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PROTEINS OF THE CELL ENVELOPE OF A MARINE PSEUDOMONAD, *PSEUDOMONAS* BAL-31

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SUMMARY

The protein composition of the envelope fraction of *Pseudomonas* BAL-31 was studied by polyacrylamide gel electrophoresis. Two major polypeptides of molecular weights 130 000 and 110 000 were found. These two polypeptides, which account for as much as 40–50% of the total protein of the envelope, are associated with the outer membrane. One of these proteins might be a glycoprotein. The inner membrane contains a more heterogeneous collection of smaller polypeptides.

INTRODUCTION

The Gram-negative marine pseudomonad, *Pseudomonas* BAL-31, is of special interest because it is the host cell of PM2, a lipid-containing bacteriophage[1]. This virus matures at the periphery of the host bacterium and there are some indications that viral specific proteins[2] are synthesized on or very close to the bacterial membrane. Furthermore, one of the viral structural components appears to be a lipid bilayer[3,4]. Current interest in cell–virus membrane systems and the nature of membrane proteins in general has prompted us to fractionate and characterize the proteins of the *Pseudomonas* BAL-31 membrane.

The envelopes of Gram-negative bacteria consist of three layers: a cytoplasmic membrane, a mucopeptide layer and an outer membrane[5]. By treatment with lysozyme and EDTA, the mucopeptide layer is digested and removed[6], but the resulting spheroplast still has two membrane components in its envelope[7]. By osmotic shock of these spheroplasts, a mixture of outer and cytoplasmic membranes are obtained. This mixture is called the envelope fraction. The enzymology and the chemical composition of the envelope fraction of *Pseudomonas* BAL-31 have been previously described[8].

All known plasma membranes are composed of lipid, protein, and a trace of carbohydrate. The lipid and protein components of animal cell membranes have been extensively studied, but in bacteria the nature of the proteins found in membranes is not well understood.

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Little is known concerning the number, size and type of proteins[9-11]. In *Escherichia coli* envelopes Schnaitman[10] reported the presence of a major protein of molecular weight 44 000. This protein, which accounted for 40% of the total protein of the envelope preparation, is localized in the outer layer of the cell membrane[11].

The proteins of the envelope fraction can be fractioned by electrophoresis on polyacrylamide gels[12-14], a procedure which is known to separate polypeptides according to their molecular weight[15]. In this paper we describe a class of membrane-specific proteins from *Pseudomonas* BAL-31 and report the presence of a possible glycoprotein in the envelope fraction. The polypeptide contents of the separated outer and inner layer of the cell membranes are also reported.

MATERIALS AND METHODS

Bacteria and growth conditions

The conditions for the growth of *Pseudomonas* BAL-31 in BAL synthetic medium containing glucose as a carbon source has been previously described[16]. Glycerol (0.4%) was substituted for glucose as the carbon source in the BAL synthetic medium[16] in order to obtain a good incorporation of glucosamine into trichloroacetic acid-precipitable cell material. The original bacterial strain was passed serially for 2 weeks to adapt it to the glycerol medium, which is unenriched.

Preparation of labeled membranes

(*1*) *Amino acid labeling of glucose-grown cells. Pseudomonas* BAL-31 was grown overnight in synthetic medium[16] at 25 °C and diluted with fresh medium to a concentration of $1 \cdot 10^8$ cells/ml. Cells were then grown for another 2 h, and during this logarithmic growth period repeated additions of the ^{3}H-labeled amino acid mixture, without carrier amino acids (at a final concentration of each addition of 0.25 μCi/ml), were made at the following times: 0, 30, 60 and 90 min. 30 min after the last addition, the cells were collected, washed, and used for the preparation of membranes.

(*2*) *Glucosamine labeling of glycerol-grown cells.* Cells were grown for 40 h in glycerol medium at 25 °C. When the cell titer reached a concentration of $1 \cdot 10^8$ cells/ml, two additions of [^{3}H]glucosamine, at a final concentration of each addition of 0.25 μCi/ml were made at 0 and 2 h. After the last addition, cells were incubated further at 25 °C for 2 h; no carrier glucosamine was added.

