Conformation of Glycopeptides

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Abstract: The presence of carbohydrate side-chains in native glycoproteins alters a number of biochemical properties of the peptide backbone. One of the most frequently studied questions is the conformation-modifying effect of sugar incorporation into asparagine, serine and threonine residues. When N-glycosylation modifies the conformation, the resulting structures are more ordered than the peptide chain without sugar addition. For O-glycopeptides the final conformations can be either more ordered or less ordered. In any event, only the innermost carbohydrates make contact with the peptide backbone. Through-space structural changes are mostly found downstream of the O-glycosylation site. In the repeat unit of epithelial mucin-1 protein, clustering of the carbohydrates results in an easily observable stabilization of the poly-proline II helix.

Key Words: Glycoproteins, N-glycosylation, O-glycosylation, mucin, secondary structure.

INTRODUCTION

A thorough review of the 75,000 protein sequences deposited to the SWISS-PROT database by 1998 indicates that approximately half of all known proteins are glycosylated [1]. Glycoproteins come in all colors and sizes in respect to both the polypeptide chain and the sugar component(s). While the sugar heterogeneity on native glycoproteins usually prevents detailed structural analysis, glycopeptide models corresponding to full-sized natural products or fragments of glycoproteins offer remarkable tools to investigate the role of carbohydrate side-chains attached to the peptide backbone in a wide range of biological and biochemical processes. Sugar addition appears to alter many properties of the peptide backbone from direct effects on the biological functions to stabilization against proteases in various environments. Based on the positive effects glycosylation makes on some properties of synthetic peptides, the use of carbohydrate addition is repeatedly suggested for manipulating various less than optimal features of peptide drug leads. We have reached a point when the combination of recent developments in carbohydrate and peptide chemistry allows the preparation of highly complex glycopeptides, ready to deepen our understanding of the principles of bioorganic and natural products chemistry as well as provide new tools for peptide-based drug design.

Regardless of the rationale for the production of glycopeptides, the first question is almost always how glycosylation affects the secondary-tertiary structure of the peptide backbone. In fact, many glycopeptides are made for no other reason than to study the conformation of the products. In general biochemistry terms the conformation-modifying effect of glycosylation is exciting in its own right; for drug design this knowledge is a must to retain the receptor-binding ability of the leads, or conversely, to modify receptor selectivity through carbohydrate-induced conformational changes. This review attempts to summarize the newest results, in light of earlier theories, on glycopeptide conformation. The subject has been repeatedly reviewed [e.g., 2-5], although the structural aspects were only mentioned in the context of other glycopeptide functions. Recently an excellent summary of the conformational preferences of oligosaccharides was published with detailed analysis of the secondary structure of T-cell antigenic glycopeptides [6]. At this point we have to establish the ground rules. To represent native glycoprotein fragments, here we are looking at 'true' glycopeptides, meaning mono- or oligosaccharides attached to serines (Ser), threonines (Thr) or asparagines (Asn) via glycosydic bonds in α or β anomeric configuration. This categorization excludes C-glycosides, sugar-oximes, or vancomycin-type glycopeptide antibiotics that do not feature natural glycosylation patterns. While N-acetyl-glucosamine (GlcNAc) and N-acetyl-galactosamine (GalNAc) are those moieties in glycoproteins that are conjugated to the peptide chain, glycoproteins are also rich in glucose (Glc), galactose (Gal) mannose (Man) and fucose (Fuc), and therefore these sugar models are included here. According to the other convention of this review, the term 'conformation' is used to define the shape of the peptides as identified by direct and physical measurements of the general fold or specific amino acid backbone or side-chain distances. Structural changes due to glycosylation if they are inferred from altered immunological, or other functional studies, are not considered. This distinction has to be made to allow direct comparison of all strictly related published data. As popular as the subject is, the existing reports are equally as controversial. We attempt to provide a representative, and fair albeit not exhaustive overview of this fascinating subject.

