A Monosaccharide Is Bound to the Sodium Pump α-Subunit†

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ABSTRACT: We have recently reported that the Na pump α-subunit has cytosolic-oriented oligosaccharides which were sensitive to cleavage by an enzyme specific for hydrolysis of N-linked glycans [Pedemonte et al. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9789–9793]. We now describe experiments that characterize the saccharides and further substantiate our previous findings. Bovine milk galactosyltransferase has been used in conjunction with radiolabeled UDP-galactose to label *N*-acetylglucosamine residues on the protein. The Na pump α-subunit contains some O-linked carbohydrates; however, the bulk (>80%) of the radioactivity was found in oligosaccharides sensitive to peptide:*N*-glycosidase F degradation but not to alkaline hydrolysis. Alkaline hydrolysis produced degradation of the protein, and the [^{3}H]Gal radiolabeled carbohydrates remained bound to peptides and were released by subsequent peptide *N*-glycosidase F treatment. The exogenously galactosylated sugars cleaved by the glycosidase were analyzed by liquid chromatography and had elution volumes identical to a galactose-*N*-acetylglucosamine disaccharide standard. Since the galactose was exogenously added, we propose that the N-linked glycans on the α-subunit of the Na pump are composed of a single sugar residue, which is probably *N*-acetylglucosamine.

There is growing evidence of the existence of glycoprotein carbohydrate moieties in the cytosol of cells (Hart et al., 1989). Glycosyltransferases that catalyze the addition of monosaccharides to oligosaccharides in a highly specific manner provide an efficient in vitro means of introducing a label of high specific activity into the oligosaccharides of glycoproteins (Holt & Hart, 1986). The use of glycosyltransferases and radiolabeled sugar nucleotide donors has allowed Hart and collaborators to demonstrate the existence of cytosolic-oriented *N*-acetylglucosamine (GlcNAc) molecules O-linked to serines or threonines of membrane proteins (Hart et al., 1989).

By using galactosyltransferase and radiolabeled galactose (Gal), we recently have demonstrated that the α-subunit of the canine renal sodium pump is glycosylated (Pedemonte et al., 1990). Due to the very low level of carbohydrate on this polypeptide and the small size of the oligosaccharides, our findings would not have been possible without the use of this highly efficient labeling method. We reported evidence of small oligosaccharide moieties covalently bound to the polypeptide in a peptide:*N*-glycosidase F (PNGase)-sensitive linkage (Pedemonte et al., 1990). We found that the Na pump α-subunit oligosaccharides present in the membrane of right-side-out microsomal vesicles are inaccessible to exogalactosylation with the impermeant galactosyltransferase and can only be labeled following detergent permeabilization of the vesicles. The composition of the sodium pump α-subunit glycans was not known. Here we provide evidence suggesting that the N-linked carbohydrate moieties attached to the Na pump α-subunit are composed of a single GlcNAc molecule.

EXPERIMENTAL PROCEDURES

Dog kidney Na,K-ATPase was isolated from outer medulla and assayed as described previously (Pedemonte & Kaplan, 1988). Polyacrylamide gel electrophoresis (PAGE) was performed using the system described by Laemmli (1970). For β-elimination, the protein of interest was separated by PAGE. The pieces of gel containing the protein, visualized by Coomassie Blue staining, were excised from the gel, dried, and treated with 50 mM NaOH and 1 M $NaBH_4$ at 50 °C (Capasso et al., 1988).

Galactosylation and PNGase Treatments. Bovine milk galactosylatransferase (EC 2.4.1.22; Sigma) was allowed to autogalactosylate in the presence of 0.4 mM UDP-galactose (Sigma), 5 mM $MnCl_2$, 50 mM Tris-HCl at pH 7.2, 1 mM 2-mercaptoethanol, and 1% aprotinin, in a total volume of 250 μL. After 30 min at 37 °C, an equal volume of glycerol was added. The galactosyltransferase was then stored in this solution (10 units/mL) at −20 °C until used. Radiolabeling of the Na^+,K^+-ATPase was performed as follows. Na^+,K^+-ATPase samples (about 0.1 mg) were resuspended in 40 mM galactose, 1 mM 5′-AMP, 0.2% aprotinin, 5 mM $MnCl_2$, 40 μM UDP-[^{3}H]galactose (0.4–4 μCi), 5 mM Tris-HCl, pH 7.2, 0.1 mM 2-mercaptoethanol, 5% (vol/vol) glycerol, and 0.1–0.6 unit of galactosyltransferase. After incubation for 30 min at 37 °C, the Na^+,K^+-ATPase was washed by centrifugation and resuspension with 50 mM Tris-HCl, pH 7.2. Glycopeptidase treatment was carried out with purified Na^+-pump protein (about 80 μg in 15 μL) dissolved with 15 μL of 1% SDS, diluted with 25 μL of buffer containing 200 mM EDTA and 4% (vol/vol) 2-mercaptoethanol, and taken to pH 7.5 with Tris base. Forty-five microliters (0–10 units) of peptide *N*-glycosidase F (PNGase; EC 3.2.2.18) from *Flavobacterium meningosepticum* (Boehringer Mannheim *N*-glycosidase F) was then added. The final SDS concentration was 0.15%. Digestion with PNGase was performed at 37 °C for 20–24 h. Similar digestion results were obtained when 0.1% SDS and 0.5% Nonidet P-40 were used. In the latter case, the protein was separated by liquid chromatography rather than SDS/PAGE. The radioactive labeling of the Na pump α-subunit was examined after separating this subunit by PAGE or HPLC (using a TSK 3000 column) and measuring the radioactivity content. In both PAGE and HPLC, the α-subunit was identified by its molecular weight and by its specific reaction with a monoclonal antibody. The labeling of the Na pump α-subunit was retained after PAGE, HPLC,