The procedure for the preparation of cell envelope has been previously described[8]. For the separation of outer and inner membrane, a procedure which sequentially strips away the bacterial surface layers has been used[17-19]. Cell envelope and inner and outer membrane preparations are free of hexokinase and glucose-6-phosphate dehydrogenase activities, two enzyme activities characteristically found in the cytoplasmic fraction[20]. The assay methods for those enzymes have been described previously[20].

Solubilization of membrane proteins

Labeled and unlabeled membranes were dissociated with 1% recrystallized sodium dodecyl sulfate containing 1% 2-mercaptoethanol by incubation at 37 °C

for 5 h or by boiling for 3 min in a sealed tube[2]. The membrane proteins were then dialyzed overnight against 0.01 M sodium phosphate buffer (pH 7.2), containing 0.1% sodium dodecyl sulfate and 0.14 M 2-mercaptoethanol.

For the isolation of membrane proteins, an extensive solubilization procedure described by Viñuela *et al.*[21] was also used. In this method, membranes were extracted with phenol and the phenol phase containing proteins was sequentially dialyzed against acetic acid, urea and sodium dodecyl sulfate.

Electrophoresis

Gel electrophoresis was carried out as previously described[22] in the sodium dodecyl sulfate-containing gel system described by Summers *et al.*[23]. The buffer used for electrophoresis was 0.05 M sodium phosphate, pH 7.2, with 0.1% sodium dodecyl sulfate and 1 mM dithiothreitol; the latter was used to maintain sulfhydryl groups in the reduced state[24]. Electrophoresis was carried out at room temperature for 3.5 h at 9 mA tube. In order to visualize the protein bands after electrophoresis, gels were fixed and stained with Coomassie brilliant blue, as previously described[22]. Gels were also stained for glycoprotein, according to a modification of the procedure of Zacharius *et al.*[25]. Gels were fixed for 30 min in 10% (w/v) trichloroacetic acid, washed briefly with several changes of water, and then immersed in 1% (w/v) periodic acid dissolved in 3% (v/v) acetic acid for 60 min. Excess periodic acid was removed by overnight washing with 3–4 changes of water and the bands visualized by immersion in fuchsin–sulfite stain for 2.5 h in the dark, followed by four 15 min washes with freshly prepared 0.5% metabisulfite. Gels containing labeled proteins were preserved by freezing and storage at −20 °C. Radioactivity was determined after solubilization of gel slices in Nuclear-Chicago Solubilizer[22].

Sources of materials

^{3}H-labeled amino acids (L-amino acid mixture), [^{3}H]glucosamine (1.3 Ci/mM), and [^{32}P]phosphoric acid were purchased from New England Nuclear Corp., Boston, Mass. Deoxyribonuclease I (Worthington Biochemical Corp., Freehold, N.J.) was treated with phenylmethylsulfonyl fluoride to inactivate traces of chymotrypsin[8]. Dithiothreitol was obtained from Calbiochem, Los Angeles, Calif., and Nuclear Chicago Solubilizer from Amersham Searle, Des-Plaines, Ill.

RESULTS

Properties of the envelope fraction

The envelope fraction used in these studies was free of cytoplasmic contamination as judged by the absence of glucose-6-phosphate dehydrogenase and hexokinase, two enzymes characteristically found in the cytoplasmic fraction[20]. This preparation has been examined extensively by electron microscopy of fixed, sectioned preparations, and consists entirely of membrane vesicles. Fresh preparations of envelope fraction were used in all of the studies reported here.

Gel electrophoresis of the envelope proteins

Fig. 1 illustrates the appearance of polyacrylamide gels of the envelope fraction, as observed by slicing the gels and measuring the radioactive label. A single major peak which accounts for 40 to 50% of the labeled protein was observed in the envelope

(Fig. 1a). A similar observation has also been reported by Schnaitman[10] in the case of the envelope fraction of *E. coli.* In some gels, this peak appears to split into two closely spaced peaks. It is significant to note that a similar splitting of an apparently homogeneous envelope protein into two closely spaced bands has been observed in the case of *E. coli*[10]. We previously reported that when the membrane proteins were denatured, reduced, and alkylated prior to electrophoresis in the presence of 10 M urea, the single predominate protein band (u) was resolved into two bands[2]. Previously we thought that the single peak might represent at least two proteins which were so similar that they could not be resolved by the sodium dodecyl sulfate–gel system. Recently, however, we have observed that by reducing the concentration of phosphate in the electrophoretic buffer from 0.1 M to 0.05 M, two large polypeptides were consistently resolved in the polyacrylamide gel electropherogram (Fig. 1b). The protein composition of the envelope fraction has also been examined by direct solubilization of the envelope protein in 1% sodium dodecyl sulfate in 8 M urea. This procedure yielded poor results, with larger amounts of protein required to produce stainable bands and excessive amounts of protein remaining near the top of the gel. But solubilization of the membrane proteins by boiling in the presence of 1% sodium dodecyl sulfate containing 1% 2-mercaptoethanol, also resolved the same two polypeptides in the electropherogram. In order to prevent aggregation of these two large polypeptides, we used an extensive solubilization procedure, which probably removes