EARLY STUDIES: DIRECT MEASUREMENT OF GLYCOPEPTIDE STRUCTURE

Detailed glycopeptide conformational studies were started at the late eighties. In a pioneering paper, the secondary structure of protected dipeptides decorated with monosaccharides was investigated by circular dichroism (CD) and infrared (IR) spectroscopies [7]. Glycosylation was found to increase the magnitude of the CD bands,

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characteristic of the type II beta-turns, and this finding was interpreted as an indication of the stabilization of the folded backbone conformation. Based on IR data, five-, seven-, or ten-membered glyco-turns were proposed to play an important role in fixing the steric orientation of the carbohydrate antennae systems in glycoproteins. Later this hypothesis was frequently debated. By using mid-sized N-glycopeptide models corresponding to a rabies-virus derived peptide antigen carrying either hydroxyl- or acetamido groups at C2, we could not verify the presence of a special H-bond system, proposed to originate from the acetamido group of sugars at C2 and the hydroxy amino acids two residues downstream [8]. The natural pentasaccharide core antennae increases the local nuclear magnetic resonance (NMR) correlation times of amino acid residues near the glycosylation site of a hen ovomucoid glycopeptide, but the pentasaccharide does not dramatically affect the average conformation of either the peptide backbone or the Asn side-chain [9]. Similar observations were made on an N-glycosylated peptide corresponding to the nicotinic acetylcholine receptor, although this peptide carries the sugar at a position close to the C-terminus [10], where no major conformational changes are expected after glycosylation [11]. The lack of special H-bonds was also observed for an O-glycosylated somatostatin analogue [12]. When structural changes were detected, these were explained on the basis that the presence of the sugar sterically excludes many possible conformations otherwise available for the peptide backbone. Finally the question was asked whether the identity of the specific sugar matters at all if the general carbohydrate size and shape are similar [12]. The effects of a naturally occurring Thr-linked fucose moiety on the structure, dynamics and stability of a proteinase inhibitor were investigated [13]. A comparison of the NMR data and the structure of the fucosylated inhibitor with those of the non-fucosylated form shows that conformational changes occur only in the vicinity of the fucose moiety. Nevertheless, a comparative analysis of the NMR exchange rates of the amide protons indicates that fucosylation is responsible for an overall decrease of the dynamic fluctuations of the molecule. We noted the lack of major conformational changes after incorporation of a disaccharide into the antibacterial peptide drosocin, and correlated the peptide's flexibility with the induced fit of glycosylated drosocin when the native peptide binds to its target molecule [14,15]. We will return to the drosocin example later.

The next question to study was how the length of the carbohydrate moiety affects the secondary structure of O-linked glycopeptides. It was found that elongation of the carbohydrate from mono- to disaccharide results in markedly different turn conformations [16]. We confirmed that major alterations in the secondary structure following elongation of a medium-sized peptide chain occur only when the starting peptide has little conformational preference [17]. Remarkably, α- or β-glycosylated versions of the same peptide exhibit very similar CD spectra. In contrast to the turn-like peptides, when the initial peptide conformation is more stable the differences between monosaccharide- and disaccharide-containing peptide conformations are negligible. Incorporation of a monosaccharide destabilized the helical conformation of another 15-mer rabies-derived peptide, an effect, that was more pronounced following elongation of the sugar, but without generating new structures. This situation is markedly similar to that of the 12-mer N-glycosylated rabies related helical peptide we mentioned earlier [8]. In summary, these early studies indicated that the conformation-modifying effect of incorporating different sugar structures depends upon both the length of the sugar and the initial secondary structure of the peptide. Since the carbohydrate antennae in natural glycoproteins are often longer than the mono- or disaccharides most frequently used in the above conformational studies, a comparative analysis of secondary structure of unmodified peptides and peptides decorated with long sugars was long overdue.