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and several other treatments (as shown in the results section), indicating that the [^{3}H]Gal was covalently bound. For fluorography, gels were immersed in EN^{3}HANCE (New England Nuclear), dried under vacuum, and exposed to preflashed film at −70 °C. For electroelution, the pieces of gel containing the proteins of interest were placed in an electroeluter (Bio-Rad) with Laemmli (1970) electrode buffer, and a constant voltage of 180 V was applied for 24 h at 4 °C. Sugars were estimated chemically in the eluates of chromatographic columns using orcinol: 30 μL of each collected fraction was placed in the well of a multiwell plate, and 170 μL of 0.2% orcinol in 60% H_2SO_4 was added. The reaction was developed at 80 °C. The presence of carbohydrates was determined in a multiwell scanner at 416 nm. Milk galactosyltransferase, fetuin, GlcNAc, GlcNAc-Gal, and GlcNAc-GlcNAc-Gal were obtained from Sigma. PNGase was obtained from Boehringer.

[^{14}C]Gal-GlcNAc-GlcNAc and [^{14}C]Gal-GlcNAc were synthesized by the reaction of authentic GlcNAc-GlcNAc and GlcNAc with UDP-[^{14}C]Gal and galactosyltransferase. The reaction products were purified in a Bio-Gel P2 column, where they eluted in the same volume as authentic Gal-GlcNAc-GlcNAc and Gal-GlcNAc, respectively, obtained commercially. After acetylation, the synthesized standards presented the same elution (R_f) in TLC as peracetylated standards commercially obtained. The samples were prepared, and TLC was performed as described by Alvarez and Touchstone (1988).

RESULTS

Purified renal Na pump protein was treated with UDP-[^{3}H]Gal and galactosyltransferase which is known to transfer Gal from UDP-Gal specifically to terminal GLcNAc's (Schanbacker & Ebner, 1970; Brew et al., 1968; Torres & Hart, 1984). A high concentration of nonlabeled Gal was always present in the medium to dilute any possible release of [^{3}H]Gal from UDP-[^{3}H]Gal that may occur during the reaction and become fortuitously attached to the protein. Controls in the absence of galactosyltransferase demonstrated that the radiolabeling of the Na pump α-subunit was dependent on the presence of the transferase. Furthermore, the attachment of [^{3}H]Gal to the α-subunit was prevented by the addition to the reaction medium of an excess of GlcNAc, which acted as an alternate acceptor for the transferase. After the labeling reaction was completed, the protein was washed by centrifugation and resuspension to eliminate all radioactive material that was not tightly bound to the protein. That the labeled protein was the Na pump α-subunit was confirmed by reaction of a monoclonal antibody against the α-subunit with the radiolabeled protein in western blots after gel permeation HPLC (data not shown). Dissolution of the labeled protein in medium containing detergents (SDS) and urea, separation of the polypeptides in SDS–PAGE (Figure 1), electroelution of the α-subunit, and separation in a Sephadex G-50 column (Figure 3B), and the enzymatic release of the radioactivity from the protein (Figure 3), demonstrated that the radioactive galactose was *covalently* bound and that it was bound to the protein and not to the lipids. Figure 1 illustrates the Coomassie Blue stained Na pump α- and β-subunits separated by PAGE and an autoradiogram of a similar gel. Both sodium-pump subunits were radiolabeled following transferase treatment, which indicates that both have carbohydrate moieties with terminal GlcNAc residues (Torres & Hart, 1984).

