get a migration of the pyronin Y-tracking dye of about 60 mm distance. The gels were scanned at 550 nm using a Gilford spectrophotometer equipped with a Model 2410 linear transport accessory. The molecular weights were estimated by converting relative mobilities to the logarithmic scale based on a calibration obtained from the mobilities of reduced molecular markers of known molecular weights.

RESULTS

It was shown (Fig. 1) that the resealed, practically hemoglobin-free phosphate ghosts retain the essential features of the glucose carrier function observable with intact cells; they exhibited a $HgCl_2$-sensitive, extremely rapid flux (with a permeability greater than 10^{-5} cm/s) specific to D-glucose, but not to D-mannitol. The glucose

carrier activity of these resealed white ghosts was extremely resistant to prolonged digestion with pronase. For example, a digestion with 1 mg pronase per ml of the reaction mixture for up to 90 min at 37 °C did not damage either the carrier activity or the diffusion barrier. This is also shown in Fig. 1, where the treated ghosts, although smaller in volume, were essentially indistinguishable from the untreated ghosts in selectively and rapidly transporting D-glucose but not D-mannitol. With pink ghosts prepared in hypotonic balanced salt solution, the carrier function demonstrated its full activity after digestion with 10 mg pronase per ml reaction mixture for up to 9 h.

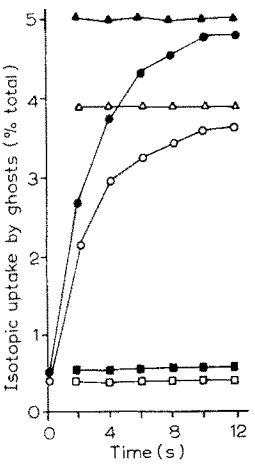

Fig. 1. Time courses of isotonic equilibration (influx) of D-glucose (circles), D-mannitol (squares), and water (triangles) by ghosts treated with (open symbols) and without (solid symbols) pronase. The treatments were carried out at 37 °C for 90 min. The pronase concentration was 1 mg/ml reaction mixture containing 5 mg of membrane protein. The zero-time points for glucose were two second-uptakes in the presence of $HgCl_2$ (2 mM) in the flux system. Calculated half-equilibration times for D-glucose in this particular set of experiments were 2.5 and 2.7 s for the untreated control and the treated ghosts, respectively.

It was subsequently shown that these pronase-treated, glucose carrier-active, resealed white ghosts were depleted of more than 50% of their original non-hemoglobin membrane protein as a result of the digestion. Figs 2 and 3 compare the reductions in iron-hemoglobin protein content of various ghosts which have been treated with pronase and then repeatedly washed. All of these treated ghosts, except unresealed ghosts, were shown to be glucose carrier-intact. With the resealed white ghosts, the membrane protein decreases progressively reaching a final level of about 50% of the non-treated value after approximately 60 min of digestion. No appreciable decrease in their lipid phosphorus contents was detected during this digestion. Unresealed leaky white ghosts are even more susceptible to the pronase digestion, releasing more than 75% of the membrane protein contents within 60 min. With the pink ghosts prepared in the hypotonic balanced salt solution, on the other hand, the depletion of their non-hemoglobin membrane protein is much less extensive even with higher concentrations of the enzyme, with the maximum level of the depletion being only 20% of the non-treated control.

