Evidence for the Presence of a Carbohydrate Moiety in Fluorescein Isothiocyanate Labeled Fragments of Rat Gastric (H^+–K^+)-ATPase

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ABSTRACT: Limited tryptic digestion of fluorescein isothiocyanate (FITC)-labeled (H^+–K^+)-ATPase from rat resting light gastric membranes produced a soluble 27-kDa polypeptide which retained the fluorescence of the parent enzyme. Its production was markedly enhanced in the presence of an amphiphilic detergent, Zwittergent 3-14, which potently inhibits the ATPase activity. This increase is probably due to protection of certain tryptic cleavage sites through conformational changes of the membrane enzyme by the detergent. The NH_2-terminal sequence of the 27-kDa polypeptide corresponded exactly to that beginning at Asn-369 of the cDNA-deduced primary structure of the rat ATPase. The presence of the phosphorylation site, Asp-385, and FITC-labeled Lys-517, which is known to be a part of the ATP-binding site, indicates that the 27-kDa polypeptide contains a major cytoplasmic portion of (H^+–K^+)-ATPase. Interestingly, the polypeptide was stained with periodate–Schiff's base, indicating its glycoprotein nature. The carbohydrate group attached to the polypeptide seems to include at least an N-linked high-mannose moiety, since the polypeptide showed Con A binding activity as detected with a Con A–biotin/avidin–peroxidase assay on nitrocellulose transblots. Also, its Con A binding activity was inhibited by excess methyl α-D-mannopyranoside and disappeared upon treatment of the polypeptide with endoglycosidase H and *N*-glycanase. Further tryptic action converted the 27-kDa polypeptide to 2 smaller FITC-labeled polypeptides of 25 and 15 kDa, which lost 18 and 96 amino acid residues, respectively, from the NH_2 terminus of the parent polypeptide. These polypeptides also showed Con A binding activity, sensitive to methyl α-D-mannopyranoside and endoglycosidase H. The association of the FITC label and Con A binding activity throughout the additional tryptic cleavage of the 27-kDa polypeptide indicates the proximity of Lys-517 (FITC labeled) to the residue bearing the N-linked carbohydrate. The amino acid sequence of rat gastric (H^+–K^+)-ATPase deduced from cDNA clones shows that only one asparagine, Asn-492, meets the minimum specificity requirement for N-linked carbohydrate attachment in this region of the ATPase. These results raise the question whether the N-linked carbohydrate moiety, most likely at Asn-492, is cytoplasmic, as proposed by hydropathy plots, or extracellular as most N-linked carbohydrates are in membrane proteins. Determination of its orientation appears to be crucial in understanding the topology of gastric (H^+–K^+)-ATPase.

(H^+–K^+)-ATPase, the primary acid pump in the stomach, catalyzes the electroneutral exchange of H^+ for K^+ (Ganser & Forte, 1973; Lee et al., 1974; Sachs et al., 1976; Fellenium et al., 1981; Forte & Machen, 1986). The enzyme is an integral membrane protein of 93 kDa (Forte & Machen, 1986) and has been proposed to contain carbohydrate on the basis of periodic acid–Schiff's base (PAS) staining (Saccomani et al., 1979). Recently, the complete amino acid sequence of rat stomach ATPase was deduced by isolation and analysis of cDNA clones (Shull & Lingrel, 1986). The ATPase consists of 1033 amino acids (M_r 114012) and has been predicted to contain 8 transmembrane domains similar to the catalytic subunit of (Na^+–K^+)- (Shull et al., 1985; Kawakami et al., 1985), Ca^{2+}- (MacLennan et al., 1985), and H^+-ATPases (Serrano et al., 1986). According to the prediction, there is a cytoplasmic stretch of 438 amino acids between the fourth and fifth hydrophobic domain which contains the phosphorylation site (Asp-385) and Lys-517 which is reactive with fluorescein 5′-isothiocyanate (FITC). Characterization of this putative cytoplasmic segment will aid in understanding not only the membrane topology of the ATPase but also possible posttranslational modifications of the ATPase. In this study, we have been able to prepare a large amount of a 27-kDa soluble, tryptic fragment of FITC-labeled (H^+–K^+)-ATPase which has Asn-369 as the N-terminus and retained the fluorescence of the parent enzyme. This putative cytoplasmic polypeptide appears to contain a high-mannose moiety, most likely on Asn-492.