To estimate the stoichiometry of labeling and hence N-glycosylation, we did the following experiment. The

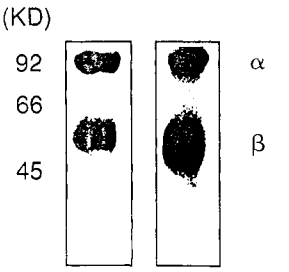

FIGURE 1: Galactosylation of the Na pump subunits. (Left Lane): Purified Na pump protein was dissolved in sample buffer, and the subunits were separated in a 7.5% Laemmli (1970) gel. The gel was then fixed and stained with Coomassie Blue. (Right Lane): The purified Na pump protein was labeled with UDP-[^{3}H]Gal and galactosyltransferase, and it was washed by centrifugation and resuspension to remove all the noncovalently bound radioactivity. The protein was dissolved in sample buffer, and the subunits were separated by PAGE. The gel was fixed, saturated with EN^{3}HANCE (New England Nuclear), dried, and put in a fluorography cassette with preflashed Kodak X-OMART AR film. A photograph of the developed film is shown. The positions where molecular weight standards migrated in the gels are indicated on the left.

purified Na pump was exogalactosylated, and the subunits were separated by PAGE. The α-subunit was electroeluted from the gel, and aliquots were used to determine protein concentration and radioactivity. The labeling was about 2.7 nmol of [^{3}H]Gal/mg of α-subunit protein, this corresponds to 1.8 nmol/mg of Na pump since the α-subunit is by mass about $^2/_3$ of the Na pump molecule. 1.8 nmol/mg is very close to the 2 nmol of phosphoenzyme/mg that we have previously measured (Pedemonte & Kaplan, 1986, 1988). This would indicate that there is about one site of galactosylation in the α-subunit per phosphorylation site.

For β-elimination of any O-linked carbohydrates, the bands corresponding to the α-subunit were excised from the gel. The pieces of gel were dried and treated with 50 mM NaOH in the presence of 1M $NaBH_4$ (Capasso et al., 1988; Spiro, 1972). Most of the radioactivity (about 90%) was released to the liquid phase by the alkaline treatment. The supernatant was concentrated in a rotary evaporator and applied to a Sephadex G-50 column. The radioactive elution profile is shown in Figure 2B. Even though the labeled oligosaccharides were eluted over a wide range of molecular weights, the bulk of the radioactivity eluted within the range of resolution of the Sephadex G-50 column. The observation that the radioactive eluates following β-elimination were in the range of resolution of the Sephadex G-50 column indicates that the exogalactosylated oligosaccharides are not single GlcNAc residues O-linked to the protein. Such single O-linked saccharides have been previously observed by Torres and Hart (1984) in their studies on lymphocytes. If the exogalactosylation of the sodium-pump α-subunit had labeled O-linked GlcNAc, a disaccharide Gal-GlcNAc would have been released by the β-elimination. Disaccharides of the type GlcNAc-Gal (molecular mass 383) fall below the lower limit of resolution of Sephadex G-50, which for dextrans is about 500 Da. If such a disaccharide had been released, the [^{3}H]Gal would have been detected at the elution volumes of the Sephadex G-50 corresponding to the salt peak (V_t in Figure 2). As we did not observe the bulk of radioactivity at or close to the salt peak of the Sephadex G-50 column, the β-elimination appears not to have released the exogalactosylated glycans.

To ensure that the β-elimination procedure was effective, the alkaline treatment of the Na pump α-subunit was compared to that of fetuin, which is known to have both N- and O-linked carbohydrates (Spiro & Bhoyroo, 1979). Purified Na pump and fetuin were treated in parallel with

