FEBS 28808 FEBS Letters 576 (2004) 51–56

N-glycosylation at Asn^{491} in the Asn-Xaa-Cys motif of human transferrin

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Received 14 July 2004; accepted 4 August 2004

Available online 11 September 2004

Edited by Sandro Sonnino

Abstract Glycopeptides derived from human transferrin were exhaustively analyzed by matrix-assisted laser desorption ionization and electrospray ionization mass spectrometry (MS). Both MS techniques clearly revealed the sequences of and the attachment sites of bi-antennary complex-type oligosaccharides, at both Asn^{432} and Asn^{630}, both of which are located in a well-known motif for N-glycosylation, Asn-Xaa-Ser/Thr, but also at Asn^{491} in the Asn-Xaa-Cys motif. The latter has been reported to be a minor N-glycosylation site in several glycoproteins. The relative abundance of this abnormal glycosylation was estimated to be approximately 2 mol% of the transferrin preparation used in this study.

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Keywords: Human transferrin; N-glycosylation; Asn-Xaa-Cys motif; Mass spectrometry

1. Introduction

It is widely recognized that the sugar chains of glycoproteins contribute significantly to the functional properties of glycoproteins, which include their stability, biological activity, and binding affinity, even though little is known concerning their biological functions. The importance of carbohydrate moieties on glycoproteins has increased along with the maturation of the protein sequence database. However, the characterization of oligosaccharide structures of glycoproteins remains a challenge, owing to their complexities, as represented by the heterogeneity, the number of isomers possible and branching structures.

Mass spectrometry (MS) has become a powerful and useful tool for the analysis of complex structures of carbohydrates such as those linked to a glycoprotein and is capable of revealing the molecular mass and sequence of the constituent sugar units. Such capabilities have been gained since the advent of ionization methods, i.e., electrospray ionization (ESI) [1] and matrix-assisted laser desorption ionization (MALDI) [2], both of which allow for the direct measurement of relatively large carbohydrates and glycoproteins with molecular masses over 50 000 Da and provide sufficient molecular ions that are amenable to a structural analysis using tandem mass spectrometry (MS/MS). In spite of the increasing MS capabilities for carbohydrate analysis, it is still not sufficient to permit a precise profile of the oligosaccharides of a glycoprotein to be acquired. One major issue to be overcome is the difficulty in isolating individual glycopeptides carrying single glycosylation from a glycoprotein, which permits the site-specific analysis of carbohydrate moieties, an issue that needs to be clarified for a multiply-glycosylated protein.

In this study, we report on the use of MALDI- and ESI-MS/MS to analyze glycopeptides derived from a commercially available human transferrin, the carbohydrate moieties of which are well known to be a bi-antennary complex-type oligosaccharide and which are located at Asn^{432} and Asn^{630}, both of which are involved in the consensus sequence (Asn-Xaa-Ser/Thr) of N-glycosylation [3–5]. During the course of the analysis, we detected, for the first time, glycosylation at Asn^{491} as a minor component, in addition to the above two glycosylation sites, which are located in the sequence, an Asn-Xaa-Cys motif, which has been reported to be a minor N-glycosylation site [6–13]. Furthermore, ESI-MS/MS of this glycosylated peptide (Ile^{490}–Lys^{508}) indicated that it is a bi-antennary complex-type oligosaccharide.

2. Materials and methods

2.1. Materials

Human transferrin (T2252) and α-cyano-4-hydroxy cinnamic acid (CHCA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *Achromobacter lyticus* protease I (lysylendopeptidase (LEP)), ^{18}O-normalized water (93.8 atom%), and Peptide-*N*-glycosidase F (PNGase F), which cleaves the β-aspartylglycosylamine linkage of Asn-linked carbohydrate, were obtained from Wako Pure Chemical Industries (Osaka, Japan), ISOTEC Inc. (Miamisburg, OH, USA), and New England Bio-laboratory (Beverly, MA), respectively.

