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Separation and characterization of the two Asn-linked glycosylation sites of chicken serum riboflavin-binding protein

Glycosylation differences despite similarity of primary structure

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Serum riboflavin-binding protein, a phosphoglycoprotein from the blood of laying hens, contains two Asn-Xaa-(Thr)Ser sequons in very similar but well-separated regions of amino acid sequence. In order to evaluate the effect of local amino acid sequence on the structure of the attached oligosaccharides, serum riboflavin-binding protein was purified to homogeneity, reduced and alkylated, digested with trypsin, and the two glycopeptides were separated by reversed-phase chromatography. After digestion with peptide-*N*-glycosidase F the released oligosaccharides were separated by high-pH anion-exchange chromatography and the oligosaccharide profiles of the two glycopeptides were compared. Although the two asparagine residues that are glycosylated are contained in pentapeptide segments in which four out of five amino acids are identical, the array of oligosaccharides present at each site show differences in both type and distribution. This suggests that local secondary or tertiary structure, or the order of glycosylation, influences the oligosaccharide structure more than does the primary structure flanking the attachment site.

INTRODUCTION

Riboflavin-binding protein (RfBP), a phosphoglycoprotein, is one of the many nutrient-binding proteins found in avian eggs [1,2]. It binds 1 mol of riboflavin with a dissociation constant of 1.28 nM at 25 °C and pH 7.0 [3], and is required for the transport of riboflavin to the developing oocyte [4,5]. RfBP is synthesized in both the liver and the oviduct during egg laying, and can be isolated from the blood (sRfBP) [6], egg yolk (yRfBP) [7], and egg white (wRfBP) [8]. While all are products of a single gene [5], each form is distinguishable from the others as a result of tissue-specific post-translational modifications.

RfBP is secreted as a single polypeptide chain of 219 amino acids [9,10], although yRfBP is 11 or 13 amino acids shorter at its *C*-terminal end due to limited proteolysis in egg yolk [11]. The protein is cross-linked by nine disulphide bonds [12], contains eight phosphoserine residues [13], and contains 14% carbohydrate by weight [14]. There are two potential asparagine glycosylation sites [15], and composition analysis indicates that both sequons possess complex-type carbohydrate structures.

Despite the fact that an Asn-Xaa-Thr/Ser sequence is required for attachment of N-linked oligosaccharides, glycoproteins which are synthesized in the same tissue often possess many different and sometimes mutually exclusive sets of carbohydrate structures (e.g. egg white proteins). This observation has led some to postulate that the polypeptide influences the structure of the attached oligosaccharide [16]. When the oligosaccharide structures of the four glycosylation sites of ovomucoid were examined, it was found that there was less branching (less processing) the closer the attachment site was to the *C*-terminus [17]. It has also been postulated that the order in which the protein encounters the oligosaccharide-processing enzymes, as it traverses the Golgi, determines the resulting carbohydrate structure(s) [18]. Perhaps the primary structure surrounding the oligosaccharide attachment site directs the protein to different compartments of the oligosaccharide-processing apparatus [19], and therefore is a determinant of the final array of oligosaccharide structures at each attachment site. Rather than the local primary structure, the three-dimensional environment at the attachment site may be the important determinant of oligosaccharide structure.

RfBP is a unique glycoprotein with which to test the effect of primary structure on glycosylation. It has two well-separated carbohydrate attachment sites which presumably encounter the same carbohydrate-processing enzymes. The amino acid sequences immediately adjacent to each of the carbohydrate attachment sites at asparagines 36 and 147 have more sequence identity than has been reported in eukaryotic glycoproteins: attachment site I, -Cys-Tyr-Ala-*Asn*-Phe-Thr-Glu-; attachment site II, -Met-Tyr-Ala-*Asn*-Gly-Thr-Asp-. This allows one to test if two similar neighbouring sequences on one protein affect oligosaccharide structure.

EXPERIMENTAL

Materials

The alkylating agent 4-vinylpyridine (4-VP) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Tos-Phe-CH_2Cl-treated trypsin was obtained from Worthington Biochemicals (Freehold, NT, U.S.A.). Tos-Lys-CH_2Cl, peptide-*N*-glycosidase F (PNGase F) and *Arthrobacter ureafaciens* neuraminidase were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). PNGase F was obtained in the glycerol-free form.