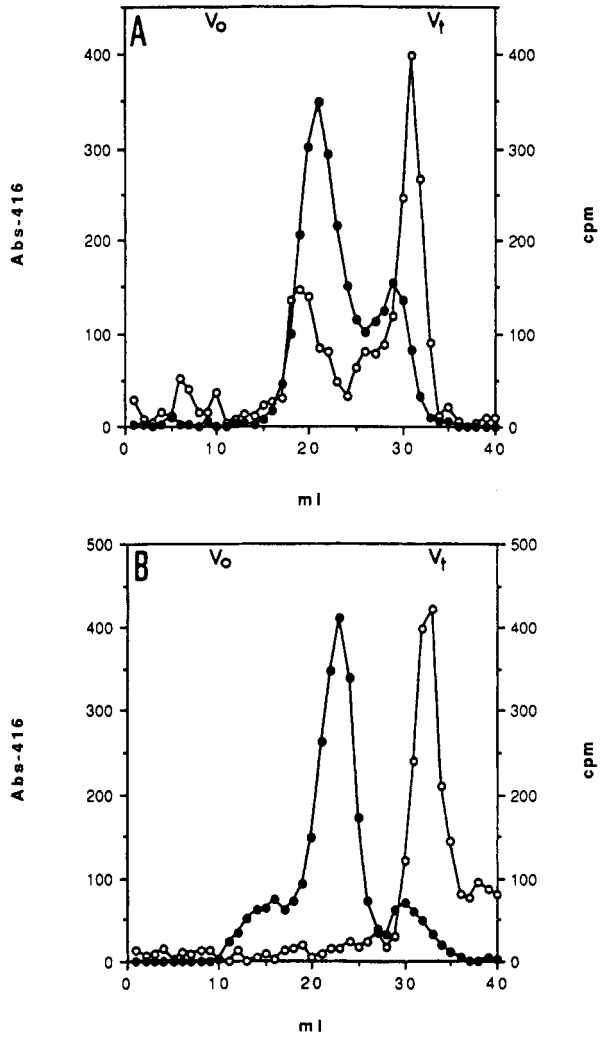

FIGURE 2: Determination of O-linked carbohydrates. Purified Na pump (B) and fetuin (A) were treated with galactosyltransferase and UDP-[^{3}H]Gal, and they were separated in a 7.5% Laemmli (1970) gel. The gel was fixed and stained with Coomassie Blue. The bands corresponding to the Na pump α-subunit (panel B) and fetuin (panel A) were excised from the gel and dried. The pieces of gel were incubated with 50 mM NaOH and 1 M $NaBH_4$ at 50 °C. The β-elimination proceeded as indicated by Capasso et al. (1988). The soluble material of each sample was concentrated in a rotary evaporator and centrifuged, and the supernatant was filtered through a Millex filter (0.45 unit). The radiolabeled samples were then chromatographed on a Sephadex G-50 column (1.1 × 35 cm) and eluted with 50 mM NH_4HCO_3 at 0.1 mL/min. Aliquots of 1 mL were collected and used for determination of radioactivity (cpm, ● - ●) and orcinol-reactive sugars (Abs-416, O - O). V_o and V_t indicate the void volume and the elution of the salt peak determined with Blue Dextran and Phenol Red, respectively.

galactosyltransferase and UDP-[^{3}H]Gal and were separated by PAGE. The Coomassie Blue stained bands corresponding to fetuin and the Na pump α-subunit were excised from the gel, and they were treated separately with 50 mM NaOH in the presence of 1 M $NaBH_4$. The supernatants of each sample were then resolved by Sephadex G-50 chromatography. We measured both radioactivity (closed symbols) and orcinol-reactive carbohydrates (open symbols) in the Sephadex G-50 eluates (Figure 2). Fetuin presented a profile of radioactivity (Figure 2A) similar to that of the α-subunit (Figure 2B), and most of the radioactivity was eluted prior to the salt peak (V_t). Even though the quantity of radioactivity is very similar in fetuin and the Na pump α-subunit samples (area under the curves with filled symbols), the main radioactive peak from fetuin is orcinol-positive whereas it is not from the Na pump α-subunit. The positive orcinol reaction of the radioactive eluates of fetuin is very likely produced by the carbohydrates contained in its three complex carbohydrate moieties (Spiro & Bhoyroo, 1974). Since the orcinol assay is negative for hexosamines (Winzler, 1955; Francois et al., 1962), the lack of orcinol reactivity of the α-subunit radioactive eluates (Figure 2B) is consistent with our hypothesis that the carbohydrate moieties of this protein are formed by single GlcNAc molecules (see below). In the volumes at or near the salt peak (around 30–35 mL in Figure 2), we obtained from both proteins evidence or orcinol-reactive nonradioactive carbohydrates. The observation that these sugars had not been labeled by the galactosylation treatment indicates that they do not contain terminal GlcNAc molecules. Even though in Figure 2 there is some overlap between the last radioactive peak and the main orcinol-positive peak, the peaks appear at elution volumes different in both fetuin (Figure 2A) and the Na pump α-subunit (Figure 2B). In fetuin (Figure 2A), the nonradioactive main orcinol-positive peak is produced by β-elimination of its three O-linked carbohydrate chains (Spiro & Bhoyroo, 1974). It is likely then, that the Na pump α-subunit also has some orcinol-positive O-linked sugars lacking terminal GlcNAc moieties. We can preclude the possibility that this peak is produced by N-linked GlcNAc bound to small peptides because the carbohydrates detected by the orcinol reaction were not labeled by exogalactosylation. Therefore, the β-elimination reaction worked properly releasing O-linked carbohydrates that were detected in the Sephadex G-50 eluate volumes close to the salt peak (V_t), but these carbohydrates were not labeled with [^{3}H]Gal. The β-eliminated carbohydrates were observed in both fetuin and Na pump α-subunit, and their elution volume was clearly different from the bulk of radioactivity.

