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Metabolic alteration of the *N*-glycan structure of a protein from patients with a heterozygous protein deficiency

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Abstract

Glycosylation, an important post-translation modification, could alter biological activity or influence the clearance rates of glycoproteins. We report here the first example of a heterozygous protein deficiency leading to metabolic alteration of *N*-glycan structures in residual secreted protein. Analysis of C1 esterase inhibitor (C1INH) glycans from normal individuals and patients with hereditary deficiency of C1INH demonstrated identical *O*-glycan structures but the *N*-glycans of patients with a heterozygous genetic deficiency were small, highly charged and lacked sialidase releasable *N*-acetylneuraminic acid. Structural studies indicate that the charge character of these aberrant *N*-glycan structures may result from the presence of mannose-6-phosphate residues. These residues might facilitate secretion of C1INH through an alternate lysosomal pathway, possibly serving as a compensatory mechanism to enhance plasma levels of C1INH in these deficient patients.
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Keywords: Glycosylation; C1 esterase inhibitor; *N*-glycan; *O*-glycan; Hereditary angioedema; C1 esterase inhibitor deficiency

1. Introduction

C1 esterase inhibitor (C1INH) is a serum protein that regulates early events in the classical pathway of complement [1]. Significantly reduced levels of this protein are seen in the clinical condition called hereditary angioedema (HAE), an autosomal dominant disorder. HAE patients typically have levels of C1INH that are less than would be predicted based upon lack of one normal allele, which has been ascribed to catabolism of the normal C1INH or to transinhibition of the normal gene [2,3]. Individuals with HAE either show an absolute decrease in the amount of circulating antigenic and functional C1INH (type I) or a dysfunctional C1INH with normal antigenic levels (type II) [1]. Patients typically suffer from episodic attacks of angioedema, abdominal pain and respiratory difficulty which may cause death in as many as a quarter of patients. Attenuated androgens, effective in preventing attacks of HAE, are presumed to work by increasing biosynthesis of C1INH [4]. Treatment with C1INH has been shown in clinical trials to be effective in preventing attacks [5]. Nevertheless, replacement therapy remains unavailable in the United States in 2004.

Abbreviations: C1INH, C1 esterase inhibitor; HAE, hereditary angioedema; AGA, (Amido G Acid), monopotassium 7-amino-1,3-naphthalenedisulfonic acid; CE, capillary electrophoresis

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C1INH is a serine protease inhibitor (SERPIN) that is the only substance known to control activity of activated subcomponents of the C1 molecule (C1r and C1s) and mannose-binding protein-associated serine protease (MASP) [1,6,7]. C1INH is one of the most heavily glycosylated plasma proteins. The molecular mass of the protein portion of C1INH is 53 kDa and the carbohydrate portion contributes an additional 18 kDa resulting in a molecular mass of 71 kDa [8,9]. The apparent molecular weight of C1INH measured by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), is considerably larger, probably the result of the rod and globular domains caused, in part, by its heavy glycosylation [10]. C1INH contains six biantennary *N*-linked glycans, the structures of which have been explored using NMR spectroscopy [11]. C1INH is the only SERPIN with *O*-linked glycans, having multiple small linear glycan chains of structures previously analyzed by NMR [11].

Glycosylation is known to play an important role in the biological activities and clearance rates of many glycoproteins. However, glycosylation does not appear to play a role in the activity of C1INH towards its target proteases in vitro [12,13]. It has been suggested that glycosylation plays a role in other biological activities of C1INH [14] and it is likely that glycosylation will influence the clearance rate of C1INH in vivo [14,15].

Our study examined, for the first time, the structure of *N*- and *O*-linked glycans of C1INH from patients with HAE and found extensive differences in glycan complexity and structure when compared to normal C1INH. These appear to result from differences in glycan biosynthesis and suggest a different pathway for the secretion of this important protein.

2. Materials and methods

2.1. Purification and characterization of C1INH from human serum

Normal C1INH was purified from pooled serum donated by normal subjects. HAE C1INH was from three male HAE patients. Two of these patients had type I HAE, one of who had been chronically treated with attenuated androgens while the second had been intermittently treated with attenuated androgens. C1INH was also purified from a patient with type II HAE who had never received attenuated androgen. C1INH was purified as previously described [16,17].

