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Biochemistry

Structural (shape-maintaining) role of the cell surface glycoprotein of *Halobacterium salinarium*

(cell morphology/surface labeling/proteolytic enzymes)

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ABSTRACT **The obligate halophile, *Halobacterium salinarium*, maintains a rod-shaped morphology under normal growth conditions. Lactoperoxidase(EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase)-catalyzed iodination and treatment with proteolytic enzymes were used to demonstrate that the recently described envelope glycoprotein [Mescher, M. F. & Strominger, J. L. (1976) *J. Biol. Chem.* 251, 2005–2014] is the only major cell surface component of this organism. The morphological changes that accompany alteration of the structure of the glycoprotein by growth in the presence of bacitracin or its removal with proteolytic enzymes strongly suggest that it forms a rigid matrix at the cell surface and is responsible for maintenance of the characteristic rod shape.**

Halobacterium salinarium has recently been shown to have an envelope glycoprotein similar to those present on the surface of eukaryotic cells with respect to the nature of the carbohydrate-protein linkages, the number, size, and composition of the carbohydrate moieties, and their localization to a limited region(s) of the protein (Fig. 1 and ref. 1). This glycoprotein accounts for all of the nonlipid carbohydrate of the cell envelope and 40–50% of the envelope protein (2). Despite the absence of a peptidoglycan layer, these bacteria are able to maintain a rod-shaped morphology under normal growth conditions (3). The large amount of glycoprotein in the cell envelope and the effect of the antibiotic bacitracin on the growth and morphology of the cells (4) suggested that the glycoprotein might play a structural role in the envelope. Evidence is presented in this report which shows that this glycoprotein is the only major envelope component present at the cell surface and which strongly suggests that it is the structural component responsible for maintenance of normal cell morphology. The structural role of the *H. salinarium* glycoprotein may have implications for the role of at least some of the cell surface glycoproteins of higher organisms.

MATERIALS AND METHODS

Halobacterium salinarium, strain 1 (ATCC 19700), was grown in the medium previously described (2), harvested by centrifugation when growth had reached midlogarithmic phase, and washed three times in basal salts solution having the same ionic composition as the growth medium.

Lactoperoxidase-catalyzed iodination (5) of intact cells was done in a reaction mixture consisting of 2×10^{10} cells in 2 ml of 0.1 M Tris·HCl, pH 7.2, having 4 M NaCl, 40 mM $MgSO_4 \cdot 7H_2O$, and 5 mM $CaCl_2$, with 50 μg of lactoperoxidase (EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) (Sigma) and 2 mCi of Na ^{125}I (New England Nuclear). Labeling was done by addition of 0.005-ml aliquots of 0.03% H_2O_2 every 5 min for a total of 30 min. The reaction was then stopped by addition of 10 ml of 10 μM Na_2SO_3, and the cells were washed extensively in the same buffer as used for labeling. Lysed cells were labeled identically with the following exceptions: NaCl, $MgSO_4 \cdot 7H_2O$, and $CaCl_2$ were omitted from the buffer, 10 ml of cold, 10% trichloroacetic acid was added at the end of the reaction time, and the precipitate was washed extensively with cold, 10% trichloroacetic acid followed by ethanol. Intact cells or trichloroacetic acid precipitates were then dissolved in 3% sodium dodecyl sulfate ($NaDodSO_4$) and examined by $NaDodSO_4$-polyacrylamide slab gel electrophoresis.

Discontinuous polyacrylamide slab gel electrophoresis (6, 7) was done with a 3% acrylamide stacking gel and a 7.5% acrylamide running gel. Gels were stained for protein with Coomassie brilliant blue (8). Samples labeled with ^{125}I by lactoperoxidase-catalyzed iodination were examined by autoradiography of dried gels using Kodak x-ray film (SB-54).

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

RESULTS

Surface location of the glycoprotein

Halobacteria require high salt concentrations not only for optimum growth, but for maintenance of structural integrity (3). As the salt concentration of the suspending medium is lowered the cells undergo morphological changes, and at less than 1–1.5 M NaCl, lysis occurs. Drastic changes in the structure of the cell envelope occur under these conditions. It is therefore necessary that experiments to determine which proteins are normally exposed at the surface of growing cells be done in medium having the same ionic composition as that used for culturing the cells. Two methods, lactoperoxidase-catalyzed iodination and proteolytic degradation, have been successfully used under these conditions to demonstrate the surface location of the *H. salinarium* glycoprotein.