The 6 M-HCl for carbohydrate composition analysis was obtained from Applied Biosystems (Foster City, CA, U.S.A.). Trifluoroacetic acid (TFA) was obtained from Sigma (St. Louis, MO, U.S.A.). Polypropylene tubes (1.5 ml) for hydrolysis reactions were purchased from Sarstedt (Princeton, NJ, U.S.A.). A monosaccharide standard consisting of six monosaccharides,

Abbreviations used: RfBP, riboflavin-binding protein (the prefixes s, y and w denote serum, egg yolk and egg white respectively); 4-VP, 4-vinylpyridine; PNGase F, peptide-*N*-glycosidase F; TFA, trifluoroacetic acid; ddH_2O, distilled/deionized water; TBA, thiobarbituric acid; HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; tPA, tissue plasminogen activator.

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Table 1. Oligosaccharide structures

Oligosaccharide	Structure
Fibrinogen monosialylated oligosaccharide	Galβ1-4GlaNAcβ1-2Manα1-6 NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNacβ1-4GlcNAc
P105	NeuAcα2-6Galβ1-4GlcNacβ1-2Manα1-6 NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc
GP03	NeuAcα2-6Galβ1-4GlcNacβ1-2Manα1-6 NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc
FT07	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6 NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3 NeuAcα2-3Galβ1-4GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc

fucose (Fuc), galactosamine (GalN), glucosamine (GlcN), galactose (Gal), glucose (Glc) and mannose (Man), and an *N*-acetylneuraminic acid (NeuAc) standard were obtained from Dionex (Sunnyvale, CA, U.S.A.).

NaOH (50%, w/v) used to prepare all NaOH-containing eluents was obtained from Fisher (Pittsburgh, PA, U.S.A.). Acetonitrile for reversed-phase eluents was obtained from Burdick and Jackson (Muskegon, MI, U.S.A.).

Oligosaccharide standards PI05, GP03 and FT07 were obtained from Dionex. The monosialylated oligosaccharide from fibrinogen was a gift from Dr. R. Reid Townsend, University of California at San Francisco.

sRfBP purifications

sRfBP (186 mg) was purified from approx. 30 litres of plasma from laying hens by the method of Miller *et al.* [6], except that the 0.5 M-sodium citrate, pH 5.5, buffer used for blood collection contained NaF (0.1 mg/ml) to inhibit phosphatases, and the purification was performed at 4 °C with the exception of the final column step [20]. The progress of the purification was monitored using the Lowry protein assay [21] and the radioligand exchange assay for RfBP [22]. Purity was ascertained by both denaturing [23] and non-denaturing gels [24] electrophoresis.

Carbohydrate composition analysis

The monosaccharide composition of sRfBP was determined using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [25]. Samples for neutral sugar analysis were hydrolysed in 400 μl of 2 M-TFA. To determine the amino sugar content, samples were hydrolysed in 400 μl of 6 M-HCl. In both cases, hydrolysis was for 4 h at 100 °C in 1.5 ml polypropylene tubes. Samples for sialic acid analysis were hydrolysed in 400 μl of 0.1 M-HCl for 1 h at 80 °C. Following hydrolysis, all samples were immediately dried in a Speedvac (Savant Model SVC100) and then redissolved in 200 μl of deionized/distilled water (ddH_2O). All samples were analysed for their component monosaccharides using a Dionex Carbohydrate Analyzer equipped with a 0.4 cm × 25 cm CarboPac Pa1 analytical column (Dionex) and a CarboPac PA1 guard column (0.4 cm × 5 cm; Dionex), a pulsed amperometric detector and cell (Dionex PAD-II) outfitted with a gold working electrode, and an autosampler (Specta-Physics Model 8880) with a Tefzel rotor seal. The pulse potentials for the PAD-II were $E_1 = 0.05$ V, $t_1 = 480$ ms (range 2, position 5); $E_2 = 0.60$ V, $t_2 = 120$ ms (position 2); and $E_3 = -0.6$ V, $t_3 = 60$ ms (position 1). The time constant was set at 3 s. Both neutral sugar and amino sugar samples were separated isocratically with 16 mM-NaOH over 25 min. The separation was followed by a 10 min elution with 200 mM-NaOH and then a return to the starting conditions (16 mM-NaOH) for 15 min prior to the next injection [25]. This was all performed at a flow rate of 1.0 ml/min. NaOH eluents were all prepared from appropriate dilutions of 50% NaOH. Eluents containing sodium acetate were filtered through a 0.2 μm filter prior to use. The hydrolysates were quantified with the six-component monosaccharide standard. Samples for sialic acid analysis were eluted isocratically with 100 mM-NaOH/150 mM-sodium acetate and quantified using a NeuAc standard.