Materials and Methods

Globulin-free bovine serum albumin, diisopropyl fluorophosphate, biotinylated wheat germ agglutinin, *Phaseolus vulgaris* erythroagglutinin (PHA-E), concanavalin A (Con A), avidin–horseradish peroxidase (HRP), methyl α-D-mannopyranoside, and fluorescein isothiocyanate (FITC) were purchased from Sigma. The HRP color development reagent, sodium dodecyl sulfate, acrylamide, nitrocellulose, and goat anti-rabbit conjugated to HRP were obtained from Bio-Rad. Endoglycosidase H was purchased from Dupont-NEN. *N*-Glycanase was purchased from Genzyme. Zwittergent 3-14 was obtained from Calbiochem. All other reagents were of reagent grade.

Rat resting light gastric membranes enriched with (H^+–K^+)-ATPase were prepared from cimetidine-treated male Sprague-Dawley rats as described previously (Im et al., 1984). Peripheral membrane proteins were removed by incubating

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the membranes with 10 volumes of a solution containing 2 mM Tris-HCl (pH 8.0)–5 mM EDTA. The washed membranes were labeled with FITC according to the method of Karlish (1980). Briefly, the membranes (~3 mg of protein) were suspended in 4 mL of 100 mM Tris-HCl (pH 9.2)–2 mM EDTA and then reacted with FITC at a final concentration of 5 μM in dimethylformamide. After incubation for 30 min at 23 °C in the dark, the reaction was terminated by adding 4 mL of 40 mM Tris-HCl (pH 7.4)–8 mM lysine. The FITC-labeled membranes were washed and resuspended in H_2O to a final concentration of 5 mg of protein/mL.

Trypsin Digestion. Typically, 20 μL of the FITC-labeled membrane was treated for 5 min at 23 °C with various concentrations of trypsin in the presence or absence of the indicated concentrations of Zwittergent. The reaction was terminated by adding diisopropyl fluorophosphate (5 mM). The reaction mixtures were analyzed over a 10% polyacrylamide slab gel using Tris/glycine/sodium dodecyl sulfate (SDS) buffer (Laemmli, 1970).

Purification of FITC–Polypeptides. Trypsin digestion (200 μg/mL) of FITC-labeled resting light membranes (5 mL) in the presence of 0.3% Zwittergent 3–14 was terminated by adding diisopropyl fluorophosphate (5 mM). The digested membrane polypeptides were applied to a column of Whatman DE-52 (1.4 × 25 cm) equilibrated with a solution containing 10 mM Tris-HCl, pH 8.0, and 10 mM mercaptoethanol (buffer A). The column was eluted with a 0–0.5 M NaCl gradient in buffer A. Effluent fractions most enriched with FITC label (0.2 M NaCl) were pooled, concentrated by ultrafiltration, and applied to a column of Sephadex G-75 (2 × 80 cm) equilibrated and eluted with buffer A. The fractions enriched with the 27-kDa polypeptide (as examined on a 10% SDS gel) were pooled, freeze-dried, and resolved electrophoretically on a 15% preparative SDS–polyacrylamide slab gel. The fluorescent band at the region of 27-kDa molecular mass was excised, mashed, and extracted with 10 mM Tris-HCl (pH 8.0)–5 mM EDTA. The extracts were freeze-dried. In some experiments, the trypsin digests of the light gastric membranes were processed as described above without diisopropyl fluorophosphate. Under these conditions, the majority of the 27-kDa polypeptide was degraded to two smaller FITC-labeled polypeptides of 25 and 15 kDa. These polypeptides were also isolated from 15% preparative SDS–polyacrylamide slab gels. The FITC-labeled polypeptide of 93 kDa representing (H^+–K^+)-ATPase was isolated from 7% preparative SDS–polyacrylamide slab gels by extracting with 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 2% SDS.

Immunization. FITC-labeled 27-kDa peptide (1 mg/mL) was mixed thoroughly with an equal volume of complete Freund's adjuvant. The emulsion was injected subcutaneously into six sites of a New Zealand White rabbit, which was boosted at 24-day intervals with the same antigen mixed with incomplete Freund's adjuvant. Blood samples were drawn weekly beginning 10 days after the booster immunization. Immunoreactivity of rabbit antisera was determined by an enzyme-linked immunosorbent assay (ELISA).