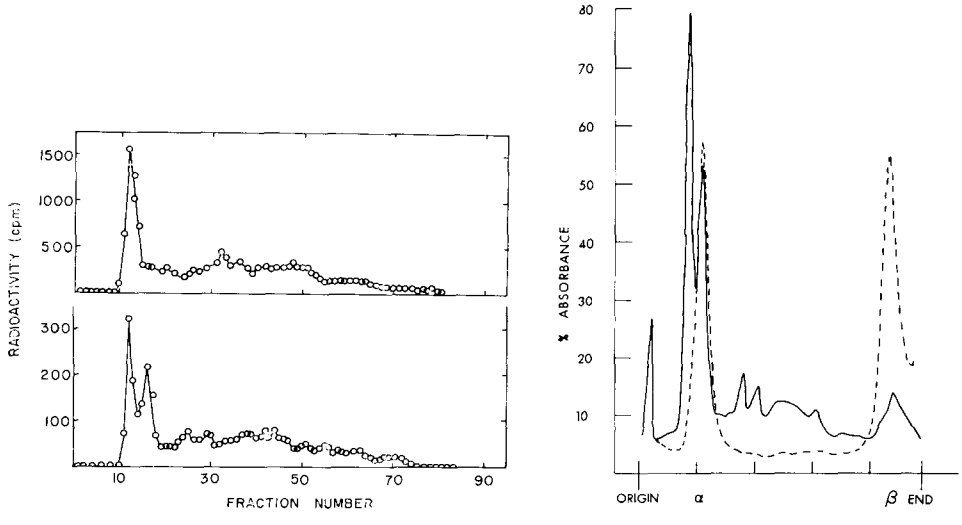

Fig. 1. Electrophoretic patterns of membrane-bound proteins labeled with ^{3}H-labeled amino acid mixtures. The membrane proteins were subjected to polyacrylamide gel electrophoresis on 7.5 cm gels according to the procedures described in the text, except that the strength of the electrophoretic buffer was varied. In case of (a) it was 0.1 M (top figure) but for (b) it was 0.05 M (bottom figure).

Fig. 2. Absorbance scan (620 nm) of a fixed and stained acrylamide gel of membrane proteins. Gels were fixed and stained with periodic acid–Schiff reagent according to the procedure described under Materials and Methods. The same gels were then restained with Coomassie brilliant blue as described in the text. The gels were then scanned with a Gilford gel scanner at 620 nm. Symbols: ———, Coomassie brilliant blue stain; — — —, PAS stain.

most of the lipids from the membranes (see Materials and Methods). A similar pattern, resolving the two major polypeptides was observed, as in Fig. 1b.

In addition to radioactive assay of labeled membrane proteins, the gels were also analyzed by using two types of staining procedures. For this purpose, unlabeled membrane proteins were electrophoresed under conditions which prevent aggregation. After electrophoresis, gels were fixed with trichloroacetic acid and stained according to the Schiff–periodate procedure, which is known to detect carbohydrates migrating with proteins[26,25]. Two bands were found in the gels; one of which migrates very close to the bottom of the gel. The gels were then washed with water and stained with Coomassie brilliant blue which stains all of the proteins in the gel. Fig. 2 represents the densitometer tracing of one such typical gel. One of the major polypeptides (band α) is stained by both staining procedures and the Schiff–periodate staining peak (β) which appears at the bottom of the gel also is stained, but weakly, with Coomassie brilliant blue. The staining of the bottom band with Coomassie blue does not mean that this band is protein since not only proteins but also lipids can be stained with Coomassie brilliant blue[14]. Lipids also migrate close to the bottom of the gel in association with the β-band[27]. The two major bands stained with Coomassie brilliant blue correspond to the two major protein bands (Fig. 1b).