Labeling of Surface Proteins. The impermeability of lactoperoxidase to cell membranes has made lactoperoxidase-catalyzed iodination of tyrosine and histidine residues an effective method for demonstrating which membrane proteins are exposed at cell surfaces (9, 10). Lactoperoxidase activity was reduced by approximately 80% at the high salt concentration necessary for labeling the *H. salinarium* surface proteins in comparison to activity in the same buffer in the absence of salt. This reduction in activity was compensated for by using somewhat higher enzyme concentrations and longer incubation times than normally used for labeling. After labeling with ^{125}I the washed cells were solubilized in 3% $NaDodSO_4$ at 100° (cells are completely solubilized under these conditions) and examined by $NaDodSO_4$-polyacrylamide slab gel electrophoresis. The very high molecular weight of the glycoprotein (200,000) made it possible to resolve it from all of the other proteins of the cell by the gel system of Laemmli (6). The glycoprotein runs as a relatively broad band on these gels and is sometimes resolved into a doublet band. This has also been observed for the

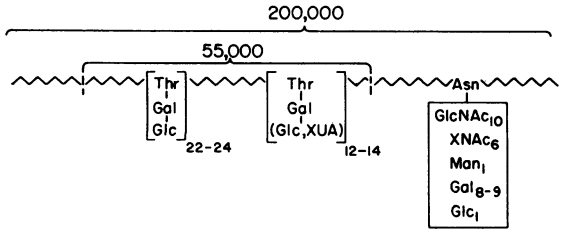

FIG. 1. Proposed structure of the cell envelope glycoprotein of *H. salinarium* (1). XUA is an unidentified hexuronic acid and XNAc an unidentified hexosamine.

purified glycoprotein (unpublished) and may be due to heterogeneity in the uronic acid content.

When labeling was done in the absence of salt (Fig. 2C), conditions under which complete cell lysis occurs, labeled bands corresponding to all of the proteins that stained with Coomassie blue (Fig. 2A) were seen. At high salt concentration, conditions under which cells remain intact, the glycoprotein was the only protein labeled (Fig. 2B). A small amount of labeled material was present at the dye front. Its absence after extraction with chloroform/methanol (2:1) indicates that it is lipid. No cell lysis occurred during the labeling and washing procedure, and all cells retained their normal rod-shaped morphology. If gels were heavily overloaded with protein and autoradiography was done for long times, some minor bands were detected (Fig. 2D). None of these bands corresponded to major protein components of the cell (Fig. 2E). *H. salinarium* is a flagellated bacterium, and some or all of these bands may be flagellar proteins. Control experiments done in the absence of lactoperoxidase or H_2O_2 resulted in no labeling (<1%) of the cells.

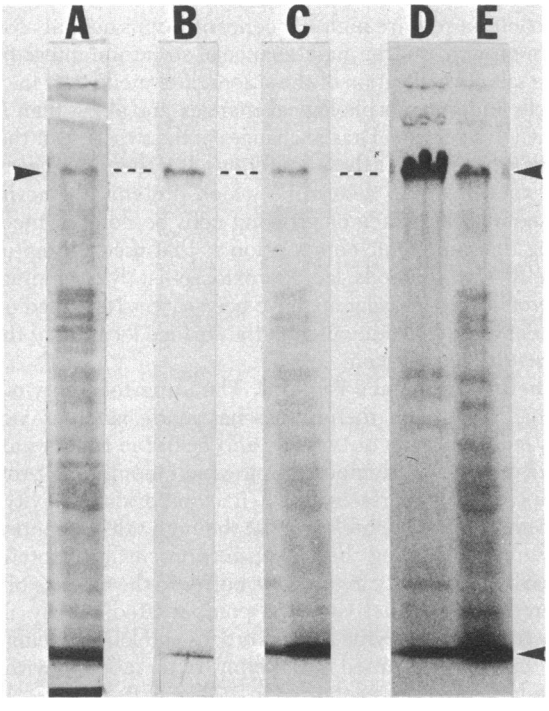

FIG. 2. Lactoperoxidase-catalyzed iodination of surface proteins. Top arrows indicate the position of the glycoprotein, and bottom arrows the position of the dye front. (A) Intact cells, stained with Coomassie blue. (B) Intact cells, ^{125}I-labeled (autoradiogram). (C) Lysed cells, ^{125}I-labeled (autoradiogram). (D) Intact cells, ^{125}I-labeled (autoradiogram). Gel is heavily overloaded in order to detect minor bands. (E) Lysed cells, ^{125}I-labeled (autoradiogram). Gel is overloaded for comparison with gel D.