CONFORMATIONAL CHANGES AFTER GLYCOSYLATION: EFFECTS ON PEPTIDE/PROTEIN PROCESSING

Glycosylation, as a post-translational modification, may affect the three dimensional structure of the nascent proteins by altering the processing of the growing polypeptide chain. Time-resolved fluorescence energy transfer was used to assess the impact of glycosylation on the conformational dynamics of flexible glycopeptides [18]. The results demonstrate that glycosylation causes the modified peptides to adopt a different ensemble of conformations. For some peptides, this change may lead to structures that are more compact and which better approximate those of the peptides in the final folded protein. Glycosylation seems able to trigger the timely formation of structural nucleation elements and thus assist in the complex process of protein folding [18]. The same authors documented that glycosylation affects the *cis/trans* amide isomer ratio of proline (Pro) near an N-glycosylation site, and alters intramolecular disulfide formation of neighboring cysteine (Cys) residues, shifting the equilibrium in favor of the disulfide [10]. Incidentally, O-glycosylated major histocompatibility complex (MHC) protein class I epitopic peptides and their non-glycosylated analogues show two distinct conformations in solution as a result of *cis-trans* isomerization about a tyrosine (Tyr)-Pro amide bond [19]. The thermodynamically preferred oxidation of Cys around N-glycosylation sites is somewhat in contrast with our experience. We found that Asn-linked glycosylation inhibits intermolecular disulfide bridge formation of proximal Cys residues in a carbohydrate length-dependent manner [11]. Rickert and Imperiali [10] explained our results as due to the process being kinetically controlled. When a disaccharide was "walked" through a peptide substrate of subtilisin Carlsberg the rate of enzymatic hydrolysis was extremely dependent upon the site of glycosylation relative to the scissile bond, pointing toward a possible new effect of glycosylation of proteins, i.e., as specific cleavage signals for enzymes [20]. In our experience, the increased protease resistance of peptides after glycosylation could be correlated with spectroscopically determined conformational changes upon sugar incorporation [21].

COMPUTATIONAL APPROACHES UNTIL NOW

When structural or distance constraints are not available from biophysical measurements, calculations can provide an estimate of glycopeptide conformation. In addition,

computer modeling can verify the experimentally determined interatomic distances. In one of the most theoretical approaches, the dependence between the anomeric carbon chemical shift and the glycosydic bond dihedral angles in glycopeptide model compounds was studied by Gauge-Inducing Atomic Orbital *ab initio* calculations [22]. Similar surfaces were obtained for Ser(GlcNAc) and Thr(GlcNAc) model glycopeptides in α and β configurations. However, sterically induced polarization by the methyl group appears to lower the chemical shift of the anomeric carbon for Thr-containing glycopeptides compared with similar analogs of the Ser series. When larger glycopeptides were studied, the energy-minimized molecular model of a 23-residue-long mucin-7 (MUC-7) fragment, decorated with a Gal-GalNAc disaccharide, revealed that the preferred helix conformation in aqueous medium is stabilized by the hydrogen-bonded salt bridge between the C3 hydroxyl of the outer Gal moiety and the lysine (Lys) amino side-chain 7 residues downstream of the sugar-bearing Ser residue [23]. It needs to be mentioned that the low intensity of the CD curve of the glycopeptide resembles to that of 3_{10}-helices or repeated turns [24] more than α-helical structures.

Even further increasing the size of the polyamides, N-glycosylated variants of the prion protein (PrP) were investigated by molecular mechanics calculations. So far the NMR structures of only recombinant, non-glycosylated prions are known. Homogeneously glycosylated proteins lacking the secondary structural effect of sugar incorporation were estimated only by computer modeling. According to these studies, glycosylation is likely to play an important role in prion conformation and biology [25]. A major mutation in PrP, suggested to be involved in the pathogenic transformation of the protein, is an Asp178→Asn residue change, near Asn181, one of two potential N-glycosylation sites. This mutation removes the salt bridge between conserved residues Asp178 and Arg164 that hold a β-sheet against helix 2, thus causing thermodynamic instability of the non-glycosylated protein [26]. According to molecular dynamics simulations, the concomitantly removed hydrogen-bonds are partially restored after glycosylation. These results are in agreement with experimental results on the instability of mutant PrP [27]. In another glycoprotein model, the presence of the bulky glycan chain on Asn86 of the class I MHC protein does not affect the average backbone fold, but induces local changes in protein structure and dynamics [28]. The major structural changes are in the solvent exposed loop regions between residues 38 and 51 as well as 85 and 93. Remarkably, there are a number of hydrogen bonds detected between protein atoms and the sugar. This is one of the best examples of direct interaction between peptide and carbohydrate, a constant point of debate in glycopeptide conformational studies. According to another interesting finding of this report, while the carbohydrate located near the substrate-binding groove does not have an influence on direct ligand (peptide epitope) binding, it can provide additional support to stabilize the residues in the protein involved in the binding process.