The purity and molecular weight of the C1INH were assessed by SDS-PAGE (12% gel) and silver-staining. Antigenically detectable C1INH was determined by dot blot and Western blot assays by probing membranes with a goat IgG anti-human C1-esterase inhibitor antibody (Dia-Sorin Inc., Stillwater, MN). Bound antibodies were detected by using alkaline phosphatase conjugated rabbit IgG anti-goat IgG antibody. Functional C1INH activity was determined by using traditional sheep red blood cell assays [16]. The carbohydrate-deficient transferrin (CDT) assay, using isoelectric focusing, immunoblotting and laser densitometer measurement [18], was performed by Specialty Laboratories, Santa Monica, CA.

2.2. N-glycan release

Before performing glycan analysis, C1INH was further purified using a Sephacryl S-200 HR (Sigma Chemical Co., St. Louis, MO) column (45×1.5 cm i.d.) in 2 mM HEPES, 0.2% SDS, pH 7.5 buffer to remove low molecular weight contaminants that might interfere with glycan analysis [19]. C1INH was precipitated by adding 9 volumes of acetone (12 h at −20 °C), recovered by centrifugation, re-suspended in 0.2 ml of buffer 1 (20 mM HEPES, 1% SDS, pH 8.2) and β-mercaptoethanol (20 mM) was added. The sample was boiled for 5 min to dissolve the protein and then combined with 0.8 ml of buffer 2 (20 mM HEPES, 1.25% NP-40, pH 8.2) containing 50 mM EDTA. PNGase F (Boehringer Mannheim, Germany, 1.0 U/ml) was added to release the *N*-glycan chains from C1INH. The reaction was carried out overnight at 30 °C and stopped by boiling for 3 min. The released *N*-glycans were recovered from the core protein by fractionation on the S-200 HR column. Protein was monitored at 280 nm and glycan was measured by reducing sugar assay [20]. After the glycan fractions were pooled, 2% (v/v) of saturated KCl was added and the sample was incubated at 4 °C overnight. The precipitated potassium-SDS salt was removed by centrifugation and the glycans were passed over a 3 ml Biobead SM2 (Bio-Rad, Hercules, CA) column to remove the residual nonionic detergent. The column was washed with 5 column volumes of water. The pooled fractions were freeze-dried and then individually desalted by using a Sephadex G-10 (Sigma) column.

2.3. O-glycan release

O-glycans were released from C1INH (after *N*-glycan release) by using mild β-elimination conditions [21]. Briefly, 1 mg freeze-dried C1INH was dissolved in 1 ml of 0.5 M lithium hydroxide (nitrogen saturated) and the solution was kept in an ice bath for 48 h under nitrogen atmosphere. The reaction was stopped by addition of 1 ml of 0.5 M acetic acid. The released *O*-glycans were recovered from the protein by fractionation on the S-200 HR column. *O*-glycan was detected at 210 nm and fractions were desalted on Sephadex G-10.

2.4. Fluorescent labeling of glycans

Fluorescent glycan conjugates were prepared by reductive amination in the presence of sodium cyanoborohydride [22,23]. Amido G Acid, monopotassium 7-amino-1,3-naphthalenedisulfonic acid (AGA, Aldrich, Milwaukee, WI, re-crystallized from water) was dissolved in 15% acetic acid (5 mg/100 μl), the purified glycan was added and the

mixture was incubated for 1 h at room temperature. Sodium cyanoborohydride (Sigma, 100 μl of 1.0 M) was added to the mixture and incubated for 12 h at 45 °C. Excess AGA was removed using a Sephadex G-25 (Sigma) column (45×1.5 cm i.d.) with detection at 247 nm.

2.5. Preparation of fluorescently labeled standards

Oligomannose-type *N*-linked oligosaccharide (Man8), di-sialylated, galactosylated, biantennary *N*-glycan (with or without fucose substitution, A2F and A2) (Oxford Glyco Systems (OGS), Wakefield, MA), NA2 and NA2F (jack bean sialidase treated A2F and A2), chitobiose (Sigma), and Man1–8 standards [24] were fluorescently tagged with AGA as described above.