2.2. Preparation of glycopeptides

Human transferrin (6 nmol) was dissolved in 100 μl of 250 mM Tris–HCl (pH 8.5) containing 6 M guanidine hydrochloride and 2 mM EDTA, and incubated at 37 °C for 30 min. The protein was treated with a 50-fold excess of DTT (1 mg) to reduce Cys residues at room temperature for 3 h in an atmosphere of N_2 followed by incubation with 2.5-fold excess of monoiodoacetic acid, relative to the amount of DTT. The reaction was allowed to proceed for 30 min at room temperature in the dark. The solution was dialyzed against distilled water and lyophilized. The resulting *S*-carboxymethylated human transferrin

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Abbreviations: MS, mass spectrometry; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; MS/MS, tandem mass spectrometry; CHCA, α-cyano-4-hydroxy cinnamic acid; LEP, lysylendopeptidase; PNGase F, Peptide-*N*-glycosidase F; RP, reverse-phase; TFA, trifluoroacetic acid; CID, collision-induced dissociation; NeuNAc, *N*-acetyl-neuraminic acid; Hex, hexose; HexNac, *N*-acetylhexosumine

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doi:10.1016/j.febslet.2004.08.061

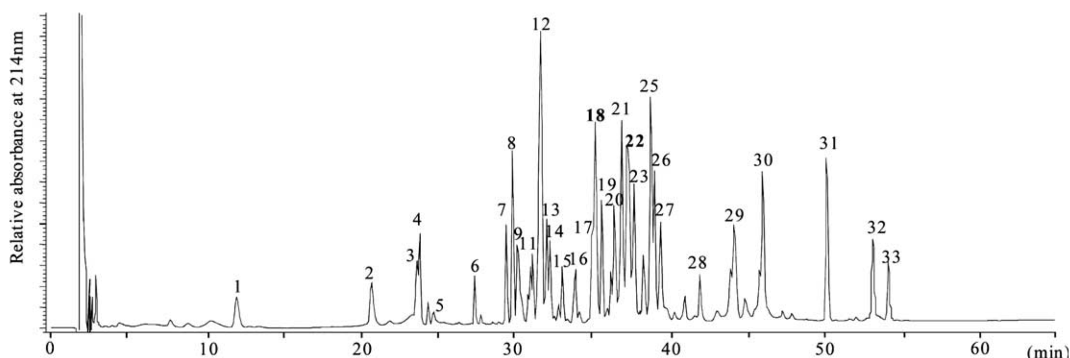

Fig. 1. RP-HPLC of a LEP digest of carboxymethylated human transferrin. The observed MH^+ values for each fraction are assigned to the sequence (see Fig. 2, Table 1). Fractions 18 and 22 contained glycopeptides and were subjected to further re-chromatography (Fig. 4(a)).

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  1 MRLAVGALLV CAVLGLCLAV PDKTVRWCAV SEHEATKCQS FRDHMKSVIP  50
      Signal peptide                  (12)        (3)
 51 SDGPSVACVK KASYLDCIRA IAANEADAVT LDAGLVYDAY LAPNNLKPVV 100
     (14)                      (32,33)
101 AEFYGSKEDP QTFYYAVAVV KKDSGFQMNQ LRGKKSCHTG LGRSAGWNIP 150
    (15)         (25)
151 IGLLYCDLPE PRKPLEKAVA NFFSGSCAPC ADGTDFPQLC QLCPGCGCST 200
     (29)                               (30)
201 LNQYFGYSGA FKCLKDGAGD VAFVKHSTIF ENLANKADRD QYELLCLDNT 250
                       (10)       (17)            (19)
251 RKPVDEYKDC HLAQVPSHTV VARSMGGKED LIWELLNQAQ EHFGKDKSKE 300
                   (8)                    (31)
301 FQLFSSPHGK DLLFKDSAHG FLKVPPRMDA KMYLGYEYVT AIRNLREGTC 350
     (20)                      (5)                 (28)
351 PEAPTDECKP VKWCALSHHE RLKCDEWSVN SVGKIECVSA ETTEDCIAKI 400
                   (12)       (16)  •        (11)
401 MNGEADAMSL DGGFVYIAGK CGLVPVLAEN YNKSDNCEDT PEAGYFAVAV 450
                                (18)              •   (23)
451 VKKSASDLTW DNLKGKKSCH TAVGRTAGWN IPMGLLYNKI NHCRFDEFFS 500
          (18)                                    (18,22)
501 EGCAPGSKKD SSLCKLCMGS GLNLCEPNNK EGYYGYTGAF RCLVEKGDVA 550
                   (22)                   (26)
551 FVKHQTVPQN TGGKNPDPWA KNLNEKDYEL LCLDGTRKPV EEYANCHLAR 600
                                  •              (21)
601 APNHAVVTRK DKEACVHKIL RQQQHLFGSN VTDCSGNFCL FRSETKDLLF 650
                                     (22)
651 RDDTVCLAKL HDRNTYEKYL GEEYVKAVGN LRKCSTSSLL EACTFRRP   698
     (24)         (4)       (13)                  (21)
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Fig. 2. Amino acid sequence of human transferrin. Double-arrowhead solid lines and the numbers in parentheses indicate the LEP peptides obtained in Fig. 1 (see Table 1). Amino acids in boldface correspond to the glycopeptides found in fractions 18 and 22. Asn (N) with filled circles denote the N-glycosylation sites verified by ESI-MS/MS (see Fig. 5).