Our group also looked at the effect of glycosylation on MHC-peptide binding, but in this case the peptide epitopes carried the sugar side-chains [29]. Our goal was to design T-cell agonist and antagonist glycopeptides by modulating the interaction surfaces with MHC or the T-cell receptor. In our models corresponding to rabies virus proteins, the natural α-linked O-glycosylation with mono- and disaccharides does not change the peptide backbone positions when bound to MHC. In contrast, β-linked N-glycosylation shifts the peptide in the MHC groove, owing to altered anomeric carbon orientation. The MHC-binding of the Asn-linked glycopeptides are less favorable than that of the Ser- or Thr-linked analogs due to steric and culombic conflicts. Finally, we tried to interpret CD and NMR data on the conformation of the C-terminal pentapeptide of Peptide T, glycosylated in the mid-chain Asn, by molecular modeling calculations [30]. Peptide T, a fragment of gp120 of the human immunodeficiency virus (HIV)-1 California isolate, carries determinants for chemotactic activity [31]. The incorporated GlcNAc moiety changes the ensemble backbone conformation of the peptide and limits the conformational space available to the peptide. An interesting effect of glycosylation, perhaps suitable to explore in receptor agonist or antagonist design, is the transformation of type I or type III β-turn structures into type II turns. This finding is in good agreement with experimental data on type II β-turn stabilization after sugar addition [7]. Together with the reduced degradation rate of glycopeptides in mammalian sera [21] glycosylation may be a viable alternative of the frequently utilized strategies aimed at creating diversity in peptide libraries [30].

N-GLYCOPEPTIDES

NMR chemical shift and nuclear Overhauser enhancement (NOE) data could not verify the secondary structure modifying effect of peptide T glycosylation as detected by CD or molecular modeling as described above [32]. Apparently, the sugar-peptide interactions in peptide T are transient in nature. If a shift in the turn structure occurs at the borderline of type I (III) – type II conformational transition, this may be picked up by CD and modeling because the signal arising from these methods are unique to the given turn type and the timescale of data acquisition is faster than that for NMR. In general, while CD is more sensitive to the changes in the general fold of the peptides after glycosylation, only NMR can provide exact information on interatomic distances and alterations thereof.

Six additional reports suggest that N-glycosylation changes the secondary structure of peptides while four papers claim the contrary. At least the six positive reports all agree that glycosylation results in a more ordered backbone conformation. Asn is a strong turn-forming amino acid residue [33], and perhaps the controversies arise from our inability to detect minor changes in the turn potential or geometry due to the already well-developed turn character of the peptides. The turn at the 243-246 fragment, located centrally in a 21-mer peptide corresponding to the human T-lymphotropic virus type 1, was not significantly altered by single GlcNAc addition [34]. Of course, the most stabilized turns are found in cyclic peptides. These are the structures for which we expect the least conformation-modifying effect of glycosylation. The bicyclic hexapeptide nepadutant, a selective tachykinin NK2 antagonist [35], is glycosylated on its single Asn residue. The Asn is located in a type II β-turn environment, and in this already stable turn system no

sugar-peptide NOE contacts are reported and therefore the carbohydrate apparently does not appear to influence the backbone conformation [36].

Returning to HIV gp120, the potential N-glycosylation site Asn at the amino-terminus of a 24-mer peptide representing the tip of the V3 loop segment was decorated with a β-linked GlcNAc moiety [37]. Neither CD nor NMR nor unrestricted molecular modeling calculations detected any conformational alteration upon carbohydrate incorporation. In contrast, concomitant O-glycosylation of Ser6 and Thr19 resulted in a total of 29 NOE crosspeaks between carbohydrate protons and the peptide backbone. Compared to the N-linked glycopeptide, a higher proportion of β-turn containing structures were sampled around the central glycine-proline-glycine-arginine (Gly-Pro-Gly-Arg) tetrapeptide segment based on the analysis of the simulation trajectories of the di-O-glycosylated analog. Considering the short sugar and the terminal position of carbohydrate incorporation (where the peptide structures are generally fraying) there is nothing surprising in the lack of conformational changes upon N-glycosylation. It needs to be mentioned that in this case CD did not support the NMR- and molecular mechanics-based turn stabilization of di-O-glycosylation. At least CD and NMR agree in that N-glycosylation of eel calcitonin does not change the three-dimensional structure, in spite of remarkable, and sugar-length dependent modifications in the hypocalcemic activity of the peptide [38]. NOE crosspeaks were also detected between the acetamido methyl group and the peptide backbone of a model glycopentapeptide, although the biological relevance of this finding is questionable as these contacts were manifested only at -12°C [39].