Separation and identification of carbohydrate attachment sites

sRfBP (7.5 mg) was reduced and alkylated with 4-VP [26]. The reduced and alkylated protein was digested with Tos-Phe-CH_2Cl-treated trypsin at an enzyme/substrate ratio of 1:100 in 0.1 M-ammonium bicarbonate, pH 8.5, for 1.5 h, with a second addition of enzyme at 1 h [27]. The digestion was terminated by addition of Tos-Lys-CH_2Cl to achieve a final concentration of 50 μg/ml. Approx. 5.6 μg of this digest was separated on either a Zorbax RP-300 C_{18} column (0.46 cm × 15 cm; Dionex) or a Zorbax Protein Plus column (0.46 cm × 25 cm; Rockland Technologies). The separation was accomplished in 60 min at a flow rate of 1.0 ml/min. The initial 5 min was isocratic at 5% eluent B (90% acetonitrile/0.1% TFA) and 95% eluent A (0.1% TFA). This was followed by a linear gradient of 5 to 52% B in the final 55 min. This separation was scaled up 10-fold and fractions were collected using a FOXY II fraction collector (ISCO) and immediately dried in the Speedvac. Each fraction was analysed for amino sugar content to identify the glycopeptides.

Sequence analysis

N-Terminal sequence analysis was performed on fractions which contained carbohydrate, in order to assign each glycopeptide in the amino acid sequence. A Porton Instruments (Tarzana, CA, U.S.A.) PI2090E Integrated Microsequencing System was used for the analysis. Approx. 50 pm of each peptide was sequenced after deposition on a peptide support (Porton). There was a 90% repetitive yield.

PNGase F digestion

The glycopeptides from the two N-linked carbohydrate attachment sites were collected from three scaled-up reversed-phase separations, dried and then dissolved in 100 μl of 50 mM-sodium phosphate buffer, pH 7.6, containing 10 mM-EDTA and 0.5 units of PNGase F. This solution was mixed and allowed to

incubate for 18 h in a 37 °C water bath. A 10 μl sample of a 10 mg/ml sRfBP tryptic peptide solution was dried and digested with PNGase F by the same protocol. This digest was applied to the Zorbax RP-300 C_{18} column under the conditions used to separate the total tryptic peptides, and the void volume peaks were collected and dried.

Arthrobacter ureafaciens neuraminidase digestion

A 120 μl sample of the remaining 150 μl of each PNGase F digestion was diluted with 120 μl of 0.1 M-sodium acetate at pH 5.0, and 10 μl of a 1 munit/μl solution of *Arthrobacter ureafaciens* neuraminidase (in 0.1 M-sodium acetate) was added. The samples were mixed and incubated at 37 °C for 24 h.

HPAEC-PAD oligosaccharide mapping

The PNGase F digestions were diluted to 200 μl with ddH_2O (the PNGase F digestion of the total sRfBP tryptic peptides was rehydrolysed in 200 μl of ddH_2O), and 50 μl was separated on a CarboPac-PA100 column (0.4 cm × 25 cm; Dionex) with a gradient of sodium acetate (50–250 mM in 60 min) and a constant concentration of NaOH (100 mM) after an initial 4 min at 50 mM-sodium acetate and a constant concentration of NaOH (100 mM). Oligosaccharides were detected with the gold electrode and a detector setting of 100 nA (full scale). Tentative identification of oligosaccharides (Table 1) was made using oligosaccharide standards. A 150 μl sample of each neuraminidase digestion was separated with the same gradient.