was dissolved in 100 μl of 100 mM Tris–HCl (pH 9.0) containing lysylendopeptidase (E/S = 1/100) and incubated for 10 h at 37 °C.

2.3. *Reverse-phase-HPLC*

The peptides produced by the LEP digestion were separated by reverse-phase (RP)-HPLC using a Develosil $5C_{18}$-UG column (4.6 × 150 mm, Nomura Chemicals, Aichi, Japan), using a Waters model LC204 system (Milford, MA). A linear gradient between solvent A (0.1% trifluoroacetic acid (TFA) in water) and solvent B (0.1% TFA in acetonitrile) was used for the separation. The peptides were eluted by increasing solvent B from 5% to 60% over a 55 min period at a flow rate of 1.0 ml/min. The eluates were monitored at both 214 and 280 nm using Waters model 441 and 440 UV absorbance detectors, respectively.

2.4. *PNGase F treatment of glycopeptides*

The glycopeptides were dissolved in 100 mM NH_4HCO_3 in 40 atom% $H_2{}^{18}O$, and treated with 1 μl of a PNGase F solution containing 500 units for 5 h at 37 °C. Deglycosylated peptides were purified by RP-HPLC using the same conditions as the above, quantified by amino acid analysis, and examined by MALDI-MS and ESI-MS/MS.

Table 1
Summary of observed masses of the LEP peptides of human transferrin and their corresponding theoretical masses

Fraction no.[a]	Observed *m/z*	Theoretical *m/z*	Position
1	–[b]	–	–
2	–	–	–
3	1209.5(m)[c]	1209.5(m)	38–46
4	1175.7(m)	1175.6(m)	660 668
5	913.2(m)	913.5(m)	324–331
6	–	–	–
7	–	–	–
8	2151.0(m)	2151.0(m)	259–278
9	–	–	–
10	978.2(m)	978.5(m)	216–225
11	1727.7(m)	1727.7(m)	385–399
12	1674.7(m)	1674.8(m)	24–37
	1437.7(m)	1437.7(m)	363–373
13	1000.3(m)	1000.5(m)	669–676
14	1416.6(m)	1416.7(m)	47–60
15	1096.4(m)	1096.6(m)	98–107
16	1281. 5(m)	1281.5(m)	374–384
17	1273.6(m)	1273.7(m)	226–236
18	1249.3(m)	1249.6(m)	454–464
	3391.7(a)[d]	3393.5 (a)	Glycopeptide (421–433)
	3682.1(a)	3684.7(a)	Glycopeptide (421–433)
	4175.0(a)	4176.2(a)	Glycopeptide (490–508)
	4465.3(a)	4467.5(a)	Glycopeptide (490–508)
19	2011.0(m)	2011.0(m)	237–252
	2742.3(m)	2742.3(m)	237–258
20	1276.6(m)	1276.6(m)	300–310
21	3999.7(a)	4000.5(a)	577–610
	1786.8 (m)	1786.8(m)	684–698
22	2260.0(m)	2260.0(m)	490–508
	5261.5(a)	5261.5(a)	Glycopeptide (619–646)
	5551.8(a)	5552.8(a)	Glycopeptide (619–646)
23	2073.0(m)	2072.9(m)	434–452
24	1566.7 (m)	1566.8(m)	647–659
25	1629.6(m)	1629.8(m)	108–121
26	1913.8(m)	1913.9(m)	531–546
27	–	–	–
28	3340.3(a)	3340.7(a)	332–359
29	3637.3(a)	3639.2(a)	136–167
30	4995.2(a)	4997.5(a)	168–212
31	2070.1(m)	2070.0(m)	279–295
32	3858.0(a)	3858.3(a)	62–97
33	4936.4(a)	4936.6(a)	62–107