Asn298 of the serine proteinase inhibitor hen egg ovalbumin is glycosylated and the glycoprotein's X-ray crystal coordinates are available [40]. A 297-301 pentapeptide fragment was synthesized and conjugated to a mannose-rich heptasaccharide moiety via the native β-linked GlcNAc unit [41]. NMR- and molecular modeling-based structures of the isolated glycopeptide were comparable with the same fragment in the full protein. A remarkable aspect of the calculations is the apparent stabilization of peptide conformation by glycosylation. This effect is mutual: this relatively short peptide is able to alter the ensemble of the structures sampled by the oligosaccharide. The proximity of the GlcNAc moiety to the peptide indicates that the possible interaction involves the acetamido group. This notion is supported by glycopeptide models corresponding to the hemagglutinin of influenza virus [42]. Modification of the hemagglutinin peptide with carbohydrates induces a β-turn structure similar to that found in the native protein. Replacement of the C2 and C2' N-acetyl groups with hydroxyls results in a less ordered peptide conformation. It was concluded that the N-acetyl groups on the sugars promote the compact turn structures through carbohydrate-peptide steric interactions.

To further investigate the influence of individual monosaccharide moieties on the structure and function of glycoproteins, the conformation of wild-type glycosylated immunoglobulin (IgG)-Fc was compared with four glycoforms bearing truncated oligosaccharides [43]. Upon oligosaccharide truncation, the only structural changes detected involved the C'E loop located near the glycosylation site. In agreement with the reports from the previous paragraph, removal of the terminal GlcNAc as well as mannose moieties resulted in the largest conformational changes in both the carbohydrate and the polypeptide components. In a newer study on the conformation of glycosylated pentadecapeptide models of the nicotinic acetylcholine receptor, the effect of sugar length was revisited [44]. Although inspection of the NOE data revealed no major structural changes between the glycosylated and non-glycosylated analogs, some longer-sugar bearing glycopeptides exhibited modest, but detectable conformational alterations. These should involve the carbohydrates located near the peptide backbone, as the outermost sugars did not have an impact on either the solution structure of the peptide or the rate of disulfide bridge formation. The structure-stabilizing role of long carbohydrates was further supported by the finding that the percentage of *cis* isomer of a proline residue in mid-chain position, and five residues away from the glycosylation site decreased as increasing number of sugar moieties were added to the peptide backbone. A survey of the existing literature suggests that by facilitating a key segment of the secondary structure, glycosylation can potentially play a crucial role in directing the protein-folding pathway [45]. Alternatively, sugar addition can rigidify the residues proximal to the N-glycosylation site. In either event, if conformational changes are found for N-glycopeptides, this means a more ordered secondary structure compared to the non-glycosylated counterparts.

O-LINKED GLYCOSYLATION

If the existing literature is confusing for the structure-modifying effects of N-glycosylation, it is even more so for Ser/Thr-linked glycopeptides. A review of approximately 25 non-mucin 1 related recent papers (due to the distinguished interest in mucins in our laboratory and outside this glycopeptide family will be studied separately) reveals an almost equal distribution for supporting or disparaging role of glycosylation to define the conformation of the peptide backbone. At least for N-glycosylation when positive data were found, these pointed in the same direction: stabilization of the structure. For O-glycosylation structure stabilizing and destabilizing effects are uniformly recorded. Let's start where we left with the N-glycopeptides, the effects of O-glycosylation on Pro *cis/trans* isomerization. α- GalNAc or β-GlcNAc moieties were incorporated to Ser side-chains in a Sendai viral nucleoprotein nonapeptide antigen, in mid-chain positions [46]. No NMR signals corresponding to the *cis* isomer of the following proline residue were detected, as opposed to the similarly bulky tyrosine, which drove a significant amount of the Pro population into *cis* orientation. This suggests that the bulk is not enough and perhaps hydrophobic interactions are required and is consistent with the observation that only an uncharged histidine (His) residue causes proline isomerization, the protonated form does not [47]. The presence of a nearby disaccharide does not seem to modify the pK of His, at least according to our studies on the 19-residue-long antibacterial peptide drosocin [15]. The charged state of the His remained unchanged in spite of a large number of NOE contacts

between the peptide and the carbohydrate indicating close association.