Western Blots and Lectin-Binding Assays. Proteins were analyzed by SDS–polyacrylamide gel electrophoresis (10%) and transferred electrophoretically (Towbin et al., 1979) onto nitrocellulose sheets. The sheets were successively treated for 1 h each with 2% bovine serum albumin (globulin-free), biotinylated lectins (1 μg/mL), and avidin–horseradish peroxidase (1 μg/mL) for lectin-binding assays (Hawkes, 1982) or with 3% gelatin, rabbit sera (1:200 dilution), and goat anti-rabbit IgG–horseradish peroxidase (1:1000 dilution) for

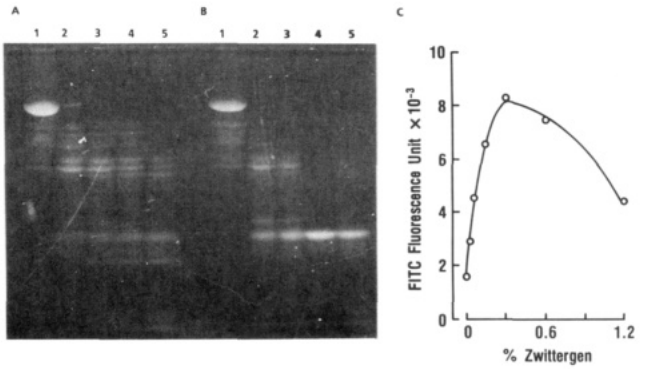

FIGURE 1: (A and B) Resolution by SDS gel electrophoresis of FITC-labeled polypeptides of rat gastric membranes before and after tryptic digestion. The FITC-labeled gastric membranes (20 μL containing 100 μg of protein) were mixed with trypsin at 0, 1, 2, 3, and 8 μg (panel A, lanes 1–5) and incubated for 5 min at 23 °C. The reaction was terminated by adding diisopropyl fluorophosphate (5 mM). An aliquot of the mixture (~50 μg of protein) was applied to a 10% SDS gel. In panel B, the conditions were the same as those shown in panel A, except that 0.3% (w/v) of Zwittergent was included during the tryptic digestion. (C) Effect of varying Zwittergent 3-14 concentrations during tryptic digestion of rat gastric membranes on formation of the FITC 27-kDa polypeptide. The FITC-labeled rat light gastric membranes were digested with trypsin (4 μg) as described above except that the detergent concentration varied from 0 to 1.2%. The tryptic polypeptides were then resolved on a 10% SDS gel. The 27-kDa polypeptide was excised and extracted with 2 mL of 10 mM Tris-HCl, pH 9.0. Fluorescence was measured in a Spex DMIB spectrofluorometer.

antibody reactions. Between each step, the sheets were washed extensively with the Tris-buffered saline. The reactive bands were visualized by horseradish peroxidase reaction with 4-chloro-1-naphthol in a solution containing 50 mM Tris, pH 7.5, and 150 mM NaCl in the presence of hydrogen peroxide (Glass et al., 1981). In some experiments, the Con A binding assay was carried out in the presence of 0.1 M methyl α-D-mannopyranoside, a high-affinity ligand for Con A.

Other Assays. The NH_2-terminal sequence of each polypeptide was determined on an Applied Biosystems Model 470 instrument fitted with an on-line Model 120 phenylthiohydantoin (PTH)-amino acid analyzer (Hewick et al., 1981). Various polypeptides were treated with endoglycosidase H (1 μg/mL) in a buffer containing 0.1 M sodium citrate, pH 5.5, and 0.1% SDS or with *N*-glycanase (8 units/mL) in 0.17% SDS, 0.2 M sodium phosphate, pH 8.6, and 1.25% (w/v) NP40 for 30 min at 37 °C. In some experiments, SDS–polyacrylamide gels were stained with Coomassie blue or with the periodic acid–Schiff's base stain for carbohydrates (Segrest & Jackson, 1972). Fluorescence measurements were made in a Spex DMIB spectrofluorometer using excitation and emission wavelengths of 493 and 530 nm, respectively. Protein was determined by Lowry et al. (1951) using bovine serum albumin as standard.