RESULTS AND DISCUSSION

sRfBP purification

sRfBP was purified 16000-fold from 30 litres of laying-hen plasma with a 25% yield [20]. A single protein band was observed after electrophoresis in both denaturing and non-denaturing gels.

Sugar analysis

Table 2 shows the Man, Gal, Fuc, GlcN, GalN and NeuAc contents of sRfBP. The amount of Man confirms the published result of two Asn-linked oligosaccharide attachment sites [15]. The value of 5.21 residues per mol (res/mol) was lower than the expected value of 6, but a low yield of Man is commonly observed with acid hydrolysis [25]. The amount of NeuAc observed was approx. 2.5 res/mol lower than that previously determined [5,9]. Previous determinations were made using the thiobarbituric acid (TBA) assay [28], which can form non-sialic-acid chromophores [29]. The presence of Fuc can result in a higher than normal reading [28]. When the TBA assay was used on our sample, the result was only one residue lower than the published values [5,9] when one uses a molecular mass of 30000 kDa ([6] used a molecular mass of 36 kDa). The distribution of mono- and di-sialylated oligosaccharides in our oligosaccharide separation suggests that the amount of NeuAc determined previously by the TBA assay was overestimated. A parallel HPAEC-PAD analysis of the NeuAc content of wRfBP yielded a value of 0.65 res/mol, which is lower than the published value of 1 res/mol. Because wRfBP does not contain Fuc, one can postulate that there are other non-sialic-acid chromophores produced when RfBP is heated in 0.5 M-H_2SO_4.

The presence of GalN was unexpected, because there has been no previous report of any RfBP containing GalN. The quantity suggests one O-linked oligosaccharide attachment site. We have located GalN in the reversed-phase map of the sRfBP tryptic peptides; this peptide contains Ser, to which a GalN could be attached. Though unlikely, it is possible that the peptide in question is converted to a compound during acid hydrolysis which elutes at the retention time of GalN and is electrochemically active. We did not find any GalN in wRfBP [20].

The amount of Fuc found was a bit higher than reported by Miller *et al.* [6], but this may be result of conducting the majority of the protein purification at 4 °C, which could minimize the loss of Fuc. Hamazume *et al.* [9] did not report any Fuc in sRfBP. The amount of Gal and GlcN is comparable to that in previous reports.

Table 2. Carbohydrate composition of sRfBP

Sugar	Content (res/mole)
Fuc	1.72±0.45
GalN	0.86±0.09
GlcN	12.75±0.65
Gal	6.75±0.14
Man	5.21±0.25
NeuAc	2.57±0.38

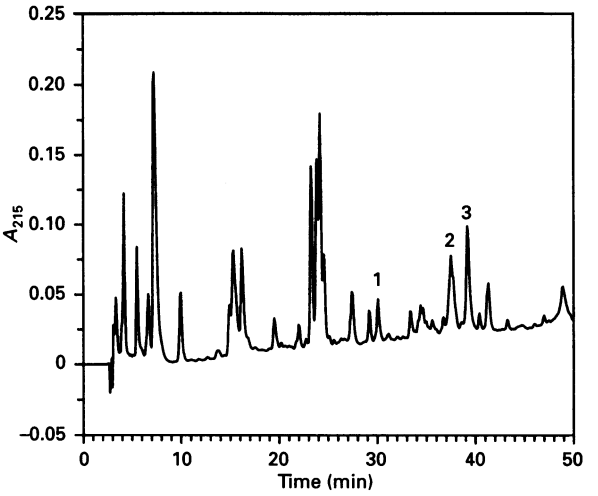

Fig 1. Reversed-phase separation of sRfBP tryptic peptides on Zorbax Protein Plus column

See the Experimental section for details

Glycopeptide analysis

The tryptic peptides of sRfBP were separated by reversed-phase chromatography (Fig. 1) and collected for amino sugar analysis. Peaks 2 and 3 were found to contain GlcN, and therefore were believed to represent the two N-linked attachment sites of sRfBP. Peak 1 is the tryptic peptide whose hydrosylate is found to contain a peak eluting with the retention time of GalN. *N*-terminal sequencing confirmed that peaks 2 and 3 were the N-linked glycopeptides. Peak 2 was the peptide containing Asn-147, and peak 3 was the peptide containing Asn-36. The primary structures of each glycopeptide are presented in Fig. 2.