In general, no substantial difference is detected between the predominantly random coil conformation of glycosylated and non-glycosylated drosocin variants, but there are subtle differences in the small population of folded conformers [15]. While the central turn becomes more extended, a terminally located turn is slightly tightened. A minor stabilization of glycosylated drosocin's turn structure was verified by CD spectroscopy [14]. Like for drosocin, the dominant conformation of another insect-derived antibacterial peptide pyrrhocoricin is largely unordered, and there is only little change in the backbone conformation upon native glycosylation [48]. Additional literature examples can be found for both turn tightening and extended structure stabilization. The conformational equilibrium between turn structures of the V3 loop of HIV-1 is shifted after glycosylation toward a more compact conformation [49]. Another example for glycopeptides in extended structure relates to a six times glycosylated decapeptide fragment of the extracellular domain of human glycophorin A [50]. NMR and molecular dynamics simulations indicate that the GlcNAc moieties are located at opposite sides of the peptide chain and reduce the flexibility of the backbone. However, this effect may not be strictly due to the presence of the carbohydrates. A similar multiglycosylated chemokine, lymphotactin, assumes largely unordered structures that become more ordered as the temperature is lowered. However, incorporation of eight GalNAc residues, clustered in an 18-residue mucin-like domain, has no impact on the NMR resonances [51].

Although no solution conformational changes were detected for an O-glycosylated analogs of the 108-144 fragment of prion protein, likely responsible for the species-to species transmission of prion diseases, the fibril formation is altered upon sugar addition [52]. While α-GalNAc at Ser135 suppresses the formation of amyloid fibrils, the same sugar at Ser132 shows the opposite effect. The sugar orientation due to the anomeric configuration must play a role because β-GlcNAc on Ser135 lacks the fibril inhibitory potential. Knowing the dynamic sheet-helix conformational equilibrium of the prion protein, the observed reduction of fibril formation can be due to either β-pleated sheet destabilization or direct α-helix stabilization. Such sheet → helix conformational transition after incorporation of a Gal-GalNAc disaccharide was noted on the C-terminal leucine zipper domain of human salivary mucin [53]. In addition to the shift in the percentage of the secondary structural elements, the position of the helical segment is also shifted by a residue downstream after glycosylation. Sugar-induced conformational changes were similarly observed in another domain of this protein, albeit no long-range peptide-carbohydrate NOE contacts could be detected [54]. Of relevance to the anomeric connections, the ϕ torsion angles of the glycosydic C1'-O1 bonds of glycopeptides corresponding to the carbohydrate-protein linkage region of connective tissue proteoglycans show a strict preference for the –sc range, whereas the ψ torsion angles exhibit dependence upon the interglycosydic linkage [55]. The predominant conformation of the glycopeptide bond is ϕ=-sc, and ψ=+ac. In spite of this strict anomeric orientation, the conclusion of the study is that glycosylation has only a marginal influence on peptide backbone conformation.

A perennial subject of arguments is the effect of sugar incorporation into helical peptides. Of course, if the helix is stabilized by multiple i, i+4 ionic bridges, glycosylation is not expected to modify the secondary structure, and indeed it does not break the helix system [56]. A perhaps more exciting proposition of the abstract of this report is that the glycopeptide retains its helicity in sodium dodecyl sulfate micelles while the unglycosylated peptide loses it. Unfortunately no experimental proof is provided for this claim. Another report asserts that a helical glycopeptide structure is identical in methanol and unilamellar vesicles [57], but thorough examination of the CD curves suggest otherwise. CD spectroscopy also documented helix stabilization by glycosylation [58]. In this case, sugar addition to a Thr and the repeated Gly-Pro-Thr sequence of collagen was required for the stable tertiary structure, and glycosylation was found to be an effective alternative to hydroxyproline incorporation for stabilizing the collagen triple helix. However, in the glycopeptide world nothing is this simple. CD spectra in an aqueous trifluoroethanol solution show that GlcNAc attachment at Thr6 or Thr21 reduces the helical content of calcitonin, indicating that the O-glycosylated residue is a stronger helix breaker than the original, non-glycosylated Thr [59]. As the final piece of this puzzle, the helix-disrupting effects of glycosylation are shown to be manifested only at the fraying edges; sugar addition to Ser13 in mid-chain position does not influence the helix potential.