Incorporation of [^3H]glucosamine into the cell

Pseudomonas BAL-31, in a glucose-containing medium, incorporates [^{3}H]-glucosamine at a low and constant rate which is dramatically increased when glycerol was substituted for glucose as the carbon source (Fig. 3). It was observed that the incorporation of [^{3}H]glucosamine was stimulated by reducing the concentration of glucose in the medium. Since in *E. coli*, glucose and glucosamine enter into cells through the same transport mechanism[28], it is reasonable to assume that glucose competitively blocks the incorporation of glucosamine. Under the same conditions, using glycerol-grown cells, the following monosaccharides did not incorporate into the cell: fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylmannosamine. The lack of incorporation of *N*-acetylglucosamine in contrast to that of glucosamine is the reverse of the situation in *E. coli*[9].

Electrophoretic profiles of [^3H]glucosamine-labeled membrane proteins

The membranes isolated from [^{3}H]glucosamine-labeled cells were analyzed by polyacrylamide gel electrophoresis (Fig. 4). Only two bands were observed in the case of [^{3}H]glucosamine-labeled membrane proteins, as was the case with Schiff–periodate stained gels (Fig. 2). Since lipids are stained with Schiff–periodate reagents, these two bands might represent glycolipids labeled with glucosamine. The presence of glucosamine in phospholipid, as a derivative of phosphatidylglycerol, has already been reported in *B. megaterium*[29]. Furthermore, the lipopolysaccharide component is localized in the outermost layers of the cell wall[30,31]. To test this hypothesis, membranes labeled with [^{32}P]phosphate were mixed with [^{3}H]glucosamine-labeled membranes (Fig. 4), dissociated and coelectrophoresed. The distribution of ^{32}P and [^{3}H]glucosamine was the same throughout the faster moving band (β). No ^{32}P has been detected in the region where protein α bands in the electropherogram. It was also observed that the number of counts in band β was considerably reduced if the dissociated

membrane proteins were dialyzed for 48 h. From these results, polypeptide α appears to be a glycoprotein and band β appears to be lipid and glycolipid.

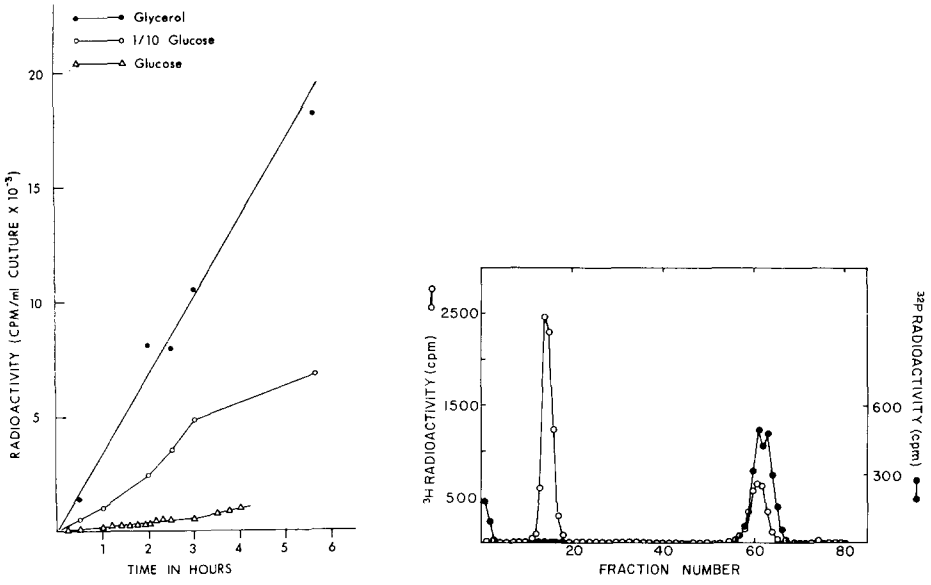

Fig. 3. Time course of [^{3}H]glucosamine incorporation into trichloroacetic acid-precipitable proteins of *Pseudomonas* BAL-31 grown in glycerol medium. The overall level of radioactivity was 0.4 μCi/ml and no carrier glucosamine was added. Samples (0.5 ml) were taken from each aliquot of cells at appropriate time intervals and diluted into 2 ml of 10% trichloroacetic acid containing 0.9% sodium pyrophosphate, collected on Millipore filters, washed three times with cold 5% trichloroacetic acid containing 0.9% sodium pyrophosphate, and then washed twice with cold ethanol. The dried filters were counted by scintillation spectrometry. ●—●, glycerol medium; △—△, glucose medium; ○—○, 1/10 glucose medium.