[a] Numbers in the chromatogram in Fig. 1.
[b] Not identified.
[c] (m), Monoisotopic mass.
[d] (a), Average mass.

2.5. Matrix-assisted laser desorption/ionization mass spectrometry

MALDI-MS spectra were obtained on a Voyager Elite XL time-of-flight mass spectrometer equipped with a delayed-extraction system (Applied Biosystems, Framingham, MA, USA) with a flight pass of 6.5 meters in the reflectron mode. The samples were mixed with the matrix solution, the supernatant of a 50% acetonitrile solution saturated with CHCA, and then air dried on the flat surface of a stainless steel plate. Ions were generated by irradiating the sample area with the output of a N_2 laser at a wavelength of 337 nm and accelerated at a 20 or 25 kV potential in the ion source with a delay of 200 ns. Mass calibration was carried out using a mixture of angiotensin I and insulin (1/5, mol/mol) for measurements of peptides.

2.6. Nano-flow electrospray ionization tandem mass spectrometry

Low-energy collision-induced dissociation (CID) MS/MS was carried out on a Q-TOF mass spectrometer (Micromass, Manchester, UK), which is a hybrid quadrupole orthogonal acceleration tandem mass spectrometer fitted with a Z-spray nano-flow electrospray ion source. Samples were dissolved in acetic acid/acetonitrile/water (1/50/50, v/v/v) at the concentration of 500 fmol/μl and ~2 μl of this solution was loaded on a nano-flow glass-capillary prior to analysis. A potential of 1.0–1.5 kV was applied to the glass-capillary tip in the ion source, which resulted in a flow rate of about 10–20 nl/min into the analyzer. Mass calibration was carried out using the cluster ions derived from NaI. MS/MS data were processed by a maximum entropy data enhancement program, MaxEnt3™ (Micromass), which is capable of deconvoluting a spectrum where peaks in a variety of charge states are present, thus producing a simplified spectrum consisting only of monoisotopic peaks in a single charge state.

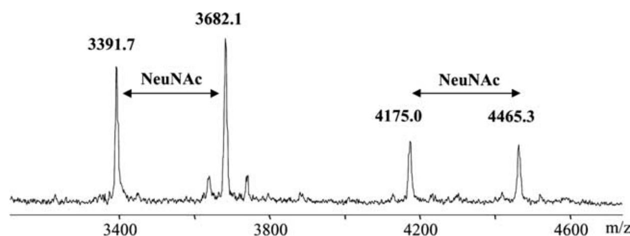

Fig. 3. MALDI-MS spectrum of fraction 18 in Fig. 1. The observed glycopeptides turned out to contain NeuNAc. The measurement was operated in the negative-ion mode.

3. Results and discussion

Nano-flow ESI-MS of intact transferrin predominantly gave the molecular mass (79 560) (data not shown), which was in agreement with the theoretical value calculated for the sum (79 556) of the protein moieties (75 144) and two oligosaccharide chains (complex bi-antennary type (2206 Da)×2). In addition, the spectrum indicated that the protein used was the fully glycosylated form with the above oligosaccharides at two potential sites, in which sialic acids were attached to the non-reducing termini. To confirm these glycosylation sites, carboxymethylated transferrin, treated with LEP, was subjected to RP-HPLC (Fig. 1). The peptides eluted were measured by both positive-ion and negative-ion mode MALDI-MS. As the result, the observed molecular masses could cover ca. 76% of the entire sequence including the two glycosylation sites (Fig. 2, Table 1). In addition, negative-ion MALDI-MS of fraction 18 revealed two glycopeptides having *m/z* 3682.1 and 4465.3 (Fig. 3). While the former value corresponds to one of the known glycopeptides, the latter could not be assigned to any peptide expected from the sequence. However, the fact that the ion at *m/z* 4465.3 was observed with a satellite ion at *m/z* 4175.0, difference in mass (290.3 Da) of which was also observed for the ion at *m/z* 3682.1, strongly suggested the presence of a sialic acid residue in this peptide, since the non-reducing terminal sialic acid in an oligosaccharide tends to be partially cleaved off, especially under conditions used in MALDI-MS measurements.