A few additional reports provide useful information on the interaction of carbohydrates and the polyamide backbone in glycopeptides. A number of NOE crosspeaks were detected between the first GalNAc moiety and the sugar-bearing Thr residue in model glycosylated hexapeptides [60]. This interaction appears to be strictly local, as neither the following Gal moiety nor other amino acid residue side-chains exhibit noticeable NOE contacts. In support, only local (Thr – GalNAc) NOE constraints were observed in the NMR spectra of a tri-glycosylated 14-residue antifreeze peptide from the Antarctic cod [61]. Nevertheless, all three Thr residues are in close contact with the sugars they are attached to. A longer-range peptide-carbohydrate interaction occurs in mannosylated analogs of the leech-derived tryptase inhibitor [62]. The single mannose moiety on Ser36 makes contact to the amide protons of Gly15 and Ser16. NMR resonances for the second strand of the β-hairpin (Gly18-Tyr21) are also affected by the presence of Man attached to Ser36, indicating a long-range structural perturbation by glycosylation. A hydrogen bond emerges between the amide proton of a GalNAc moiety (the inner sugar of a disaccharide) and Thr10 of contulakin-G, a 16-residue long glycopetide, but such a peptide-carbohydrate interaction is absent when the same disaccharide is attached to Ser7 of the sequence [63]. NMR and infrared spectroscopies indicate unordered structures for Thr oligomers without carbohydrate and turn conformations in the presence of Gal moieties [64]. The appearance of γ-turns upon sugar addition is noteworthy, although this happens in dimethyl sulfoxide (DMSO), a solvent hardly having biological relevance. Finally even DMSO could not induce any ordered structure from a glycosylated analog of a major T-cell epitopic peptide

corresponding to hen egg lysozyme [65]. At least this is consistent with the lack of any structure-inducing effect of N-glycosylation of Asn homooligomers [66].

MUCIN-1

Perhaps the most extensively studied glycopeptides correspond to the tandem repeat unit of the human epithelial mucin 1 protein [67-69]. Mucins are major epithelial luminal surface proteins and function as a physical and biological barrier protecting mucous epithelia. When mucins are associated with malignant epithelial cells, they not only protect these cells from the host environment during metastatic dissemination, but also generate immunogenic epitopes depending upon their status of glycosylation. The core sequence of mucin 1 consists of a 20-mer fragment tandemly repeated 6 times [70].

The core mucin 1 and other mucin sequences, major epitopes for cancer-specific antibodies and T-cells, were reported to be abnormally glycosylated during cancer development [71]. Due to the aberrant glycosylation in certain tumors which results in the exposure of the mucin tandem repeat protein core on the cell surface [72], and the ability of the immune system to respond to these structures [73], the 20-amino acid repeat units of mucins are currently being heavily investigated as a key to possible mucin-based vaccines for immunotherapy of tumors [74]. Some of the mucin 1-specific monoclonal antibodies and cytotoxic lymphocytes recognize the peptide sequence PDTR within the tandem repeat portion exposed by decreased degree of nearby glycosylation compared to healthy mucin. Recent results strongly suggest that variation in not only the glycan structures [75] but also the distribution of glycans on the core polypeptides give mucins unique and diverse biological functions that play essential roles in carcinoma-host and other cellular interactions [76]. To study the immunological properties and secondary structure of glycosylated mucin repeats, we synthesized a series of synthetic peptide and glycopeptide models that contained no sugars thus mimicking the core sequence; peptides with short carbohydrate side-chains (3 or 4 sugar moieties each) attached to the tandem repeat that mimic the carcinoma-originated mucin protein fragments [77,78]; and finally peptides with extended carbohydrate chains (at least 6 sugar moieties each) attached that mimicked the mucin secreted by normal cells [79]. The following mucin peptides were synthesized; Fig. (**1**) shows the relevant structures of the added carbohydrates.