In contrast, the radioactive eluates showed significant absorption at 280 nm and positive reactions with two protein assays (Lowry et al., 1951; Bradford, 1976), suggesting that the exogalactosylated carbohydrates may be bound to peptides that were produced by alkaline cleavage of the α-subunit. The radiolabeled fractions recovered from the Sephadex G-50 chromatography of the β-eliminated Na pump α-subunit were pooled, concentrated, and treated with PNGase to release N-linked carbohydrates. After treatment, half of the sample was separated again in the Sephadex G-50 column. All the radioactivity was recovered with an elution volume corresponding to the salt peak at about 33 mL (Figure 3A). Thus on PNGase treatment, all the radioactivity shifted from the molecular weight range of resolution of the Sephadex G-50 column to the salt peak. This indicates that the radioactive galactosylated carbohydrates were bound to peptides and were freed by the PNGase treatment. Therefore, the Na pump α-subunit appears to have two different kinds of carbohydrates, one sensitive to β-elimination, and another sensitive to PNGase. Only the latter were labeled with radioactive galactose. The results of the β-elimination experiments and the documented specificity of PNGase, which cleaves only N-linked oligosaccharides (Tarentino & Plummer, 1982), rule out the possibility that the radiolabeled galactose had been bound to a GlcNAc on O-linked oligosaccharides.

We also observed the PNGase sensitivity of the [^{3}H]Gal-labeled carbohydrates using an alternative approach, the ^{3}H-galactosylated Na pump protein was treated with FITC to label the α-subunit (Karlish, 1980), and the subunits were separated by PAGE. The α-subunit, localized in the gel from its FITC-derived fluorescence, was excised and electroeluted. The eluted solution containing the α-subunit (which was fluorescent and radioactive) was applied to the Sephadex G-50

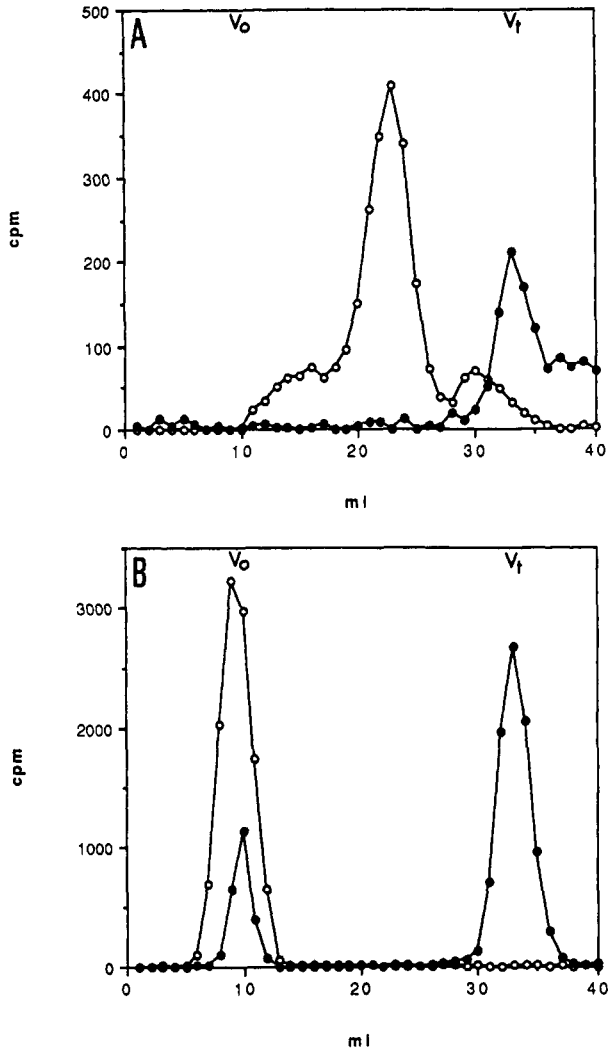

FIGURE 3: α-Subunit carbohydrates were released by PNGase. (Panel A) The radioactive eluates from the chromatography shown in Figure 2B (Na pump α-subunit) were pooled and dried in the rotary-evaporator. The sample was resuspended in a small volume and treated with PNGase. Half of the sample was then rechromatographed in the Sephadex G-50 column. Aliquots of the eluates collected every 10 min were used for cpm determination (● - ●). The graph corresponding to the radioactivity in Figure 2B is reproduced for comparison (○ - ○). (Panel B) The radioactive galactosylated Na pump protein was treated with FITC, and the α- and β-subunits were separated in a 7.5% gel. The α-subunit band, detected by the bound fluorescence, was excised from the gel and the protein was electroeluted. The α-subunit extract was chromatographed in the Sephadex G-50 column (○ - ○) with 50 mM NH_4HCO_3/0.1% SDS as the elution buffer. The radioactive eluates collected in the void volume were pooled, dried in the rotary/evaporator, and resuspended in a small volume. After treatment with PNGase, the α-subunit sample was rechromatographed in the Sephadex G-50 column (● - ●). Before and after the PNGase treatment, the fluorescence was visually observed in the tubes collected at the void volume.

column. The intact α-subunit was excluded from this gel because of its molecular mass (about 92 kDa) and emerged in the early eluates corresponding to the void volume (V_o), around 10 mL (Figure 3B). The radioactive fractions were pooled and treated with PNGase. After the hydrolysis with the glycosidase, the complete incubation mixture was applied to the Sephadex G-50 column. Most of the radioactivity was then recovered in the eluates corresponding to the salt peak volume (Figure 3B). A small fraction (18%) of the total radioactivity was recovered as nonhydrolyzed glycans in the void volume. The β-elimination and glycosidase data together clearly indicate that the bulk of the radioactive exogalactosylated oligosaccharides bound to the α-subunit are sensitive to PNGase, i.e., they behave like N-linked carbohydrates.