Results and Discussion

Rat resting light gastric membranes have been proposed to represent intracellular tubulovesicles containing reserve (H^+–K^+)-ATPase and consist of mostly inside-out membrane vesicles which are enriched with a 93-kDa polypeptide representing the catalytic unit of the ATPase (Forte & Machen, 1986; Im et al., 1984). Figure 1 shows that FITC labeling of the light membranes occurred almost exclusively in the 93-kDa polypeptide. Others have shown that FITC labeling takes place at Lys-517 of the ATPase (Shull & Lingrel, 1986; Farley & Faller, 1985) and that it is blocked by ATP (Jackson et al., 1983). Limited trypsin digestion of FITC-labeled resting

Table I: NH_2-Terminal Sequences of FITC-Labeled Tryptic Fragments from Rat Gastric (H^+–K^+)-ATPase[a]

poly-peptides	N-terminal sequence	ATPase alignment in 93 kDa
27 kDa	N-L-E-A-V-E-T-L-G-S-F-S-V-I-C-S-D-K-T	N-369
25 kDa	T-G-T-L-T-Q-N-R-M-T-V-S-H-L	T-387
15 kDa	I-V-I-G-D-A-S-T-A-L-L-K-F-S-E-L-T-L-G-N-A-M	I-455
	From cDNA Studies	
residues	N-terminal sequence	
369–400	N-L-E-A-V-E-T-L-G-S-F-S-V-I-C-S-D-K-T-G-T-L-T-Q-N-R-M-T-V-S-H-L	
455–476	I-V-I-G-D-A-S-T-A-L-L-K-F-S-E-L-T-L-G-N-A-M	

[a] The sequences were determined by automated Edman degradation and compared with the authentic primary structure of the relevant regions of rat gastric (H^+–K^+)-ATPase from cDNA studies (Shull & Lingrel, 1986).

light membranes (1–2 μg of trypsin/100 μg of membrane protein at 37 °C for 5 min) produced five readily detectable FITC–polypeptides of 60, 58, 56, 27, and 20 kDa. However, the majority of the FITC label was quenched because of its association with smaller peptides comigrating with the tracking dye (bromophenol blue). Higher concentrations of trypsin (4–8 μg/100 μg of membrane protein) only slightly affected the general pattern of FITC fragments, as reflected by a minor increase in the fluorescence intensity associated with the 27-kDa and 20-kDa polypeptides. In the presence of Zwittergent 3-14 (0.3% w/v), a detergent which potently inhibits (H^+–K^+)-ATPase activity, however, tryptic production of the FITC-labeled 27-kDa polypeptide was markedly increased. At the optimal concentration of trypsin (4 μg/100 μg of membrane protein; Figure 1B, lane 4), the detergent (0.3%) produced a 6-fold increase in the fluorescent intensity associated with the 27-kDa polypeptide as measured after extraction of the peptide from gel slices. Figure 1C further shows that lowering or raising the detergent concentration from 0.3% (w/v) reduced the yield of the 27-kDa polypeptide as measured by the fluorescent intensity of FITC. It appears that, in the presence of 0.3% detergent, the (H^+–K^+)-ATPase undergoes conformational changes which may lead to protection of certain tryptic cleavage sites of the membrane-bound enzymes.

The FITC-labeled polypeptide of 27 kDa and its two smaller tryptic fragments (25 and 15 kDa) were purified by ion-exchange and molecular sieve column chromatography and preparative SDS–polyacrylamide gel electrophoresis (see Materials and Methods). Table I lists their N-terminal sequences identified by automated Edman degradation. The 27-kDa polypeptide can be aligned beginning at Asn-369 of the cDNA-deduced primary structure of rat (H^+–K^+)-ATPase (Shull & Lingrel, 1986). This would be the 11th residue into the cytoplasmic side from the fourth hydrophobic transmembrane doamin predicted from hydropathy plots. Thus, the polypeptide includes the very early portion of the predicted cytoplasmic domain and, judging from its molecular weight on SDS gels, would appear to extend beyond residue 600 of (H^+–K^+)-ATPase. The two smaller FITC-labeled polypeptides of 25 and 15 kDa correspond to fragments beginning at Thr-387 and Ile-455, respectively. The fact that they are at least 18 and 96 amino acid residues shorter, respectively, than their parent 27-kDa polypeptide correlates nicely with what would be expected from their molecular weights.