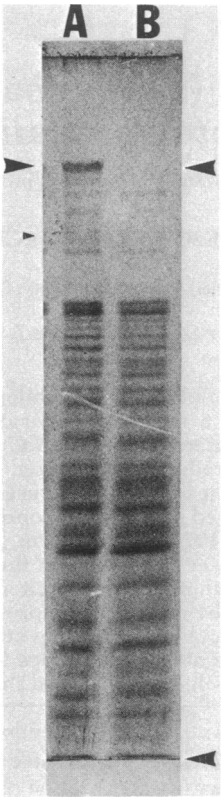

FIG. 3. Removal of the glycoprotein from the surface of intact cells by treatment with insoluble protease. Cells (2×10^{10}/ml, midlogarithmic growth) were washed and resuspended in 0.1 M Tris·HCl, pH 7.2, containing 4 M NaCl, 40 mM $MgSO_4 \cdot 7H_2O$, and 5 mM $CaCl_2$. Ten milligrams of insoluble protease (Sigma) were added, and samples were incubated with shaking for 52 hr. Samples were then centrifuged at low speed to remove the protease. The cells were washed in buffer, solubilized in 3% $NaDodSO_4$ at 100°, and examined by $NaDodSO_4$-polyacrylamide slab gel electrophoresis. Top arrow indicates the glycoprotein, bottom arrow the dye front. Protein was stained with Coomassie brillant blue. (A) Control cells, no insoluble protease (small arrow indicates a minor degradation product of the glycoprotein). (B) Cells treated with insoluble protease.

These results demonstrated that the glycoprotein is exposed at the cell surface and suggested that it is the only major external component of the cell envelope. However, they did not rule out the possibility that there are other proteins at the surface having no exposed iodinatable residues, nor did they demonstrate that all of the glycoprotein is at the surface. Experiments using proteolytic enzymes were done in order to answer these questions.

Removal of Surface Proteins by Proteolytic Enzymes. As in the case of lactoperoxidase, the impermeability of cells to proteolytic enzymes has made them useful reagents for studying external membrane proteins (11, 12). Insolubilized protease (Sigma; enzyme from *Streptomyces griseus* attached to carboxymethyl-cellulose) retains activity at high salt concentrations and was used to examine further the surface proteins of *H. salinarium*. The reason for using the insolubilized enzyme will be discussed below. Treatment of intact cells with the enzyme resulted in complete removal of the glycoprotein with no detectable loss of other proteins (Fig. 3). The minor band present in normal cells (Fig. 3A, small arrow) and absent in treated cells is a degradation product of the glycoprotein that appears upon incubation of the control cells in the absence of added protease. It was previously shown that isolated cell envelopes have an

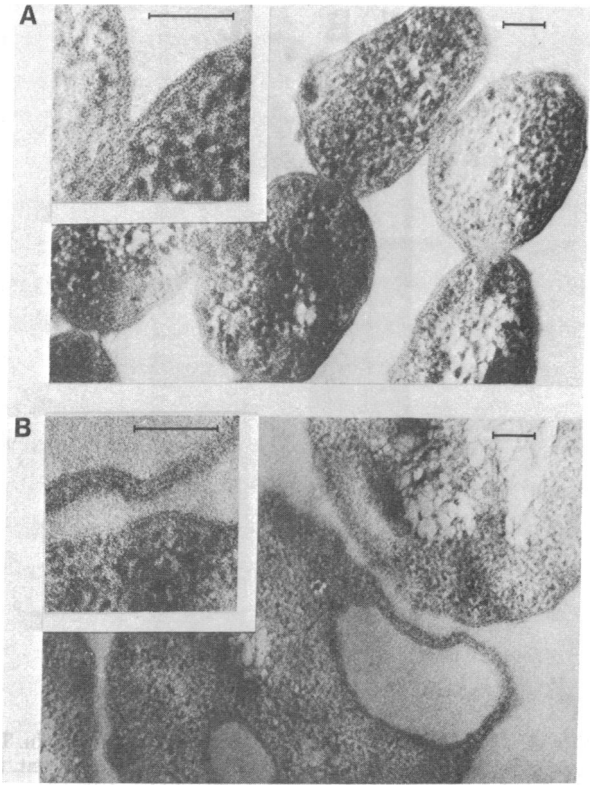

FIG. 4. Cell envelope structure of normal and treated cells. Cells were prepared for thin sectioning by fixing in 4% formaldehyde in 4 M NaCl, 40 mM $MgSO_4 \cdot 7H_2O$, 5 mM $CaCl_2$, pH 7, followed by 2% osmium tetroxide in the same salt solution, and embedding in Epon-Araldite. Bars are 100 nm. Insets are higher magnification views of the envelope structure. (A) Normal cells; (B) cells treated with insoluble protease (treatment as described in legend of Fig. 2).