Each glycopeptide was collected by reversed-phase chromatography, dried and then digested with PNGase F. Upon completion of digestion, each sample was diluted to lower the concentration of phosphate ions. We have found that phosphate

36
-PSPEPNMHECTLYSESSCCYANFTEQLAHSPIIK-

147
-CVPYSEMYANGTDMCQSMWGESFK-

Fig 2. sRfBP glycopeptides

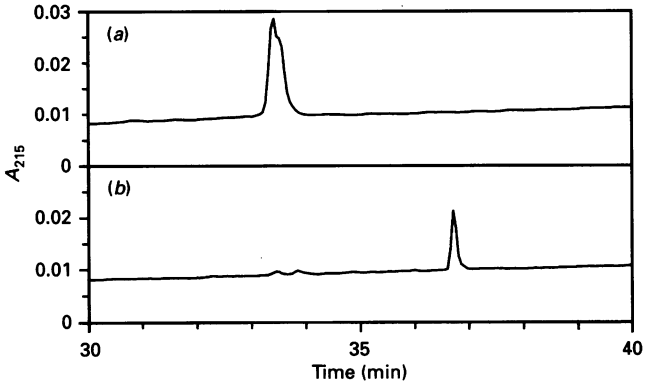

Fig 3. Reversed-phase separation of the sRfBP tryptic glycopeptide containing Asn-147, before (*a*) and after (*b*) PNGase F digestion

Separation was carried out using a Zorbax C_{18} 58-300 column (see the Experimental section).

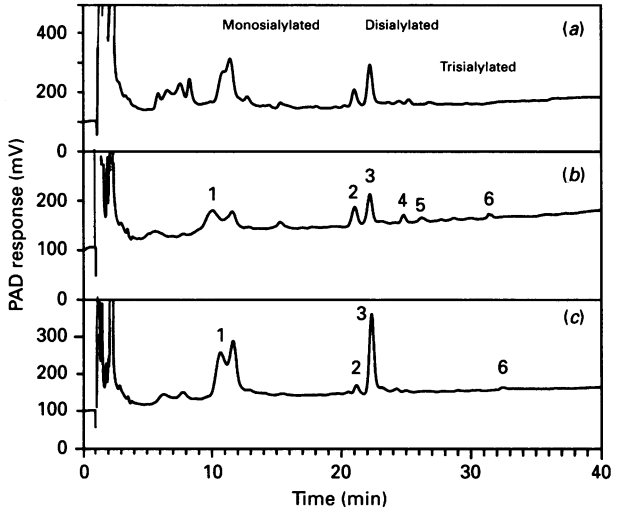

Fig 4. Oligosaccharide maps of total sRfBP oligosaccharides (*a*), Asn-147 attachment site oligosaccharides (*b*) and Asn-36 attachment site oligosaccharides (*c*)

The numbered peaks are discussed in the text.

ions limit the resolution of other oligosaccharides on the CarboPac PA100 column. A 10 μl sample of the PNGase F digest (from a total of 200 μl) was analysed by reversed-phase chromatography using the same elution program employed to separate the sRfBP tryptic peptides (Fig. 3) Comparison with an aliquot removed prior to digestion (Fig. 3*a*) demonstrates that the release of oligosaccharide caused the peptide to be retained longer (Fig. 3*b*) and decreased the peak width significantly. Using this technique we were able to assay the extent of oligosaccharide release from each of the two N-linked glycopeptides. For both sites, complete (> 95%) release of oligosaccharides were obtained (data from the Asn-36 site not shown).