2.6. Enzymatic digestions

Susceptibility of normal and HAE C1INH to Endo H (OGS, 50 mU/ml) was tested in buffer (100 mM sodium citrate, pH 5.5) at 37 °C for 24 h. For complete desialylation, the AGA-tagged glycans were digested with jack bean sialidase (OGS, 2 U/ml) in 10 mM sodium acetate (pH 5.0) buffer at 37 °C for 18 h. Linkage-specific sialidase L (V-Labs, New Orleans, LA, 1 U/ml) was incubated in 5 mM sodium phosphate buffer (7.0) at 37 °C for 24 h to release α 2→3 linked sialic acid from AGA-labeled glycans. β-Galactosidase from *S. pneumoniae* (OGS, 80 mU/ml) was incubated in 100 mM sodium acetate (pH 6.0) buffer at 37 °C for 18 h to release galactose from glycan chains. Bacterial alkaline phosphatase (Worthington Biochemicals, Lakewood, NJ, 100 U/ml) in 5 mM Tris–HCl pH 8.0 buffer at 37 °C for 48 h was used to remove phosphate esters from AGA-labeled glycans [25].

2.7. Glycan analyses

Labeled glycan was analyzed by capillary electrophoresis (CE) (Dionex, Sunnyvale, CA) at 25 kV using fluorescence detection (λ_{ex} of 250 nm and λ_{em} of 420 nm). Separation and analysis were carried out in a reversed polarity mode using a fused silica capillary tube (55 cm in length and 50 μm i.d.). The separation buffer contained 20 mM sodium phosphate, pH 3.5. The sample was pressure injected (5 s, 5 psi) resulting in an injection volume of 0.5 μl. Fluorophore-assisted carbohydrate electrophoresis (FACE) [26] used a Mini-Protean II electrophoresis system (Bio-Rad) and an UV transilluminator. ^{31}P NMR spectroscopy used a Bruker DRX 400 spectrometer at 298 K with 70% H_3PO_4 external reference (δ=0 ppm).

3. Results

SDS-PAGE (Fig. 1A) shows that C1INH purified from the serum of normal subjects [17] gave a single band on silver-staining with apparent molecular mass ~104 kDa. The apparent molecular mass of C1INH from all three HAE patients (Fig. 1A, B and C), purified using the identical method, was about 95 kDa. The C1INH activity in the serum of all three patients ranged from about 14% to 19% of normal levels. The one patient with type II HAE occasionally had antigenic values of 20, which were about 65% of normal levels. In both cases, the C1INH was immunoreactive, giving a Western blot identical to the silver-stained gel and had functional activity, although activity in serum from patients was considerably lower. These results suggested that aberrant glycosylation might account for some of the observed difference in apparent molecular mass.

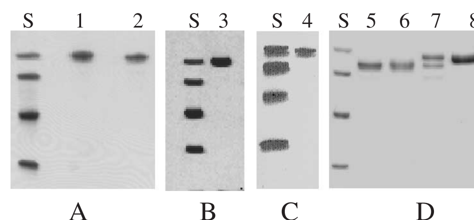

Fig. 1. SDS-PAGE analysis. The gel was visualized by silver-staining. Lane S contains standards (molecular weight from top to bottom: 97.4, 66.2, 45.0, 31.0 kDa). Panel A: Lane 1 contains C1INH from a normal individual. Lane 2 contains C1INH from an HAE patient (type I) who had been chronically treated with attenuated androgens. Panel B: Lane 3 contains C1INH from an HAE patient (type I) had been intermittently treated with attenuated androgens. Panel C: Lane 4 contains C1INH from an HAE patient (type II). Panel D: C1INH digested with PNGase F and Endo H. Lane 5 contains PNGase F digested normal C1INH. Lane 6 contains PNGase F digested C1INH from an HAE patient (type II). Lane 7 contains Endo H digested normal C1INH. Lane 8 contains Endo H digested C1INH from an HAE patient (type II).

N-glycans and *O*-glycans were released from C1INH by PNGase F digestion and β-elimination, purified [23,24] and fluorescently labeled with AGA, an anionic fluorophore [22], so that any neutral oligosaccharides would be charged for migration under electrophoresis and also to improve detection. The labeling efficiency was nearly 100% [22]. The amounts of *N*-glycans and *O*-glycans, released from C1INH obtained from normal subjects or HAE patients, were approximately the same.