endogenous protease activity that degrades the glycoprotein (2); this is presumably responsible for the small amount of degradation that occurs upon incubation of whole cells under conditions where growth does not occur. No cell lysis occurs during incubation with protease, but the treated cells undergo a morphological change from rods to spheres, an observation that led to the experiments presented in the following sections. When cells were lysed at low salt concentration prior to treatment with protease, no intact proteins were detectable by NaDodSO$_4$-polyacrylamide slab gel electrophoresis. The removal of the glycoprotein from intact cells by protease confirmed the results obtained with lactoperoxidase labeling and demonstrated that all of the glycoprotein lies external to the plasma membrane.

As expected from the lack of peptidoglycan and other complex polysaccharides, Halobacteria do not have a cell envelope structure of the type characteristic of normal Gram-negative bacteria. Instead, electron microscopy of thin sections shows a plasma membrane of normal appearance and, external to it, a densely staining outer envelope layer (13–16). It had previously been shown that this layer is composed largely of protein, and the finding that the glycoprotein is the only major protein component on the cell surface indicated that it comprises this densely staining outer layer. When normal cells of *H. salinarium* (Fig. 4A) were compared to cells that had been treated with protease to remove the glycoprotein (Fig. 4B), it was found that the outer layer was missing from the treated cells and only the plasma membrane remained. The absence of the outer layer in protease-treated cells indicates that the glycoprotein is the major, if not the only, component of this layer.

The amount of glycoprotein present in the cell envelope is consistent with this conclusion. The densely staining outer layer of the normal cell envelope is approximately 10 nm thick. Assuming the cell to be 5 μm long by 0.5 μm in diameter (the average value for cells in midlogarithmic phase), the total volume occupied by the outer layer is 0.13 μm^3 per cell. The amount of glycoprotein per cell is estimated to be approximately 4×10^{-14} g, based on the amount of glycoprotein in the cell envelope (2) and the yield of cell envelopes isolated from a known number of cells. Using this value and assuming a partial specific volume of 0.7 g/liter, the total volume occupied by the glycoprotein is 0.06 μm^3 per cell. These values are in good agreement, considering the uncertainty of the estimates, especially of the value used for the volume occupied by the glycoprotein in its native state.

In addition to no longer having the outer envelope layer, protease-treated cells appeared to have internal, membrane-bound vesicles (Fig. 4B) not seen in normal cells. It is possible that these vesicles form as a result of the invagination of excess membrane which results from the change in surface area as the cells are converted from rods to spheres upon treatment with protease. In initial attempts to demonstrate the surface location of the glycoprotein using soluble proteolytic enzymes, it was found that the cells could not be washed free of the proteases after treatment, probably as a result of the enzymes being trapped as the vesicles formed. Use of the insolubilized protease overcame this problem, since the large particle size of the carboxymethyl support prevented trapping.

The glycoprotein is the major shape-maintaining component

Protease Treatment and Cell Morphology. Treatment of *H. salinarium* with a variety of proteolytic enzymes, including pepsin (Sigma), papain (Sigma), Pronase (Calbiochem), and insolubilized or soluble protease (Sigma), causes a morphological change from the normal rod shape to spherical cells with no resulting lysis. The conversion from rods to spheres occurs in the absence of cell growth and with a time course dependent on enzyme concentration, cell density, and temperature.

Spherical cells having no remaining glycoprotein (as determined by NaDodSO$_4$-polyacrylamide slab gel electrophoresis) remain viable and will grow when washed free of the protease and placed in fresh growth medium. Growth is slower than that of normal cells, but proceeds to the same final cell density. As the spherical cells grow they regain the normal rod-shaped morphology, with approximately 50% of the cells being rods after one doubling. The morphological changes accompanying treatment with protease and subsequent regrowth are shown in Fig. 5. Control cells, in the absence of protease, retained their normal rod shape at all times. The glycoprotein content of the cells was examined by NaDodSO$_4$-polyacrylamide slab gel electrophoresis (Fig. 5) at the same times that the micrographs were taken. A comparison of control and treated cells showed that the treated cells lose the glycoprotein from the surface concomitantly with the conversion from rods to spheres and that it reappears concomitantly with the reappearance of the normal rod morphology. No change in other detectable proteins occurred. The glycoprotein appeared as a doublet in both control and treated cells during regrowth (Fig. 5D and E), possibly as a result of heterogeneity in uronic acid content. These results strongly suggest that the glycoprotein is the major envelope structural component responsible for maintenance of cell shape in *H. salinarium*. Further evidence in support of this conclusion was obtained using an antibiotic that specifically alters the structure of the glycoprotein.