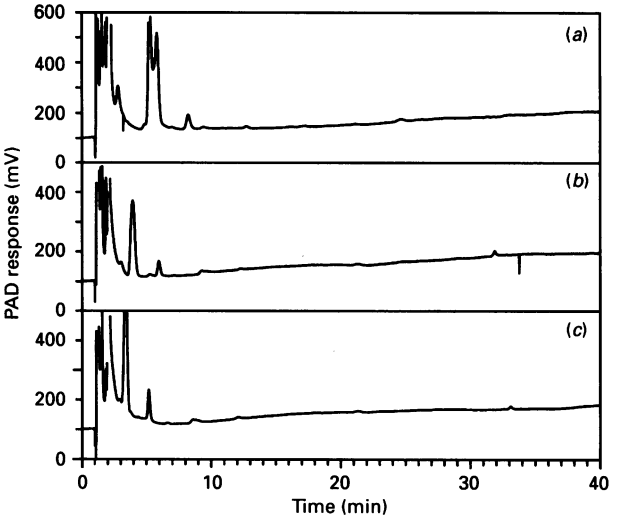

Fig 5. Oligosaccharide maps of total sRfBP oligosaccharides (*a*), Asn-147 attachment site oligosaccharides (*b*) and Asn-36 attachment site oligosaccharides (*c*) after *Arthrobacter ureafaciens* neuraminidase digestion

A 50 μl sample of the PNGase F digest was used to analyse the oligosaccharides from each site. Because HPAEC separates oligosaccharides according to charge, linkage and branching configuration (for review, see reference [30]), the detailed structures of the oligosaccharides at the two attachment sites could be compared. We found that both sites contained peaks in regions of the chromatogram where monosialylated, disialylated and trisialylated oligosaccharides were eluted, as judged from the retention times of oligosaccharide standards, but the distribution of oligosaccharides at the two sites was very different (see Fig. 4). A comparison of the three chromatograms in Fig. 4 and an enzyme digest without substrate (results not shown) suggests that the peaks at 2 min represent neutral oligosaccharides. Oligosaccharide standards were used to suggest the types of oligosaccharide structures that might be present at the two carbohydrate attachment sites of sRfBP. Peak 1 in Fig. 4, which occurs at both attachment sites, has the same retention time as the monosialylated biantennary structure found in fibrinogen (see Table 1). Peak 2, found at both attachment sites but predominantly at Asn-147, has the same retention time as a monofucosylated disialylated biantennary oligosaccharide (GP03). Carbohydrate compositional analysis of the two glycopeptides indicates that both contain fucose. The same analysis revealed that the two attachment sites have an identical GlcNAc/Man ratio. Peak 3 is also found at both attachment sites, but is present in great abundance at Asn-36 and has the same retention time as a disialylated biantennary oligosaccharide (PI05). Finally, both attachment sites contain a small amount of oligosaccharide (peak 6) with the same retention time as a trisialylated triantennary oligosaccharide isolated from bovine fetuin (FT07). This peak did not disappear when the total oligosaccharides from each glycopeptide were digested with *Arthrobacter ureafaciens* neuraminidase, and therefore we do not believe that it is a trisialylated oligosaccharide. The disappearance of the peaks thought to be mono- and di-sialylated oligosaccharides confirmed that those peaks were sialylated (Fig. 5). The PNGase F digestion of the total sRfBP tryptic peptides required a clean-up with the reversed-phase column, because of peaks in the mono- and disialylated regions of the chromatogram which were not susceptible to the neuraminidase digestion. The peaks in the void volume of the reversed-phase column (approx. 2–4 min) were collected and dried. The amount of the void volume to be

collected was determined after inspection of the chromatograms of the total tryptic peptides with and without PNGase F treatment. This separation successfully removed the neuraminidase-resident peaks and the peak originally thought to be a trisialylated oligosaccharide. Unfortunately, it appears that the reversed-phase clean-up may have added new peaks in the 5–10 min region of the chromatogram (see Fig. 4). Some or all of these new peaks are also neuraminidase-resistant (see Fig. 4 and 5). The reversed-phase clean-up was chosen because the neuraminidase-resistant peaks were hypothesized to be electrochemically active peptides.