CE mapping showed that the AGA labeled *O*-glycans (Fig. 2A, B) were nearly identical, whether prepared from C1INH obtained from normal subjects or from patients with type I or type II HAE. Electropherograms of the *O*-glycan mixture contained two prominent peaks at ~5.2 and 6.0 min. To determine the glycan size, co-injection experiments with AGA-labeled sialyl-lactose (NeuAc (α 2→3 or 2→6) Gal (β 1→4) Glc) and the *O*-glycan mixture showed that the peak at 6.0 min, while migrating very close to AGA-labeled sialyl-lactose, corresponded to a different trisaccharide. PAGE analysis (Fig. 3, lane 5) of the *O*-glycan mixture showed two major bands migrating ahead of chitobiose labeled with AGA, corresponding in size to a sialated trisaccharide and as a second much smaller product. Both glycans were sensitive to nonspecific sialidase, but only the peak at 6 min was sensitive to a NeuAc α 2→3 Gal linkage

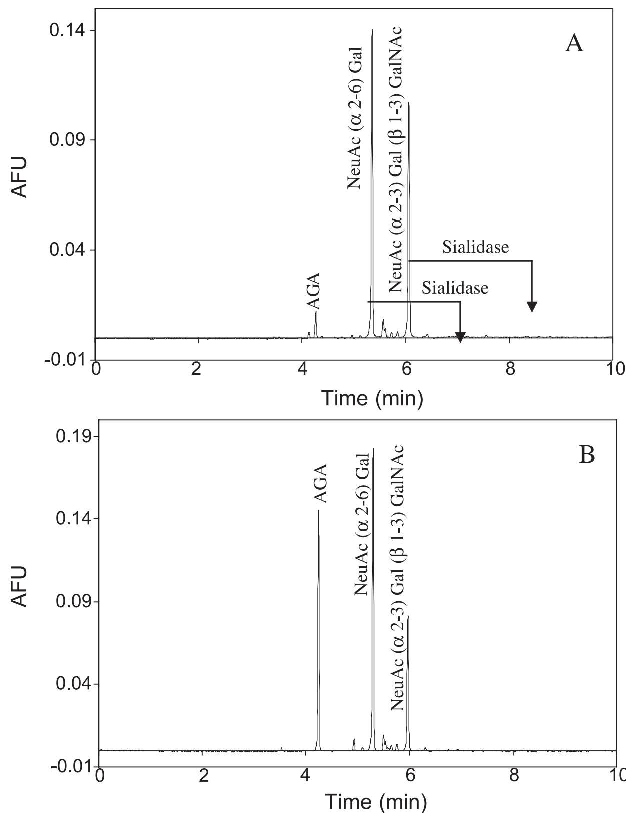

Fig. 2. CE mapping of *O*-glycans released form C1INH and fluorescently labeled with AGA. Panels A and B show *O*-glycan maps of C1INH obtained from a normal individual and an HAE (type II) patient, respectively. The arrows show the peaks that shift on sialidase treatment.

specific sialidase, sialidase L. These data demonstrate that the *O*-glycans migrating at 5.2 and 6.0 min are terminated with α 2→6 and α 2→3 linked sialic acid, respectively. These results are consistent with the peak at 6.0 min resulting from an AGA-labeled *O*-glycan of the structure NeuAc (α 2→3) Gal (β 1→3) GalNAc, previously described [11]. To identify the peak at ~5.2 min, the AGA-labeled *O*-glycan mixture was treated with non-specific sialidase and the resulting peak was identified by its co-migration with AGA-labeled galactose standard, suggesting that the peak at ~5.2 min in the *O*-glycan mixture corresponded to the AGA-labeled sialated disaccharide, NeuAc (α 2→6) Gal. These results confirmed the trisaccharide structure [11] and established the structure of a new disaccharide as the *O*-linked glycan in C1INH. The *O*-linked glycan structures of C1INH from the normal subjects and from the HAE patients were very similar.

CE maps of the *N*-glycans from the C1INH samples prepared from normal subjects were all identical, showing a prominent major component at ~8.5 min and approximately 10 minor components (Fig. 4). This major *N*-glycan was easily identified by co-migration with di-sialylated biantennary standard glycan A2 on CE. A second minor peak at ~8.7 min was identified as a fucosylated di-sialylated biantennary by co-migration on CE with a standard glycan A2F. These two structures were further confirmed by sialidase digestion, which shifted these peaks to exactly the same positions where the desialated standards NA2 and NA2F migrated. The glycan mixture from C1INH also showed minor peaks; only one at ~6 min was sialidase sensitive. The remaining minor compounds were sialidase insensitive corresponding to NA2, NA2F and other unidentified desialated structures. The results of CE mapping, thus, were in general agreement with the *N*-glycan structures of C1INH established using NMR spectroscopy [11], except that the observed ratio of fucosylated to non-fucosylated di-sialylated biantennary glycan (A2F and A2 of 1:7.7) was substantially different from the previously reported 1:2.3 ratio [11]. PAGE analysis of the AGA labeled *N*-glycan mixture showed a major band co-migrating with di-sialylated biantennary standard A2 consistent with the CE results (Fig. 3, lane 3).