To determine the amino acid sequence and glycosylation site of the newly found glycopeptide, fraction 18, treated with PNGase F in a buffer containing 40 atom% $H_2{}^{18}O$, was subjected to RP-HPLC (Fig. 4(b)), which gave two new peak fractions 1 and 2 after the deglycosylation. MALDI-MS of

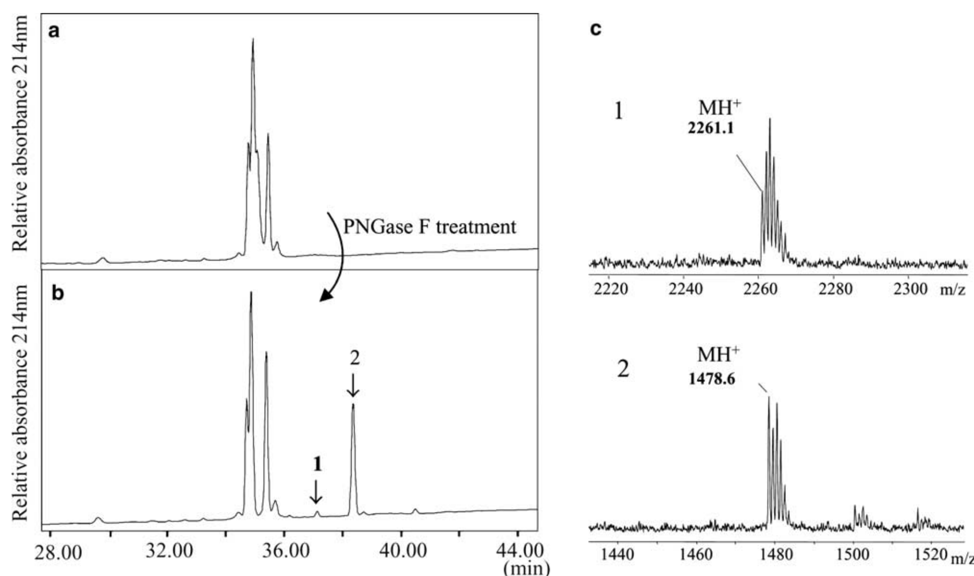

Fig. 4. Re-chromatograms of fraction 18 in Fig. 1 before (a) and after (b) PNGase F treatment, and MALDI-MS (c) of newly emerged fractions 1 and 2 in (b). Fraction 18 was treated with PNGase F in 40 atom% $H_2{}^{18}O$ (see Section 2). Isotopic ion distributions obtained for the fractions 1 and 2 in (b) indicate the partial incorporation of ^{18}O atoms into these two peptides (see Section 3).

these fractions gave signals at *m/z* 2261.1 and 1478.6 (Fig. 4(c)), respectively, both of which are deficient in mass by 2206 Da (corresponding to the residual mass of a sialylated complex-type bi-antennary oligosaccharide) from each of the original glycopeptides. ESI-MS/MS of these deglycosylated peptides revealed not only the amino acid sequences, Cys^{421}–Lys^{433} (MH^+:1478.6) and Ile^{490}–Lys^{508} (MH^+:2261.1) (upper panel of Fig. 5), but also the N-glycosylation sites at Asn^{432} and Asn^{491} in the sequence, based on the fact that these Asn residues were converted to Asp stamped with ^{18}O atoms at the β-carboxyl groups during the hydrolysis of the glycosidic bonds in the $H_2{}^{18}O$ buffer with PNGase F (see y″17 and y″18 ions, the latter of which contained the converted Asp residue and was observed as a doublet signal with a spacing of 2 u in the lower panel of Fig. 5) [14]. As a result, glycosylation at Asn^{432} could be confirmed to be one of the two known glycosyalation sites, whereas, Asn^{491}, located in the Asn-Xaa-Cys motif, a minor glycosylation site of a glycoprotein, was a novel glycosylation site, which has not been reported in human transferrin to date. Based on the absorbance at 214 nm and an amino acid analysis of fraction 1 in Fig. 4(b) in regard to the non-glycosylated form with the same sequence (Ile^{490}–Lys^{508}) eluting as fraction 22 in Fig. 2, the degree of the glycosylation at this Asn^{491} was estimated to be ca. 2 mol%.