Overall, the oligosaccharide distribution of the two sites differed in their ratios of peaks 2 and 3, as well as in the higher abundance of peaks 4 and 5 at Asn-147. The carbohydrate compositional analysis of sRfBP suggests that peaks 4 and 5 may be more highly branched disialylated structures, including bisected structures. This is supported by the oligosaccharide structures of wRfBP determined by Piskarev *et al.* [31]. They found tetra-antennary as well as triantennary complex-type oligosaccharide structures. We were unable to find published HPAEC-PAD separations of highly branched mono- and disialylated oligosaccharide structures containing a bisected GlcNAc to substantiate this hypothesis. In an earlier analysis, based on the carbohydrate composition of each of the oligosaccharide attachment sites of sRfBP, we were unable to conclude that there were any differences in oligosaccharide structure [20].

Site-specific oligosaccharide structures on multiple-glycosylated proteins

Increased interest in glycoproteins as protein pharmaceuticals along with advances in analytical capability has led to more detailed analyses of site-specific glycosylation in a number of glycoproteins. For example, the glycan distributions at the three N-linked attachment sites of tissue plasminogen activator (tPA) [32–35] and the two N-linked sites of CD4 [36,37] have been well characterized. For both proteins the glycan distribution at each site is different and, in the case of tPA, important for the enzyme's specific activity [38]. Despite the increase in the amount of site-specific glycosylation data, the determinants of glycosylation differences between attachment sites, on the same protein as well as on different proteins, are unknown. A number of studies [17,19,39–41] suggest that the protein matrix surrounding the oligosaccharide attachment site affects the processing of N-linked glycans. The effect of the protein matrix could reside in the immediate primary structure surrounding the glycosylation site or in the entire three-dimensional structure of the attachment site region of the protein [17]. Attachment sites which are inaccessible to the enzyme endoglycosidase H are not processed to complex-type structures in some higher eukaryotes [39,40], or to higher molecular mass polymannose structures in yeast [41–43]. This suggests that accessibility of the attachment site (defined by endoglycosidase H accessibility), which is the result of the three-dimensional structure of the protein, is important with regard to the extent of glycan processing at a given attachment site. Model studies implicate protein in both short- and long-range effects of glycan processing [19]. Dahms & Hart [44] have demonstrated that a protein's quaternary structure can affect its glycosylation.

The Asn-Xaa-(Thr)Ser sequon is required but not sufficient for N-linked glycosylation. An *in vitro* study of 13 unglycosylated proteins containing potential acceptor sequences found that six of the thirteen could be glycosylated after reduction and alkylation [45]. Changes in the alkylating agent, with one exception, did not allow the remaining seven proteins to be glycosylated. After treating two of the seven with cyanogen bromide, the two proteins were glycosylated *in vitro*. This suggests that the denatured protein contained some domains that prevented the interaction of the oligosaccharide transferase with the acceptor site. Peptides of varying length from lactalbumin containing the acceptor sequence could be glycosylated *in vitro*, except for a hexapeptide in which the Asn was at the *N*-terminus [46]. Studies with small peptides from RNAase A digestion and synthetic peptides revealed that the tripeptide Asn-Xaa-(Thr)Ser was sufficient for glycosylation providing that the *N*-terminus of the Asn residue is blocked with an acetyl group and the *C*-terminus is blocked by the formation of an amide [47]. Altogether, these studies suggest that both the tertiary structure and the secondary structure of the protein determine whether the Asn-Xaa-(Thr)Ser is glycosylated.

sRfBP allowed us to investigate the effect of the immediate structure surrounding the attachment site. Four of the five amino acids centred around the Asn of each of the two attachment sites are identical. The flanking amino acid on the *C*-terminal side is a conservative replacement (Asp to Glu). A search of the GenBank database for sequence similarities [48] for each of the five amino acid sequences yielded only one 100% match (the Tyr-Ala-Asn-Gly-Thr sequence of the Asn-147 attachment site is found in bacteriophage T2 receptor-recognizing protein). Therefore, with the exception of a single prokaryotic sequence, the two sRfBP carbohydrate attachment site pentapeptides are more similar to each other than to any other known sequence. We have shown that, despite some similarity in the type of oligosaccharide present at each site, the distribution is different. This suggests that the protein tertiary structure is an important determinant of glycan distribution, even in a protein containing two carbohydrate attachment sites with nearly identical local primary structure. It will be of interest to determine if similar differences are found when the protein is synthesized in a different tissue, as is the case with wRfBP.

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