The CE maps (Fig. 5A and B, and Fig. 6) of the *N*-glycans obtained from the C1INH samples prepared from the three HAE patients were similar to each other with most of the peaks migrating <8 min, but were remarkably different from the *N*-glycans found in the C1INH of normal individuals. The CE maps of *N*-glycans obtained from the C1INH of HAE patients were complex, showing over 20 peaks, none of which was clearly prominent. None of the biantennary *N*-glycan structures (A2, A2F, NA2 and NA2F) identified in the C1INH from normal subjects, were present in the CE maps of C1INH *N*-glycans obtained from the type I HAE patients. No detectable change in the CE electropherogram was observed on treatment with nonspecific sialidase, suggesting that no terminal sialic acid was present.

The *N*-glycans (Fig. 6) from the type II HAE patient showed minor amounts of the two biantennary *N*-glycan

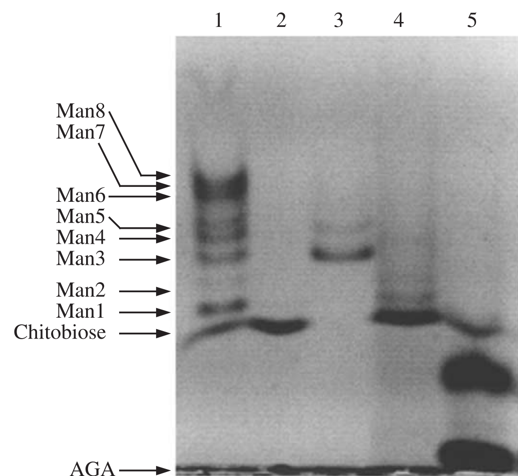

Fig. 3. PAGE analysis of *O*-glycans and *N*-glycans released form C1INH and fluorescently labeled with AGA. Lane 1 contains AGA, AGA-labeled chitobiose and AGA-labeled oligomannose (Man1–8) standards. Lane 2 contains AGA-labeled chitobiose standard. Lane 3 contains AGA-labeled *N*-glycan from C1INH obtained from a normal individual. Lane 4 contains AGA-labeled *N*-glycan from C1INIH from a patient with HAE (type II). Lane 5 contains *O*-glycan from C1INH from a normal individual. The gel was visualized by UV transillumination and is shown as a reverse image.

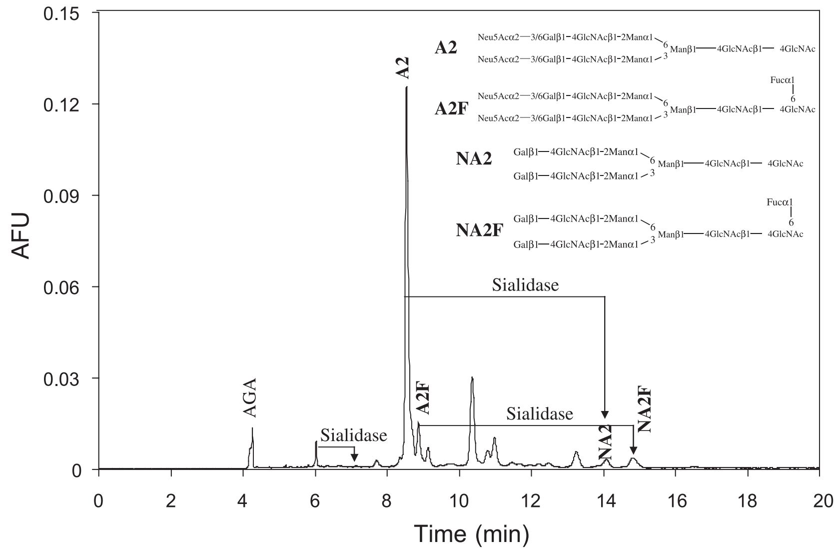

Fig. 4. CE mapping of *N*-glycans released from C1INH obtained from a normal individual and fluorescent labeled with AGA. Chemical structures of complex-type biantennary *N*-glycan standards (A2, A2F, NA2 and NA2F) are shown.