The gastric polypeptide of 93 kDa (on SDS gel) representing the (H^+–K^+)-ATPase has been proposed to be a glycoprotein on the basis of periodate–Schiff's base staining (Saccomani et al., 1979). Figure 2 shows that the 27-kDa polypeptide was

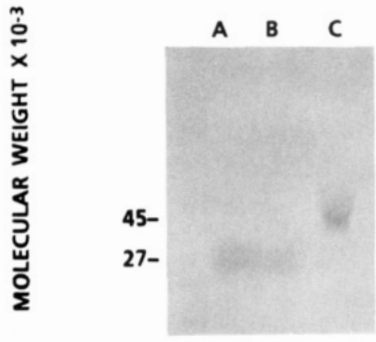

FIGURE 2: Periodate–Schiff's base staining of the tryptic 27-kDa polypeptide from gastric (H^+–K^+)-ATPase. The 27-kDa polypeptide (~200 and 100 μg in lanes A and B, respectively) and ovalbumin (100 μg in lane C) were resolved in a 10% SDS–polyacrylamide gel and stained with the periodate–Schiff's base stain.

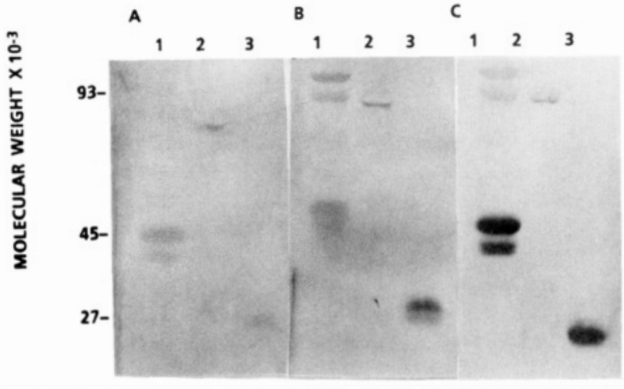

FIGURE 3: Lectin-binding properties of the FITC-labeled 93-kDa polypeptide from the resting light gastric membranes, its tryptic fragment of 27 kDa, and ovalbumin (45 kDa). The nitrocellulose transblots of ovalbumin (lane 1), the 93-kDa polypeptide (lane 2), and the 27-kDa tryptic fragment (lane 3) from a 10% SDS gel were reacted with biotinylated wheat germ agglutinin (panel A), PHA-E (panel B), and Con A (panel C). The transblots were then treated with the avidin–horseradish peroxidase conjugate and were visualized with the horseradish peroxidase color developing agent (Bio-Rad). About 10 μg of protein was applied to the gels.

also stained with periodate–Schiff's base. Furthermore, the 93-kDa and the 27-kDa polypeptides showed lectin-binding activities. Figure 3 shows that ovalbumin and the 93- and 27-kDa polypeptides show variable reactivities with biotinylated wheat germ agglutinin, PHA-E, and Con A. Although the level of biotin per mole of lectin follows the order of PHA-E (17.7 mol of biotin), wheat germ agglutinin (5.3 mol of biotin), and Con A (1.7 mol of biotin), the biotinylated Con A shows the most intensive reactivity with the polypeptides of 93 and 27 kDa. This is also seen with ovalbumin, a high-mannose-containing glycoprotein. Also, in previous studies with a variety of glycoproteins (Chu et al., 1981; Faye et al., 1985), Con A has been shown to be reactive specifically with the mannosyl groups of glycoproteins in nitrocellulose affinity blots. Their sensitivity allows estimation of the mannose contents of glycoproteins at levels which cannot be detected by conventional methods (Chu et al., 1981). To obtain further information on the oligosaccharide of the 27-kDa polypeptide, we have employed endoglycosidase H and *N*-glycanase. Endoglycosidase H specifically hydrolyzes the glycosidic bond between the adjacent *N*-acetylglucosamine residue linking the high-mannose glycan moiety to the asparagine of the protein backbone (Tarentino & Maley, 1974). *N*-Glycanase is an amidase specific for asparaginyl–oligosaccharide bonds of all types of N-linked oligosaccharides present in a variety of glycoproteins (Tarentino et al., 1985; Hirani et al., 1987).