The radioactive fractions (free carbohydrates) eluted from PNGase-treated samples were pooled, concentrated, and analyzed in a Bio-Gel P2 column. The elution volume of the radioactivity was compared to two standards [^{14}C]Gal-GlcNAc and [^{14}C]Gal-GlcNAc-GlcNAc. The elution volume of the ^{3}H-labeled experimental sample (Figure 4A) corresponded to that of the [^{14}C]Gal-GlcNAc standard (Figure 4B), and it was higher than the elution volume of [^{14}C]Gal-GlcNAc-GlcNAc (Figure 4D). Co-chromatography of a mixture of equal amounts of radioactivity of the eluted fractions corresponding to the main peak (around 60 mL) of the experimental ^{3}H-labeled sample (Figure 4A) and those of the [^{14}C]Gal-GlcNAc standard (Figure 4B) showed double labeling at the identical elution volume (Figure 4C). Therefore, the radiolabeled carbohydrates released by PNGase behave in liquid chromatography as the disaccharide Gal-GlcNAc. As [^{3}H]Gal was exogenously added, the endogenous sugar must be a single GlcNAc molecule.

The ^{3}H-galactosylated saccharides released from the α-subunit by PNGase treatment (Figure 3) were also analyzed by thin-layer chromatography (TLC) as reported by Alvarez and Touchstone (1988). The samples were acetylated and separated on TLC plates of Silica-Gel HP-K (Whatman 250 u), predeveloped in chloroform–methanol (1:1) and developed in hexane–acetone (1:1). The plates were stained with orcinol–sulfuric acid. Several authentic standards were acetylated and chromatographed with the sample: maltose, lactose, Gal-Gal-Glc, dextran hydrolyzate, lactoneotetraose, and GlcNAc-Gal. Authentic peracetylated Gal-GlcNAc and Gal-GlcNAc-GlcNAc were also used. The radioactive sample migrated with an identical R_f to the peracetylated GlcNAc-Gal standard (data not shown). This supported the liquid chromatography analysis and our hypothesis that a single GlcNAc molecule is linked to the α-subunit.

DISCUSSION

We have previously reported that the Na pump α-subunit has PNGase-sensitive glycans facing the cytosol which can be galactosylated by UDP-Gal and galactosyltransferase (Pedemonte et al., 1990). In this paper we have analyzed further the Na pump α-subunit sugar composition and the characteristics of the sugar–peptide linkage.

One interesting conclusion of our results is that the Na pump α-subunit has O-linked carbohydrates. The alkaline treatment of the Na pump α-subunit produces sugar molecules, with an orcinol-positive reaction, which eluted close to the salt peak from the Sephadex G-50 column. Fetuin, which contains O-linked carbohydrates (Spiro & Bhoyroo, 1974) was used as a positive control for the β-elimination reaction and gave the same result under similar circumstances. The O-linked carbohydrates bound to the Na pump α-subunit were not exogalactosylated by the UDP-Gal/galactosyltransferase system; thus, they do not appear to be formed by single GlcNAc molecules of the type previously described by Hart et al. (1989). The presence of O-linked carbohydrates in the α-subunit explains previous reports of glycosylation of the α-subunit by sugars other than GlcNAc (Churchill et al., 1979; Omori et al., 1983; Munakata et al., 1982; Peters et al., 1981).

Insensitivity of the exogalactosylated carbohydrates to digestion with NaOH is in line with the experiments with PNGase. PNGase cleaves the linkage between the GlcNAc of N-linked glycans and the Asn residue of the peptide backbone without any effect on O-linked sugars (Tarantino