Finally, in order to characterize the oligosaccharide structure attached to Asn^{491}, the glycopeptide observed at *m/z* 4465.3 in Fig. 3 was directly subjected to low-energy CID ESI-MS/MS, obtained from the quadruply-charged precursor ion $[M + 4H]^{4+}$ at *m/z* 1117.0. Fig. 6 shows the spectrum, as processed using MaxEnt3™ (see Section 2), based on the raw spectrum. The oxonium ions in the low mass region were useful for elucidating the outer non-reducing terminal structure involving a sialic acid. The ions observed in the high mass region, whose positive charges reside in the peptide

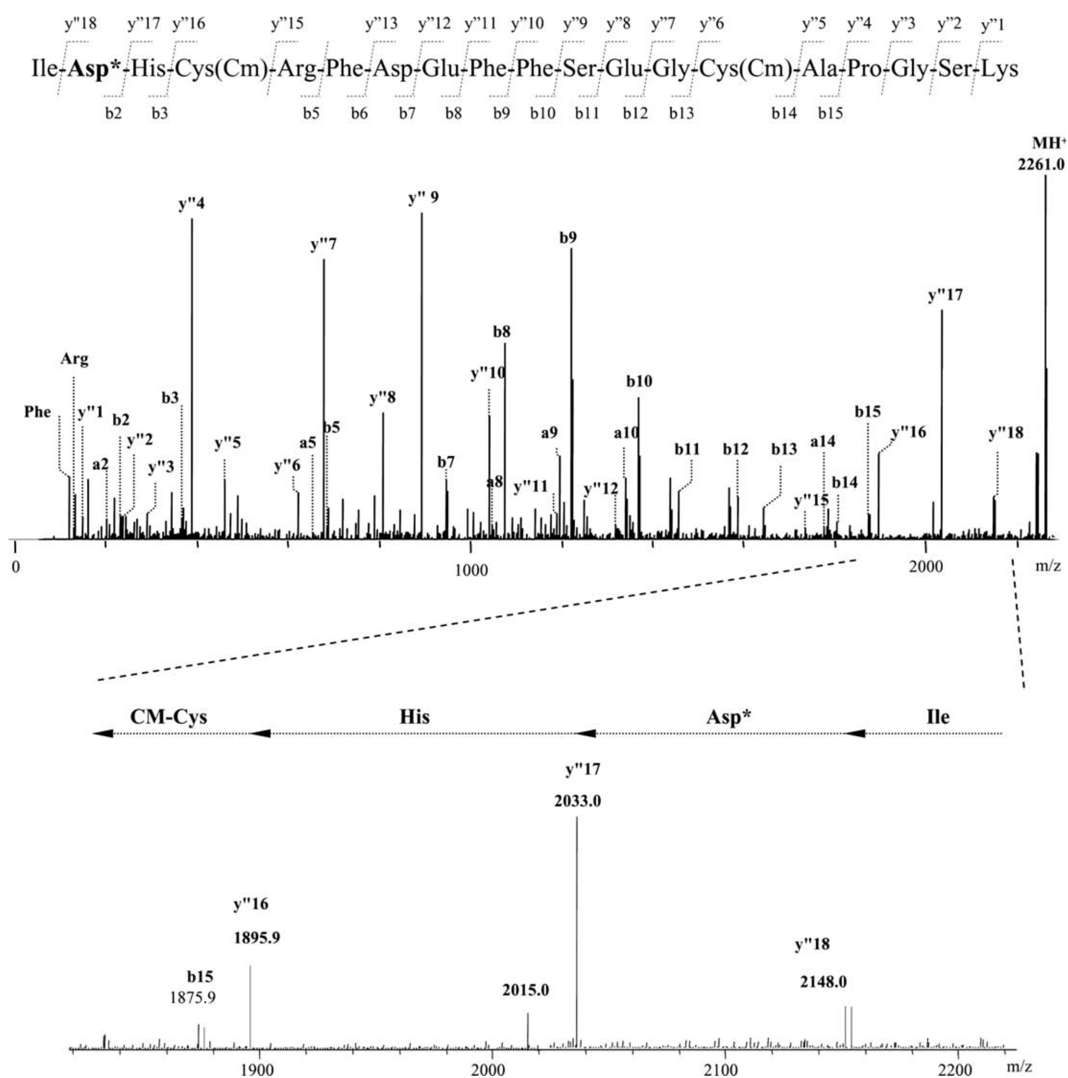

Fig. 5. Low-energy CID ESI-MS/MS spectrum from $[M + 3H]^{3+}$ at *m/z* 754.3 of a deglycosylated peptide of fraction 1 in Fig. 4b. Multiple-charged ions in the raw data were transformed to single-charged ones using MaxEnt3™ (see Section 2). The MS/MS spectrum was interpreted by "SeqMS", a software aid for de novo sequencing by MS/MS (http://www.protein.osaka-u.ac.jp/rcsfp/profiling) [16]. The sequence was constructed mainly based on b_n and y_n ions, where *n* denotes the arbitrary positions counted from the N and C terminus, which were produced by cleavage of peptide bonds upon low-energy CID. Amino acids in three-letters in the spectrum denote immonium ions. Asp* indicates an Asp residue partially labeled with ^{18}O at the β-carboxyl group. The arrows (←) show the sequences from the N terminus based on y_n ions. The nomenclature of these ions is in accordance with previous work [17].