structures, as demonstrated by co-migration with glycan standards (A2 and A2F) and their sensitivity to sialidase. Insufficient C1INH from type I HAE patient was available for the same analysis. The majority of the *N*-glycans released from the C1INH of all HAE patients migrated early (from 4 to 7.8 min), suggesting that these *N*-glycans were very small and/or carried one or more negative charges [23]. The results from SDS-PAGE analysis (Fig. 1D) on the PNGase F and endo H digested C1INHs showed that both normal or HAE C1INH proteins are susceptible to PNGase F; the majority of normal C1INH proteins containing di-sialylated biantennary *N*-glycan are not susceptible to endo H, whereas HAE C1INH proteins containing small N-glycan are susceptible to endo H. The presence of *N*-glycans of small size was confirmed by PAGE analysis (Fig. 3, lane 4). The short migration time and absence of sialidase releasable sialic acid led us to look for other charged groups in these glycans. Treatment with alkaline phosphatase and CE analysis showed the decrease of several peaks including the major peaks at ~ 5.8 and 7.8 min (Fig. 6) suggesting that a phosphate monoester was present, such as that found in mannose-6-phosphate containing glycans [27]. The pres-

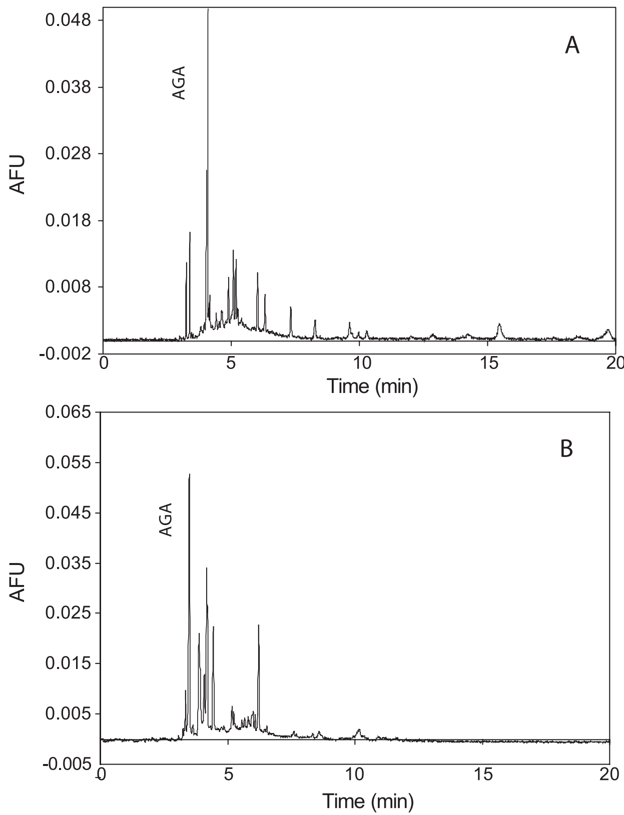

Fig. 5. CE mapping of *N*-glycans released from C1INH and fluorescent labeled with AGA. Panel A shows an *N*-glycan map of C1INH obtained from an HAE (type I) patient who had been chronically treated with attenuated androgens. Panel B shows an *N*-glycan map of C1INH obtained from an HAE (type I) patient that had been intermittently treated with attenuated androgens.

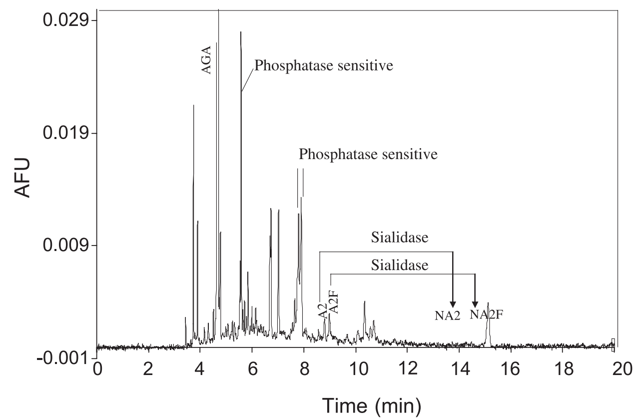

Fig. 6. CE mapping of *N*-glycans released from C1INH obtained from an HAE (type II) patient and fluorescent-labeled with AGA. The arrows show the peaks that shift on sialidase treatment. Phosphatase sensitive peaks are labeled.