Fig. 4. Co-electrophoresis of [^{3}H]glucosamine-labeled and ^{32}P-labeled membranes. Membranes were mixed, treated with 1% sodium dodecyl sulfate containing 1% 2-mercaptoethanol and subjected to polyacrylamide gel electrophoresis as described in the text.

Distribution of proteins in inner and outer membranes

For analysis of the proteins of the outer and inner layers of the membranes, cells were labeled with the ^{3}H-labeled amino acid mixture or [^{3}H]glucosamine. The isolated inner and outer membranes from ^{3}H-labeled amino acid- or [^{3}H]-glucosamine-labeled cells were analyzed by polyacrylamide gel electrophoresis (Figs 5 and 6). The pattern from amino acid-labeled outer membrane is similar to the profile obtained from whole cell envelopes (Fig. 1b) but with a greater fraction of the label in the large proteins, indicating that both large molecular weight proteins are associated with the outer membrane (Fig. 5a). The patterns of Figs 5a and 5b suggest that the smaller polypeptides are associated with the inner membrane. The pattern from glucosamine-labeled outer membrane (Fig. 6a) suggests that one of the major proteins of the cell envelope is present in this fraction as a glycoprotein (*cf.* previous section).

Analysis of glucosamine-labeled inner membranes indicates the virtual absence of the slow moving component from this fraction (Fig. 6b). The small amount

remaining is probably contamination. The fast-moving glucosamine-labeled material at the bottom of the gel has not been characterized, but it co-migrates with phospholipid. It is also found in unfractionated envelopes and outer envelopes (Fig. 6a).

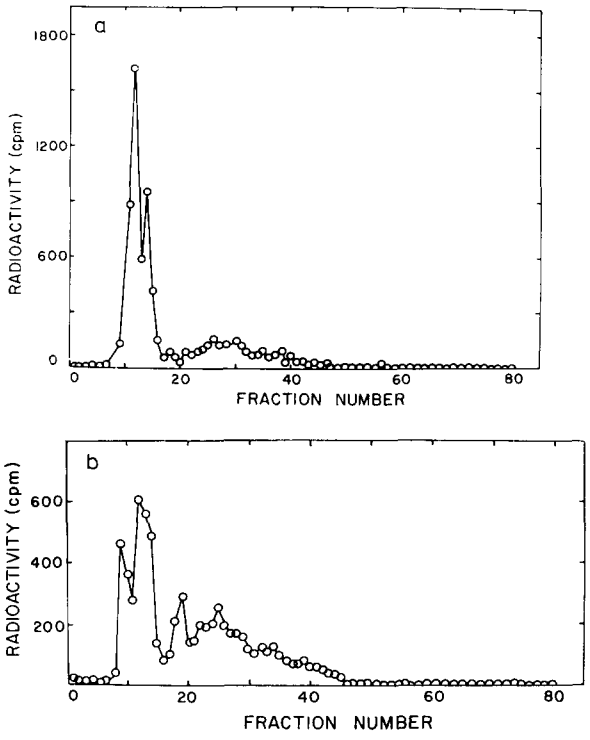

Fig. 5. Polyacrylamide gel electrophoresis pattern of isolated membranes from cells labeled with ^{3}H-labeled amino acid mixture. (a) Outer membrane, (b) inner membrane.

Determination of molecular weights

The molecular weights of the two large polypeptides associated with the membranes of *Pseudomonas* BAL-31 were estimated by the method of Shapiro *et al.*[32] by comparison with the migration of the proteins of bacteriophage PM2[22]. Molecular weights of 130000 for the polypeptide and 110000 for the glycoprotein were obtained.

DISCUSSION

A variety of proteins are present in the envelopes of *Pseudomonas* BAL-31, as is the case for *E. coli* envelopes[10] and red blood cells[33–36]. These can be fractionated according to their molecular weights in polyacrylamide gels: under favorable conditions two major components of molecular weights 130000 and 110000 (band α) can be seen, along with several minor components. These two bands, which account for roughly 40–50% of the total envelope fraction, are of particular interest.