Sodium dodecyl sulfate gel electrophoresis on these carrier-intact, pronase-

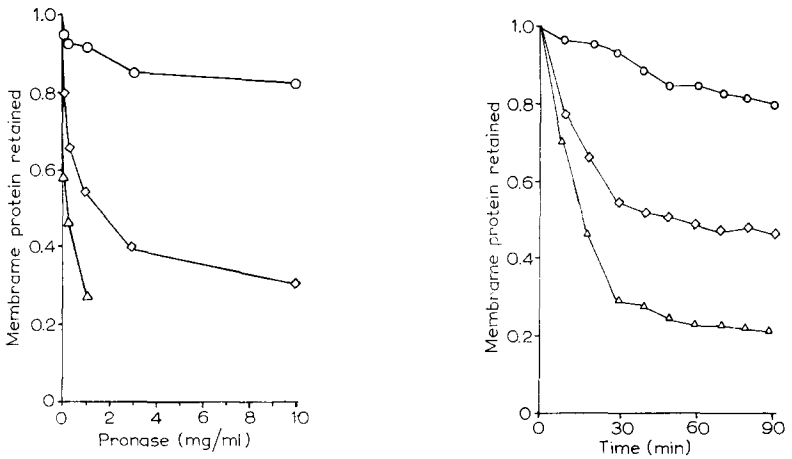

Fig. 2. Depletion in non-hemoglobin membrane protein contents of ghosts treated with pronase as a function of the enzyme concentration. The pronase treatments were at 37 °C for 30 min, then the ghosts were washed extensively as detailed in Methods. Non-hemoglobin protein was obtained by taking a difference between Lowry's protein and hemoglobin contents. Contributions in Lowry's protein of pronase were corrected in each case. The membrane proteins of ghosts were normalized to the quantity per unit membrane-lipid phosphorus (as estimated by total membrane phosphorus), and expressed in terms of a quantity (ordinates) relative to that of the untreated control ghosts. Symbols are 1/10 balanced salt solution ghosts (○), Resealed 1/15 phosphate ghosts (◇), and unresealed leaky 1/15 phosphate ghosts (△).

Fig. 3. Time courses of depression in non-hemoglobin membrane protein contents of ghosts treated with pronase. The ghosts were treated with pronase at 37 °C for a given time interval as shown in abscissa, then washed extensively as detailed in Methods. The pronase concentrations used were 10 mg/ml for 1/10 balanced salt solution ghosts and 1 mg/ml for both resealed and unresealed 1/15 phosphate ghosts. Data presentation and symbols are the same as those of Fig. 2.

digested, resealed white ghosts was next studied in some detail. It was repeatedly demonstrated in the electrophoretic pattern that proteins retained in these carrier-intact pronase-treated resealed white ghosts were grossly degraded by the digestion. The sodium dodecyl sulfate gel electrophoresis of the untreated control ghosts (Fig. 4A) revealed at least eleven major coomassie blue-stainable peaks. With the treated ghosts (Fig. 4C) none of these major peaks remained evident. With the control untreated ghosts, more than 98% of the total coomassie blue-stainable area was found in the molecular weight region of the hemoglobin monomer or higher. After the pronase treatments, more than 70% of the stain was found in molecular weight regions of the hemoglobin monomer or lower. Three broad peaks at around 25000, 40000, and 70000 accounted for the remaining 30%. These peaks were so poorly defined that it was not practical to identify them in terms of the major peaks of the control ghosts. Also noted is that the region of molecular weights higher than 150000 was essentially cleared of any appreciable peak. Such a digestion pattern was already evident at a 30-min treatment of the resealed ghosts with the pronase concentration of 1 mg/ml at 37 °C. Further increase in the incubation time up to 90 min did not change the pattern perceptably. In order to reproduce such a digestion pattern, the effective termination of the enzyme activity at the end of the

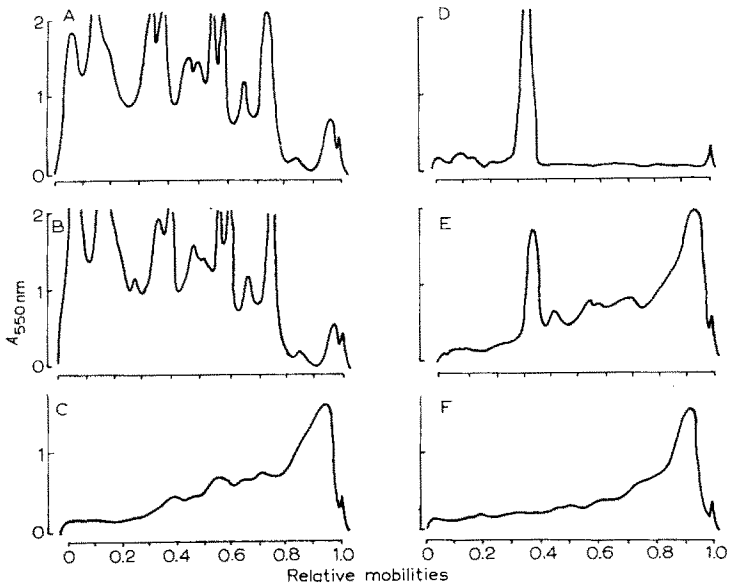

Fig. 4. Change in sodium dodecyl sulfate–acrylamide gel electrophoretic pattern of 1/15 phosphate ghosts by pronase treatments. The pronase treatments were carried out at 37 °C for 30 min using a pronase concentration of 1 mg/ml reaction mixture containing 5 mg of membrane protein. The reaction was terminated by heating and subsequent washing as described in text. The electrophoretic mobility (abscissa) was expressed as a relative quantity to that of tracking dye, pyronin Y. A, a control, resealed ghosts treated without pronase added; B, resealed ghosts treated with the pronase preheated at 100 °C for 10 min prior to the addition; C, resealed ghosts treated with pronase; D, bovine serum albumin treated without pronase; E, reselaled ghosts treated with pronase and bovine serum albumin which was added into the ghosts after the termination of digestion by heating; F, unresealed ghosts treated with pronase.