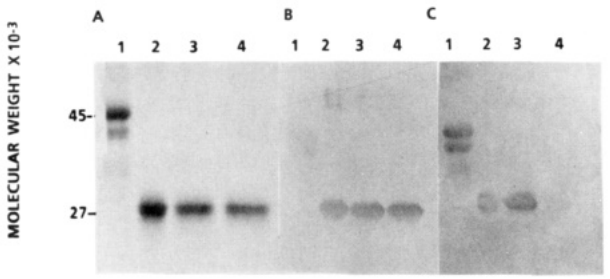

FIGURE 4: Disappearance of the Con A reactivity of the 27-kDa tryptic fragment from gastric (H^+–K^+)-ATPase upon treatment with endoglycosidase H. Ovalbumin (lane 1) and the 27-kDa tryptic polypeptide (lane 2) after incubation without (lane 3) or with endoglycosidase H (lane 4) were resolved on 10% SDS gels. The gels were either stained with Coomassie Blue (panel A) or transblotted to nitrocellulose papers. The transblots were reacted successively with rabbit sera prepared against the 27-kDa polypeptide and goat anti-rabbit IgG–horseradish peroxidase (panel B) or with biotinylated Con A and avidin–horseradish peroxidase (panel C). The transblots were stained with the horseradish peroxidase color developing agent (Bio-Rad). About 10 μg of protein was applied to the gel.

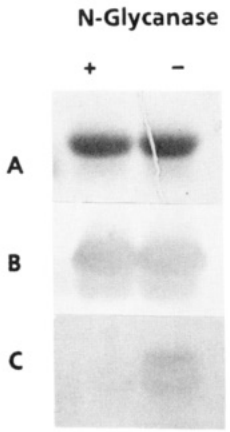

FIGURE 5: Effect of *N*-glycanase treatment of the 27-kDa tryptic polypeptide on its mobility on the SDS gel and its reactivities with rabbit antisera and Con A. The FITC-labeled 27-kDa polypeptide was incubated with or without *N*-glycanase at 37 °C for 30 min. Aliquots from each sample were resolved on 10% SDS–polyacrylamide gels. The region of 27-kDa molecular mass was shown after staining with Coomassie blue (A) and after transblotting to nitrocellulose and reacting with rabbit antisera against the polypeptide (B) or biotinylated Con A (C) as described in the legend of Figure 3.

Figure 4 shows that treatment of the 27-kDa polypeptide with endoglycosidase H produced no detectable changes either in its molecular weight (on SDS gels stained with Coomassie blue) or in its reactivity with the rabbit polyclonal antibodies prepared against the 27-kDa polypeptide. The Con A binding activity was abolished, however. Since endoglycosidase H is known to require the tetrasaccharide structure, Manα1→3Manα1→6Manβ1→4GlcNAc (Tai et al., 1977), the carbohydrate moiety may contribute a mass of at least 689 Da to the polypeptide. *N*-Glycanase, another glycosidase specific for asparagine-linked carbohydrate moieties, also abolished the Con A binding activity of the 27-kDa polypeptide (Figure 5). The 93-kDa polypeptide which showed immunoreactivity with the polyclonal antibodies against the 27-kDa polypeptide (Figure 6A) similarly lost its Con A reactivity upon treatment with endoglycosidase H in a dot-blot assay (Figure 6B). The two smaller FITC–polypeptides of 25 and 15 kDa were also reactive with Con A, immunoreactive with the polyclonal antibodies against the 27-kDa polypeptide (Figure 7), and again lost their Con A reactivity upon treatment with endoglycosidase H (data not shown). The Con A binding activity of all these polypeptides was blocked in the presence of methyl

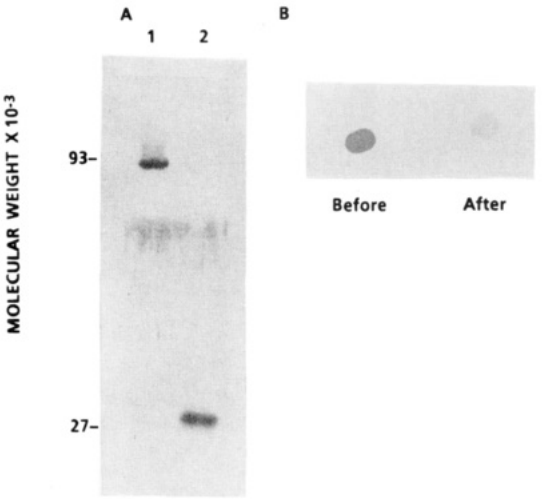

FIGURE 6: Reactivity of the FITC-labeled 93-kDa polypeptide with rabbit sera prepared against the 27-kDa polypeptide and its reactivity with Con A. The 93-kDa polypeptide representing gastric (H^+–K^+)-ATPase was obtained from FITC-labeled rat resting light gastric membranes after resolution on preparative SDS gels. Panel A shows Western blots of the 93-kDa polypeptide (lane 1) and its 27-kDa tryptic fragment (lane 2) using rabbit antisera against the 27-kDa polypeptide. In panel B, the FITC-labeled 93-kDa polypeptide (2.5 μg) was dotted onto nitrocellulose paper before or after treatment with endoglycosidase H. The dot assay was developed with Con A–biotin as described in the legend of Figure 1.