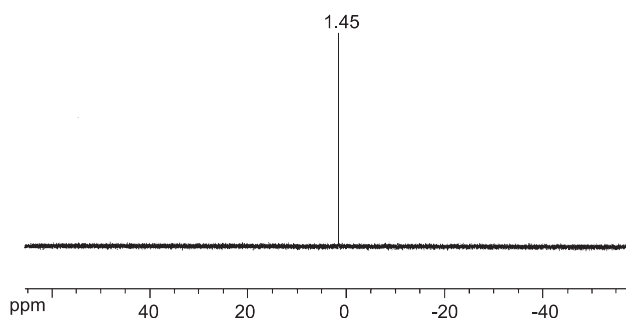

Fig. 7. ^{31}P NMR analysis of *N*-glycans released from C1INH of a type II HAE patient.

ence of this phosphate ester was confirmed by the presence of a peak at 1.45 ppm in the ^{31}P NMR (Fig. 7) in the unlabeled *N*-glycans of C1INH from HAE patients.

4. Discussion

HAE is a serious genetic disorder resulting in a patient's inability to produce sufficient functional C1INH. Our laboratory is actively investigating new methods for the production of sufficient quantities of appropriately glycosylated recombinant C1INH to treat HAE patients through *intravenous* replacement [5,24]. In carrying out this project, it was necessary to confirm the structure of C1INH glycans from healthy individuals. We found that the *N*-glycan and *O*-glycan structures in C1INH of normal subjects are similar to those previously reported [11]. In the course of these structural studies, we also had the opportunity to examine the structures of *N*-glycans and *O*-glycans obtained from C1INH purified from serum of patients with C1INH deficiency. Although the structures of *O*-glycans derived from the C1INH of HAE patients were apparently identical to those derived from C1INH from normal individuals, the structures of the *N*-glycans were remarkably different and these differences were observed in all three HAE patients. The HAE patients appeared to have a more complex mixture of *N*-glycans with shorter and/or more highly charged glycan chains. Initially, we suspected that *N*-glycan biosynthesis in these patients might have been influenced by androgen therapy that they had been receiving, but examination of C1INH *N*-glycans from an untreated HAE patient gave identical results, suggesting instead that these unusual *N*-glycan structures either resulted from aberrant *N*-glycan biosynthesis or catabolism. However, the catabolism of the *N*-glycans in patients with HAE through the action of tissue sialidases was unlikely because the *O*-glycan chains were intact, containing both α 2→3 and β 2→6-linked terminal NeuAc residues.

Further examination of the structures of these *N*-glycans showed that they were indeed shorter than the *N*-glycan structures found in C1INH of normal individuals and that they contained very little terminal sialic acid. The lack of sialidase releasable sialic acid caused us to consider other functional groups that might impart charge to these *N*-glycans. Mannose-6-phosphate residues are well known to play a role in the lysosomal and subcellular trafficking of glycoproteins [27,28]. The *N*-glycan chains of lysosomal glycoproteins often contain one or two mannose-6-phosphate residues, which are sensitive to alkaline phosphatase treatment unless they are blocked as phosphodiesters through attachment of an α-linked *N*-acetylglucosamine residue [29]. The C1INH *N*-glycans from a type II HAE patient contained three major alkaline phosphatase sensitive components and the presence of a phosphate ester was clearly identified in these purified *N*-glycans using ^{31}P NMR spectroscopy. These results suggest that C1INH in HAE patients has *N*-glycan structures containing mannose-6-phosphate residues.

Mannose-6-phosphate residues have been reported in a variety of non-lysosomal proteins some of which are hydrolytic enzymes, such as uteroferrin, that take a predominately secretory route out of the cell [27]. Thus, it is possible that HAE patients utilize this secretory route as a secondary pathway for transport of C1INH out of the cell, enhancing its release. Analysis of the serum transferrin from a type I HAE patient, providing a sensitive probe of liver protein glycosylation, was normal, suggesting that metabolically altered *N*-glycan structures are unique to the residual secreted C1INH. Thus, lysosomal secretion might correspond to an alternate route representing a compensatory mechanism to ensure the cellular release of the maximum level of C1INH in HAE patients. This novel observation suggests a somewhat speculative new secretion mechanism, since degradation usually takes place in the lysosome. Only additional studies will elucidate the importance of these observations.

Acknowledgements

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