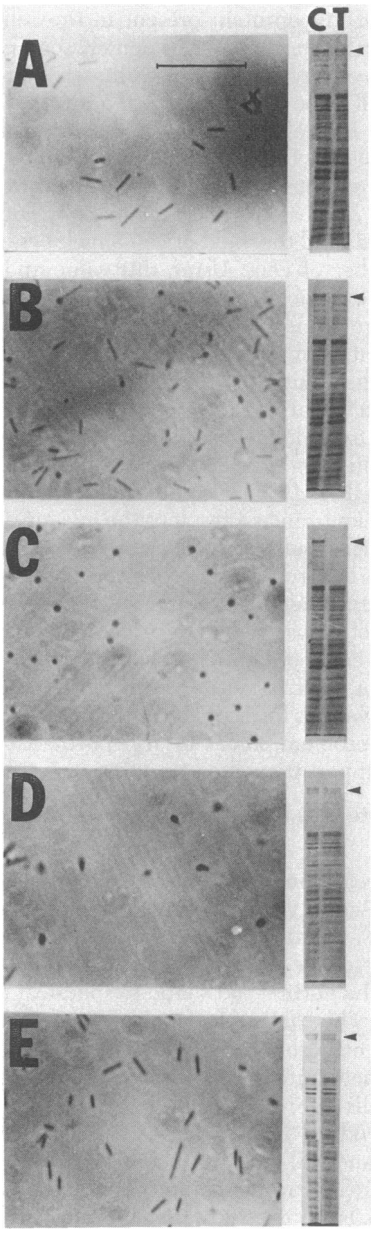

FIG. 5. Morphology and glycoprotein content of cells during treatment with insoluble protease and regrowth. Cells (2×10^{10}/ml, midlogarithmic phase) were suspended in 0.01 M Tris·HCl, pH 7.2, containing 4 M NaCl, 40 mM $MgSO_4 \cdot 7H_2O$, 5 mM $CaCl_2$, and 10 mg of insoluble protease (Sigma) was added to the test sample. The control sample was treated identically, but no insoluble protease was added. Samples were incubated at 37° with shaking. When conversion of protease-treated cells to spheres was complete the cells were harvested, placed in fresh growth medium, and again incubated at 37° with shaking. Aliquots were removed at various times during conversion and regrowth for photomicrography and $NaDodSO_4$ gel electrophoresis. Only photomicrographs of protease-treated samples are shown; control cells were rod-shaped at all times. $NaDodSO_4$ gels of control (C) and treated (T) cells are shown for samples taken at times corresponding to the photomicrographs. (A) 0-hr treatment; (B) 24-hr treatment; (C) 52-hr treatment (cells were washed and placed in growth medium at 52 hr); (D) 24-hr regrowth; (E) 42-hr regrowth.

Effect of Bacitracin on Glycoprotein Structure and Cell Morphology. Glycosylation of at least some of the glycoproteins of eukaryotic cells occurs via lipid-linked intermediates (17, 18) similar to those involved in peptidoglycan synthesis (19); this

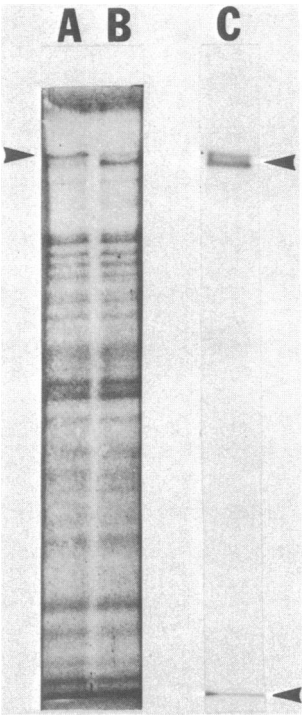

FIG. 6. The effect of bacitracin on the surface glycoprotein. Top arrows indicate the glycoprotein, bottom arrows the dye front. (A) Normal cells, stained with Coomassie brilliant blue; (B) cells grown for approximately one doubling time in bacitracin (10 μg/ml), stained with Coomassie brilliant blue; (C) lactoperoxidase-catalyzed iodination of intact, bacitracin-grown cells (^{125}I autoradiogram). Labeling was done as described in legend of Fig. 1.