In *E. coli* envelopes, Schnaitman[10] reported the presence of one major protein of molecular weight 44000. This accounted for 40% of the total protein of the envelope

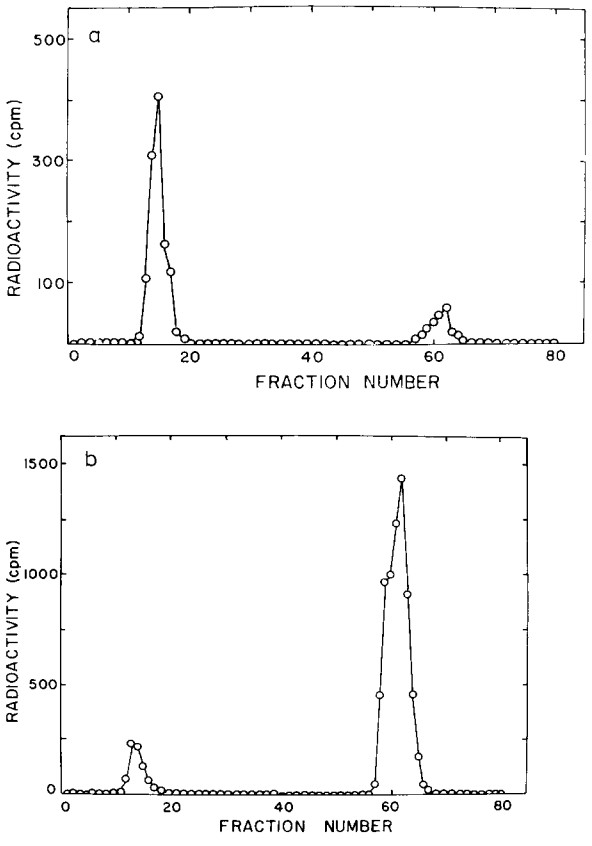

Fig. 6. Polyacrylamide gel electrophoresis pattern of isolated membranes from cells labeled with [^{3}H]glucosamine. (a) Outer membrane, (b) inner membrane.

fraction. In some gels there were two protein bands, leading Schnaitman to speculate that the single band was due to an aggregate of two or more proteins. The outer surface of human erythrocyte membranes contains two major polypeptides of molecular weights 105000 and 90000[35]. The molecular weights, which were determined by the polyacrylamide gel procedure, are similar to those we observed for *Pseudomonas* BAL-31 envelope-specific proteins. In *Bacillus brevis* P1 the T-layer protein found external to the cell wall forms 90–95% of the bulk of protein in the cell wall and it is interesting that this polypeptide subunit also has a very high molecular weight, over 100000[37]. In contrast, the reported molecular weight of the major protein in *E. coli* envelope is much smaller than that of the proteins of *Pseudomonas* membrane.

In order to separate outer and inner membranes of *Pseudomonas* BAL-31, techniques previously described for another Gram-negative marine pseudomonad[17–19] have been successfully applied in this laboratory. The major protein fraction of outer cell membrane consists of two polypeptides of 130000 and 110000 molecular weights. Inner membrane, although contaminated with outer membrane, has a more heterogeneous polypeptide content. In *E. coli*, protein analysis of separated outer and inner membrane fractions has demonstrated the presence of a 44000 molecular weight

protein in the outer membrane[10,11]. This protein constituted about 70% of the protein in the outer membrane. The inner membrane from *E. coli* also displayed a very heterogeneous polypeptide pattern. Apparently, then, the outer membrane of at least two Gram-negative bacteria consists of one or two major structural proteins integrated with lipids to form a bilayered structure. Therefore the outer membrane should be amenable to more detailed analysis of the lipid–protein interactions involved in membrane architecture.

The presence of carbohydrate in band α has been suggested by its selective staining with Schiff–periodate reagent and by labeling with [^{3}H]glucosamine. Furthermore, thin-layer chromatography of an acid hydrolyzate of [^{3}H]glucosamine-labeled membranes shows that all of the label could be recovered as amino sugars[27]. All these results suggest that band α might represent a typical glycoprotein. A strong tendency toward aggregation of the two major proteins has been noted; this might be due to an aggregating tendency of glycoprotein[38].

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