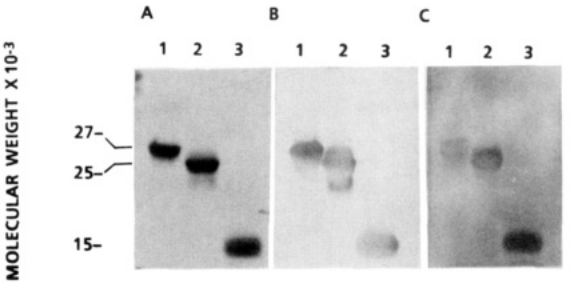

FIGURE 7: Reactivities of the FITC-labeled 25- and 15-kDa tryptic fragments from the 27-kDa polypeptide with rabbit antisera against the parent polypeptide and with Con A. The 27-kDa (lane 1), 25-kDa (lane 2), and 15-kDa (lane 3) fragments were resolved on 10% SDS gels. The gels were either stained with Coomassie blue (panel A) or transblotted to nitrocellulose paper. The transblots were reacted successively with rabbit sera against the 27-kDa polypeptide and goat anti-rabbit IgG–horseradish peroxidase (panel B) or with biotinylated Con A and avidin–horseradish peroxidase (panel C). The reactions were developed with the horseradish peroxidase color developing agent. About 10 μg of protein was applied to the gel.

α-D-mannopyranoside (0.1 M). These data, taken together, strongly indicate the occurrence of an N-linked carbohydrate moiety containing high mannose in the region of (H^+–K^+)-ATPase comprised by the 27-kDa polypeptide. It appears that the uses of Con A affinity blots, methyl α-D-mannopyranoside sensitivity, endoglycosidase H, and *N*-glycanase in combination provide sensitive, specific, and reliable information on the basic structure of oligosaccharides in glycoproteins. Recently, lectin blots and the glycosidases have been extensively used to study biosynthesis and structure–function relationships of glycoproteins containing N-linked carbohydrates. Furthermore, association of the FITC label and Con A binding activity throughout the additional tryptic cleavage of the 27-kDa polypeptide indicates that FITC-labeled Lys-517 is located near the residue bearing the carbohydrate group. The sequence information of (H^+–K^+)-ATPase deduced from cDNA clones reveals that only one asparagine residue between residues 369 and 728, Asn-492, meets the minimal specificity requirement for N-linked carbohydrate attachment, Asn-X-Ser(Thr)

(Kornfeld & Kornfeld, 1985). The presence of an N-linked carbohydrate group at Asn-492, which is only 25 residues away from a part of the cytoplasmic ATP binding site, Lys-517 (Jackson et al., 1983), raises an interesting question. Does the carbohydrate moiety face the cytoplasmic side, as proposed by hydropathy plots, or the extracellular side, where most N-linked carbohydrate groups are located? Although hydropathy plots have provided useful frameworks for studying the disposition of integral membrane proteins, one may not make definitive assignments until studies with antibodies specific for various regions of membrane proteins and other biochemical characterizations confirm the proposed orientations. Generally, ion transporters are expected to contain polar membrane-spanning segments which may not be detected with hydropathy plots. In this regard, the N-glycosidation site of the soluble 27-kDa polypeptide of gastric (H^+–K^+)-ATPase appears to be a logical starting point in searching for such polar transmembrane regions. Although O-linked *N*-acetylglucosamine has been detected in band 4.1, a cytoplasmic membrane protein in erythrocytes, and in nucleoplasmic faces of the nuclear pore complex (Holt et al., 1987a; Davis & Blobel, 1980; Finlay et al., 1987; Hanover et al., 1987; Holt et al., 1987b), so far no N-linked high-mannose group has been found to be associated with the cytoplasmic side of integral membrane proteins. In any event, the carbohydrate moiety in the 27-kDa polypeptide is relatively small, since no noticeable molecular weight reduction was observed upon its removal with endoglycosidase H or *N*-glycanase (Figures 4 and 5). Also, its accessibility to Con A in the parent polypeptide (93 kDa) appears to be somewhat limited, since the majority of the 93-kDa polypeptide, except the lower resolving component in the transblot, was marginally reactive with Con A, whereas the 27-kDa polypeptide displayed uniform and intense Con A binding activity (Figure 3). Future efforts will be directed to determine the structural and functional significance of the detected N-glycosidation in gastric (H^+–K^+)-ATPase and its implications to other analogous ATPases. While this work was in progress, Saccomani and Mukidjam (1987) reported the finding of a 28-kDa FITC-labeled soluble peptide by prolonged papain digestion of hog gastric membranes enriched with (H^+–K^+)-ATPase. Since its NH_2-terminal sequence or yield has not been reported, no comparisons could be made with the tryptic 27-kDa polypeptide.