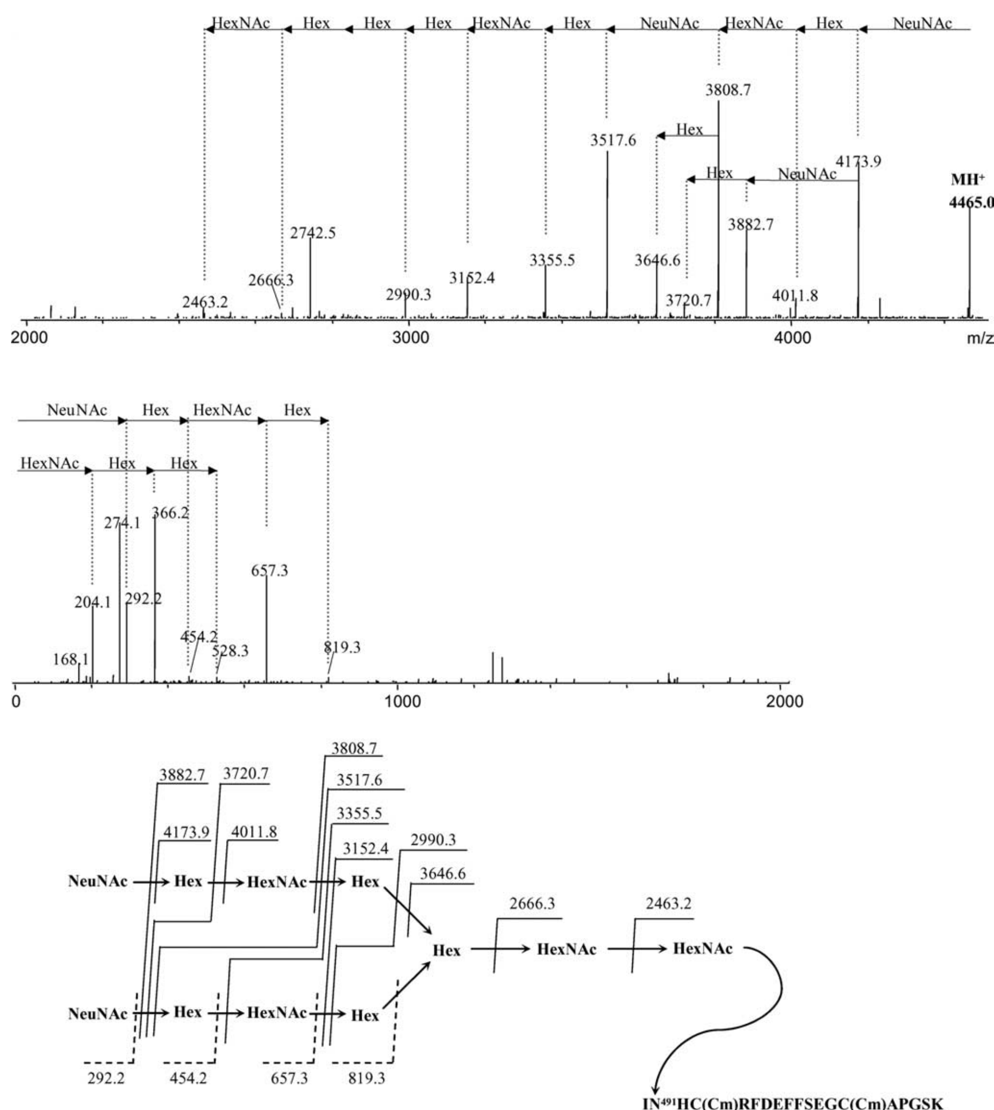

Fig. 6. Low-energy CID ESI-MS/MS spectrum from $[M + 4H]^{4+}$ at *m/z* 1117.0 for the glycopeptide of fraction 18 in Fig. 1. Multiple-charged ions in the raw data were transformed to single-charged ones using MaxEnt3™. The arrows (← and →) indicate the carbohydrate sequences from the non-reducing termini based on the ions produced upon the cleavage of glycosidic bonds (see Section 3). One of the possible assignments of the observed masses (monoisotopic) to the carbohydrate structure is depicted.

portion, arose from the cleavage of glycosidic bonds and readily revealed the relative alignment of the constituent sugars from the non-reducing termini. In addition, information on the degree of branching in the non-reducing termini could be obtained through observing the overlapped signals at *m/z* 4173.9 and 3882.7, and 4173.9 and 4011.8, the differences of which correspond to *N*-acetyl-neuraminic acid (NeuNAc) and a hexose, respectively, which resulted from the truncation of the non-reducing terminal residues from either of the two branches [15]. It is noteworthy that the low-energy CID of a glycopeptide, even with a high-molecular mass over 4 kDa, provides a simple fragmentation pattern that predominantly arises from the cleavage of glycosidic bonds and permits the direct structural analysis of the sugar moiety of a glycopeptide, while the fragmentation of the peptide portion is poor.

Since Bause and Legle [6] first demonstrated the Asn-Xaa-Cys motif for N-glycosylation by using several synthetic peptides in 1981, as far as we are aware, N-glycosylation in the Asn-Xaa-Cys motif, even including the case of the minor components in glycoproteins, has been found in