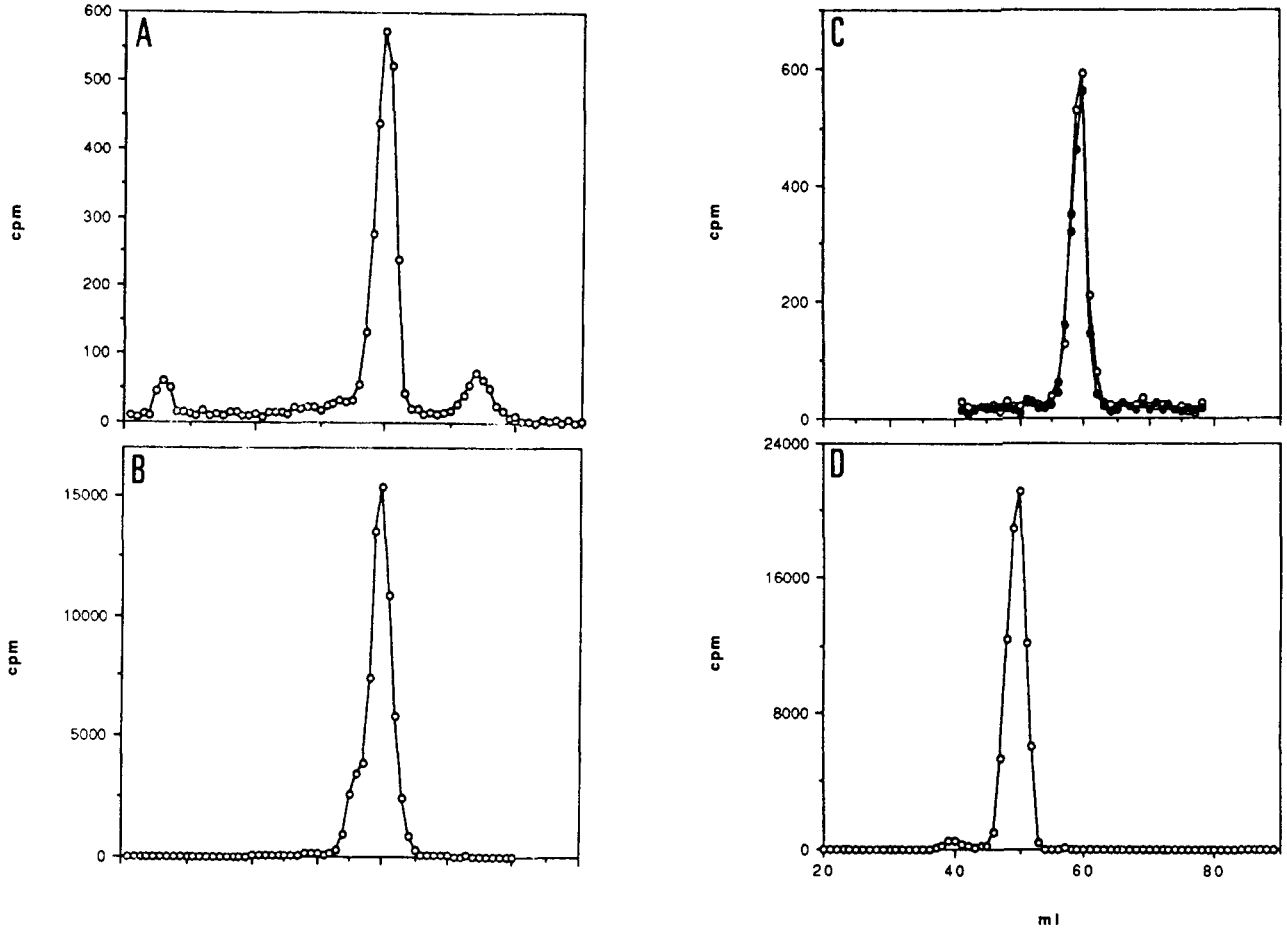

FIGURE 4: Single GlcNAc monosaccharide covalently bound to the α-subunit. (Panel A) The radioactive galactosylated carbohydrates freed by PNGase treatment and recovered in the salt peak of the Sephadex G-50 column in the experiment illustrated in Figure 3 were rechromatographed in a Bio-Gel P2 column (115 × 1 cm). The sample was eluted with 50 mM NH_4HCO_3 at a rate of 4 mL/h. Aliquots were collected every 15 min and used to determine the radioactivity. (Panel B) Authentic GlcNAc monosaccharide (obtained from Sigma) was galactosylated with UDP-[^{14}C]Gal and galactosyltransferase and chromatographed in the Bio-Gel P2 column under the conditions described above. (Panel C) Radioactive aliquots collected as described in panels A and B were separately pooled and concentrated. Equal amounts of ^{3}H-labeled samples (A) and ^{14}C-labeled samples (B) were mixed and rechromatographed in the Bio-Gel P2 column. ^{3}H-labeled and ^{14}C-labeled samples were measured in two different channels of a liquid scintillator counter. (Panel D) Authentic GlcNAc-GlcNAc (obtained from Sigma) was galactosylated with UDP-[^{14}C]Gal and galactosyltransferase and chromatographed in the Bio-Gel P2 column under the conditions described above.

et al., 1985). The PNGase-released exogalactosylated glycans of the Na pump α-subunit eluted in liquid chromatography on Bio-Gel P2 at the same volume as a disaccharide standard composed of GlcNAc and Gal moieties (Figure 4). Since we have exogenously added [^{3}H]Gal to the endogenous glycan bound to the α-subunit, the endogenous glycan must be a single GlcNAc residue. The occurrence of a glycan moiety composed of a single carbohydrate molecule accounts for our previous observation that PNGase treatment deglycosylates the α-subunit without any change in its apparent molecular weight in PAGE (Pedemonte et al., 1990).