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REFERENCES

Chu, F. K., Maley, F., & Tarentino, A. L. (1981) *Anal. Biochem. 116*, 152–160.

Davis, L. I., & Blobel, G. (1980) *Cell 45*, 699–709.

Farley, R. A., & Faller, L. D. (1985) *J. Biol. Chem. 260*, 3899–3901.

Faye, L., & Chrispeels, M. J. (1985) *Anal. Biochem. 149*, 218–224.

Fellenium, E., Berglindh, T., Sachs, G., Olbe, L., Glander, B., Sjostrand, S. E., & Wallmark, B. (1981) *Nature 290*, 159–161.

Finlay, D. R., Newmeyer, D. D., Price, T. M., & Forbes, D. J. (1987) *J. Cell. Biol. 104*, 189–200.

Forte, J. G., & Machen, T. E. (1986) in *Physiology of Membrane Disorders, Ion Transport by Gastric Mucosa* (Andreoli, T. E., Hoffman, J. F., Fanestil, D. D., & Schultz, S. G., Eds.) pp 535–558, Plenum Medical Book Co., New York.

Ganser, A. L., & Forte, J. G. (1973) *Biochim. Biophys. Acta 307*, 169–180.

Glass, W. F., II, Briggs, R. C., & Hnilica, L. S. (1981) *Science 211*, 70–72.

Hanover, J. A., Cohen, C. K., Willingham, M. C., & Park, M. K. (1987) *J. Biol. Chem. 262*, 10384–10391.

Hawkes, R. (1982) *Anal. Biochem. 123*, 143–146.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7989–7997.

Hirani, S., Bernasconi, R. J., & Rasmussen, J. R. (1987) *Anal. Biochem. 162*, 485–492.

Holt, G. D., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L., & Hart, G. W. (1987a) *J. Cell. Biol. 104*, 1157–1164.

Holt, G. D., Haltiwanger, R. S., Torres, C.-A., & Hart, G. W. (1987b) *J. Biol. Chem. 262*, 14847–14850.

Im, W. B., Blakeman, D. P., Fieldhouse, J. M., & Rabon, E. C. (1984) *Biochim. Biophys. Acta 772*, 167–175.

Jackson, R. J., Mendlein, J., & Sachs, G. (1983) *Biochim. Biophys. Acta 731*, 9–15.

Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111–136.

Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. & Numa, S. (1985) *Nature 316*, 733–736.

Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lee, J., Simpson, G., & Schales, P. (1974) *Biochem. Biophys. Res. Commun. 60*, 825–832.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

MacLennan, D. H., Brandl, C. J., Korczak, B., & Green, N. M. (1985) *Nature 316*, 696–700.

Saccomani, G., & Mukidjam, E. (1987) *Biochim. Biophys. Acta 912*, 63–73.

Saccomani, G., Daily, D. W., & Sachs, G. (1979) *J. Biol. Chem. 254*, 2821–2827.

Sachs, G., Chang, H. H., Rabon, E., Schackman, R., Lewin, M., & Saccomani, G. (1976) *J. Biol. Chem. 251*, 7690–7698.

Segrest, J. P., & Jackson, R. L. (1972) *Methods Enzymol. 28*, 54–63.

Serrano, R., Kielland-Brandt, M. C., & Fink, G. R. (1986) *Nature 319*, 689–693.

Shull, G. E., & Lingrel, J. B. (1986) *J. Biol. Chem. 261*, 16788–16791.

Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature 316*, 691–695.

Tai, T., Yamashita, K., & Kobata, A. (1977) *Biochem. Biophys. Res. Commun. 78*, 434.

Tarentino, A. L., & Maley, F. (1974) *J. Biol. Chem. 249*, 811–817.

Tarentino, A. L., Gomez, C. M., & Plummer, T. H., Jr. (1985) *Biochemistry 24*, 4665–4676.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.