appears to be the case for glycosylation of the *H. salinarium* glycoprotein. The cells have enzymatic activities for formation of lipid-linked sugar compounds having the properties expected of such intermediates (M. F. Mescher *et al.*, manuscript in preparation). In addition, growth of *H. salinarium* is inhibited by bacitracin (4), an antibiotic that stops peptidoglycan synthesis in normal bacteria by complexing with the lipid pyrophosphate released after transfer of the lipid-linked subunit to the growing peptidoglycan chain (20, 21), thus making the carrier lipid unavailable for formation and transfer of additional subunits. Preliminary studies indicate that bacitracin inhibits growth of *H. salinarium* by blocking glycosylation of the envelope glycoprotein*.

Bacitracin not only inhibits the growth of *H. salinarium*, but it also causes a morphological change from rod-shaped to spherical cells (4). When cells grown in bacitracin are examined by $NaDodSO_4$-polyacrylamide gel electrophoresis, the glycoprotein is found to have a higher mobility than that of control cells, consistent with its having a decreased carbohydrate content (Fig. 6A and B). No changes in other proteins are seen, and no differences in major lipid components were detectable by thin-layer chromatography and iodine staining. The altered glycoprotein is located at the surface of the bacitracin-grown cells, as shown by lactoperoxidase labeling of intact cells (Fig. 6C). The specific effect of bacitracin on the glycosylation of the glycoprotein strongly suggests that the change in morphology is a result of the alteration of the glycoprotein, thus supporting the conclusion that it is the major shape-maintaining component

* Preliminary results have been published in the *Abstracts of the Third International Symposium of Glycoconjugates*, Brighton, England, July 1975.

of the cell envelope. The fact that the altered glycoprotein is still located on the cell surface but is apparently unable to maintain a rod shape indicates that the presence of the carbohydrate units is necessary to this function.

DISCUSSION

The cell envelope glycoprotein of *H. salinarium* is present at the cell surface, where it forms a relatively thick external layer. Removal of the glycoprotein or alteration of its structure results in a conversion from rod-shaped to spherical cells, strongly suggesting that it forms a rigid structural matrix at the cell surface which allows the cell to maintain a stable, characteristic morphology. Further study of the glycoprotein will be necessary in order to determine the kinds of interaction that are involved in stabilizing this structure. The glycoprotein aggregates strongly in the presence of divalent cations (ref. 1 and unpublished results), suggesting that protein–protein interactions via salt bridges may be involved, a suggestion supported by the observation that placing the cells in low magnesium medium results in the loss of the normal rod shape (3, 22). The effects of bacitracin on cell morphology and glycoprotein structure indicate that the carbohydrate units may be involved; they possibly have a role in maintaining the necessary conformation of the glycoprotein. Alternatively, the carbohydrate units on adjacent molecules may interact and bind to each other or to protein.

The structural role of the glycoprotein and its extremely acidic nature may account for the requirement for an extremely high concentration of monovalent cations (approximately 4 M NaCl) for maintenance of normal cell morphology and for structural integrity of isolated cell envelopes (3). The total envelope protein of *H. salinarium* has a 20 mole % excess of acidic over basic residues (uncorrected for amides) (16). It has been suggested that a high concentration of monovalent cations is necessary to shield negative charges and prevent repulsion within a protein or between adjacent proteins (23, 24). The glycoprotein has a 33 mole % excess of acidic over basic residues (uncorrected for amides) (1), thus accounting for a major portion of the excess acidity of the cell envelope protein.

The shape-maintaining role of the *H. salinarium* glycoprotein and its structural similarities to the glycoproteins of eukaryotic cell surfaces raise the possibility that some of these glycoproteins might have a similar structural role. Bretscher (12) has suggested that the major erythrocyte glycoprotein (glycophorin) might form a matrix on the cell surface to provide the membrane with increased mechanical stability. Evidence to support such suggestions is difficult to obtain, however, due to the complex interactions of cell surface proteins with the cytoskeletal system, which appears to play a dominant role in maintaining the morphology of eukaryotic cells (25, 26). Further examination of how the *H. salinarium* glycoprotein is able to impart rigidity to the cell envelope and of what role the carbohydrate moieties play in this function should contribute to an understanding of how surface glycoproteins interact and allow a better assessment of the possibility that some of those present on eukaryotic cells may have a structural role.

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