Protein with a glycan formed by a single N-linked GlcNAc has been reported previously in a fraction of Taka-amylase by Hase et al. (1982). However, these workers hypothesized that the monosaccharide moiety was not native but the result of endoglycosidase degradation of a native complex N-linked carbohydrate. The similar possibility that our results arise from PNGase contamination by endoglycosidases which could have cleaved an internal glycosidic linkage releasing an exogalactosylated terminal GlcNAc in a branch of either O- or N-linked oligosaccharides can be ruled out. On the one hand, there was a clear separation in liquid chromatography between the radiolabeled galactosylated glycans released by PNGase and those released by the β-elimination reaction, which were not labeled; thus, the bulk of the radioactivity was not associated with O-linked oligosaccharides. On the other hand, the carbohydrate released by PNGase from the α-subunit cannot have its origin in the hydrolysis of a typical N-linked high mannose or complex oligosaccharide by a contaminant glycosidase since (i) no evidence for such glycosylation has been seen in studies of α-subunit biosynthesis (Zamofing et al., 1988; Tamkun & Fambrough, 1986); (ii) consistent with this, deglycosylation by PNGase of the Na pump α-subunit does not alter the protein apparent molecular weight in PAGE (Pedemonte & Kaplan, 1990); and (iii) such carbohydrate chains with elevated molecular weight would have been detected in the liquid chromatography experiments reported in this paper. The radioactive carbohydrate released by PNGase (Figure 3) has a molecular mass smaller than 500 (the lower limit for dextrans in Sephadex G50) and eluted from a P2 column (Figure 4) at the same volume as a disaccharide of about 383 Da (molecular mass of Gal-GlcNAc). Thus, we saw no evidence of high mannose or complex carbohydrates being labeled by UDP-Gal/galactosyltransferase and released by PNGase (or a contaminant), which is in line with previous reports of the lack of such glycosylation of the Na pump α-subunit (Zamofing et al., 1988; Tamkun & Fambrough, 1986).

We have not unequivocally established the structure of the monosaccharide bound to the Na pump α-subunit. However,

several pieces of evidence strongly suggest that it is a GlcNAc. Firstly, galactosyltransferase is perhaps the most widely used enzyme in studies related to sugar metabolism, and in the experimental conditions we have used, it has been reported to transfer Gal from UDP-Gal specifically to glycoproteins which have GlcNAc at their nonreducing terminus (Schanbacher & Ebner, 1970; Brew et al., 1968; Torres & Hart, 1984). Secondly, several previously published studies (Tamkun & Fambrough, 1986; Zamofing et al., 1988) have shown that the α-subunit has no high mannose or complex carbohydrates. Our own experiments (Pedemonte et al., 1990; this paper) confirmed this conclusion and also have shown that the exogalactosylation occurred in a sugar structure of low molecular weight. And thirdly, the exogalactosylated monosaccharide bound to Na pump α-subunit was insensitive to β-elimination but it was released by PNGase. This enzyme specifically cleaves oligosaccharides at the asparaginyl-oligosaccharide linkage: Asn-GlcNAc bond (Plummer & Tarantino, 1981; Tarantino et al., 1985; Chu, 1986). Therefore, the specificity of this enzyme also points to a GlcNAc molecule as the monosaccharide bound to the α-subunit. The possibility that the highly purified preparation of PNGase we used was contaminated with other glycosidases can be ruled out. The only glycosidase activity that has been reported in less pure preparations of PNGase F was *endo-β-N*-acetylglucosaminidase F which cleaves high mannose oligosaccharide chains at the di-*N*-acetylchitobiosyl linkage, leaving a single GlcNAc linked to an asparagine residue in the peptide backbone and release of a high molecular weight sugar (Plummer et al., 1981; Tarantino et al., 1985). Even if we assume the presence of a contaminant glycosidase and that the α-subunit has complex or high mannose oligosaccharides, the products of degradation by the putative contaminant glycosidase would be carbohydrates of molecular weight very much higher than we have observed.

In summary, we have characterized the saccharide moiety on the Na pump α-subunit which acts as an acceptor for the UDP-Gal transferase. The hitherto unrecognized N-linked glycans, obtained in their free form after PNGase treatment of the Na pump α-subunit or connected to segments of the peptide chain after NaOH treatment, were shown to be composed of a disaccharide. The disaccharide, composed of the native sugar and the attached galactose, behaves in liquid chromatography as a Gal-GlcNAc molecule. All our evidence points to a single GlcNAc as the unique component of the PNGase sensitive glycan bound to the Na pump α-subunit. We are now studying which amino acid of the polypeptide is glycosylated to determine its precise location.

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Registry No. ATPase, 9000-83-3; Na, 7440-23-5; *N*-acetylglucosamine, 7512-17-6.