only five types of glycoproteins [7–13]. The glycosylation in this motif of human transferrin, even as a minority, is suggestive of some functional role of this glycosylation in vivo but it might represent a side-product that is formed uniquely in human transferrin during its biosynthesis. It will be interesting to pursue this minor structural alteration in human blood, which might lead to an understanding of the physiological role of this minor glycosylation in transferrin. These studies are currently under way.

The glycopeptides were successfully analyzed by MALDI-MS and ESI-MS/MS, and provided relevant structural data on the sugar moieties present. The peptide portion, which retains a positive charge in a positive-ion-mode measurement, not only permits the highly sensitive detection of glycopeptides, with the actual sample consumption in an MS/MS study

at the sub-picomole level, but also provides a useful structural stamp of a sugar moiety upon low-energy CID albeit the peptide portion fragments poorly. In addition, MS/MS sequencing of a peptide labeled with ^{18}O at N-glycosylation sites arguably provides a powerful analytical method for the site-specific characterization of carbohydrates in a glycoprotein. The above MS-based techniques, used for the analysis of glycopeptides, permitted the detection and characterization of the minor glycosylation at Asn-Xaa-Cys motif in human transferrin.

Acknowledgements: This study was supported by a Grant-in-Aid for Creative Scientific Research (15GS0320 to T. T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a project on "Research on Proteomics" from the Ministry of Health, Labour and Welfare of Japan.

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