treatment appeared to be important. The termination by repeated wash with cold buffer alone was found not to be effective, and resulted in even more extensive degradation in the electrophoretic pattern than that of Fig. 4C. This may be due to a small amount of pronase tightly bound to the ghost membrane, which may have gained accessibility to more membrane proteins during the subsequent sodium dodecyl sulfate gel electrophoresis (unpublished observation by F. A. Green and C. Y. Jung). The termination by heating the digested sample at 100 °C for 10 min followed by cold washes was finally adopted with the following supporting evidence: First, a treatment of the ghosts with the pronase pretreated at 100 °C for 10 min did not change the sodium dodecyl sulfate gel electrophoretic pattern (Fig. 4B) from that of the non-treated sample, indicating that heating effectively inactivates the pronase activity. Secondly, a molecular marker, albumin, added to the digested ghost samples after the termination procedure but prior to the sodium dodecyl sulfate solubilization procedure, was not affected in the electrophoretic pattern (Fig. 4D *versus* Fig. 4E). This observation apparently rules out the possible artifact[10] which may be introduced during the sodium dodecyl sulfate solubilization step due to an activation of protease native to the erythrocyte membranes, if the membrane protein is not more susceptible to the protease than albumin. Similar electrophoretic patterns were observed with pink ghosts prepared in the hypotonic balanced salt solution after digestion with

pronase concentration of 10 mg/ml at 37 °C for 60 min. With unresealed leaky white ghosts, the digestion pattern on the electrophoresis was even more drastic (Fig. 4F) and less reproducible, being often much more drastic than Fig. 4F. A 30-min treatment with a pronase concentration of 1 mg/ml at 37 °C often resulted in virtually a total disappearance of any coomassie blue-stainable peak throughout the region of the molecular weights higher than the hemoglobin monomer. The digested products accumulated as a massive peak around the molecular weight region of the hemoglobin monomer or lower.

DISCUSSION

It is shown in the present study that pronase treated human erythrocyte ghosts which retain less than 50% of the original membrane protein can perform the glucose carrier function in its full activity. Furthermore, it is also shown that even these proteins which were retained in carrier-active pronase-treated ghosts were grossly altered in their sodium dodecyl sulfate gel electrophoretic mobilities. At least 70% of them were found to be degraded into smaller molecular weight entities. Taking these two observations together it is evident that the glucose carrier function, or more specifically the structural integrity of the carrier in the ghost membrane, is intact even after approximately 85% of the membrane proteins were degraded by the pronase digestion. This unexpected resistance could be taken as a suggestion that the transport carrier, contrary to the general belief[1], may not be an enzyme-like protein. It should be recalled that no currently known property of this carrier definitely proves that the carrier is a protein. It also appears unlikely, with reasons reported elsewhere[11], that the carrier function is subserved by lipid components alone. If the carrier is indeed a protein, it must be of an unusual nature, in that it is extremely resistant to extensive proteolytic digestion. One example of such a resistance is with protein moieties of the proteolipids[12]. The possibility that the glucose carrier may be a proteolipid-like structure has been suggested[13,14]. A further example is that of alamethicin, a cyclic peptide that can transport cations and induce action potential in an *in vitro* lipid membrane, and which exhibits an extreme resistance to proteolytic digestion[15].

The maximum number of the glucose carrier sites in human erythrocytes has been estimated to be about $3 \cdot 10^4$ per cell (W. D. Stein, personal communication, 1971). Using this figure, amounts of the carrier per 10^{10} cells can be calculated for each hypothetical molecular weight of the carrier. They are roughly 0.005 and 0.15 mg for the hypothetical molecular weights of 10000 and 300000, respectively. Since the carrier-active, practically hemoglobin-free white ghosts used in this study contained roughly 5 mg of protein per 10^{10} ghosts, the carrier, assuming that it is a protein, would amount to only 0.1 and 3% of the total membrane protein respectively. It would be quite probable that this amount of carrier protein could have been present but undetected even in the clear region of the gel electrophoresis of the pronase-treated ghosts. Nevertheless, the pronase-treated carrier-active, practically hemoglobin-free ghost preparation studied here would provide valuable material in further attempts to identify and isolate the glucose carrier in human erythrocytes.

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