Sequence	Side-chain modification (X)
HGVTSAPDTRPAPGSTAPP	-
HGVT(X)SAPDTRPAPGSTAPP	Glc(α1-4)-Glc(β1-4)-GalNAc(α1-0)
HGVTSAPDTRPAPGST(X)APP	Glc(α1-4)-Glc(β1-4)-GalNAc(α1-0)
HGVTSAPDTRPAPGS(X)TAPP	Glc(α1-4)-Glc(β1-4)-GalNAc(α1-0)
HGVT(X)SAPDTRPAPGST(X)APP	Glc(α1-4)-Glc(β1-4)-GalNAc(α1-0)
HGVT(X)SAPDTRPAPGSTAPP	$[Glc(\alpha 1\text{-}4)\text{-}Glc]_3$-GalNAc(α1-0)
HGVTSAPDTRPAPGS(X)TAPP	$[Glc(\alpha 1\text{-}4)\text{-}Glc]_3$-GalNAc(α1-0)
HGVT(X)SAPDTRPAPGST(X)APP	$[Glc(\alpha 1\text{-}4)\text{-}Glc]_3$-GalNAc(α1-0)

Here we detail the results of the CD studies and discuss our data in light of other mucin-1 conformational studies. All epithelial mucin 1 repeat peptides exhibited a negative CD band around 199 nm. While no additional band was detected for the non-glycosylated variant, a small positive band around 223 nm emerged in the CD spectra of some of the glycopeptides [Fig. (**2**)]. This positive band was not present in the Ser15-substituted analog, regardless of the length of the carbohydrate side-chain. As is evident from the spectra, glycosylation of Ser15 did not appear to modify the conformation of the mucin peptide at any level. In contrast, the 223 nm positive CD band was noticeable for the Thr4 and Thr16 glycosylated mucin repeat peptides but its intensity was not dependent upon the length of the carbohydrate moiety attached to Thr4 alone or Thr4 and Thr16 simultaneously. Trisaccharide- and heptasaccharide-bearing peptides exhibited almost identical CD spectra. Apparently, the size of the sugar side-chain did not play any role in defining the secondary structure. What made a difference was the number of carbohydrate side chains. The 223 nm positive CD band was the most intense for the Thr4, Thr16 bis-glycosylated peptides, followed by the Thr4 monoglycosylated analogs. These findings support the idea that the potential mucin 1-repeat glycosylation sites are better utilized (i.e., a higher number of Ser and Thr residues carry carbohydrate side-chains) in cancerous cells than in normal ones [80,81].

The next question was what secondary structure these CD features corresponded to. In a classical viewpoint, the CD spectra of the mucin peptides greatly resembled that of polylysine at pH 5.7 in unordered conformation [82]. However, similar spectra were measured for polyproline in a left-handed polyproline II helix [83]. Considering the large number of proline residues in the mucin repeat sequence, the polyproline II helix assignment did not seem too far-fetched.

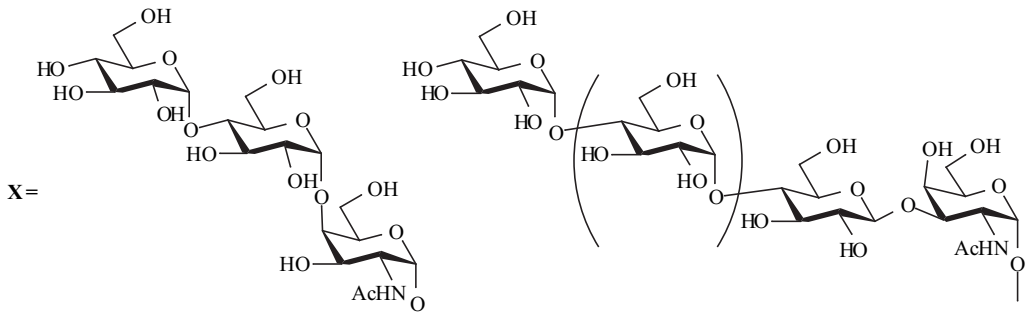

Fig. (1). Model carbohydrates added to the repeat unit of the epithelial mucin-1 protein.

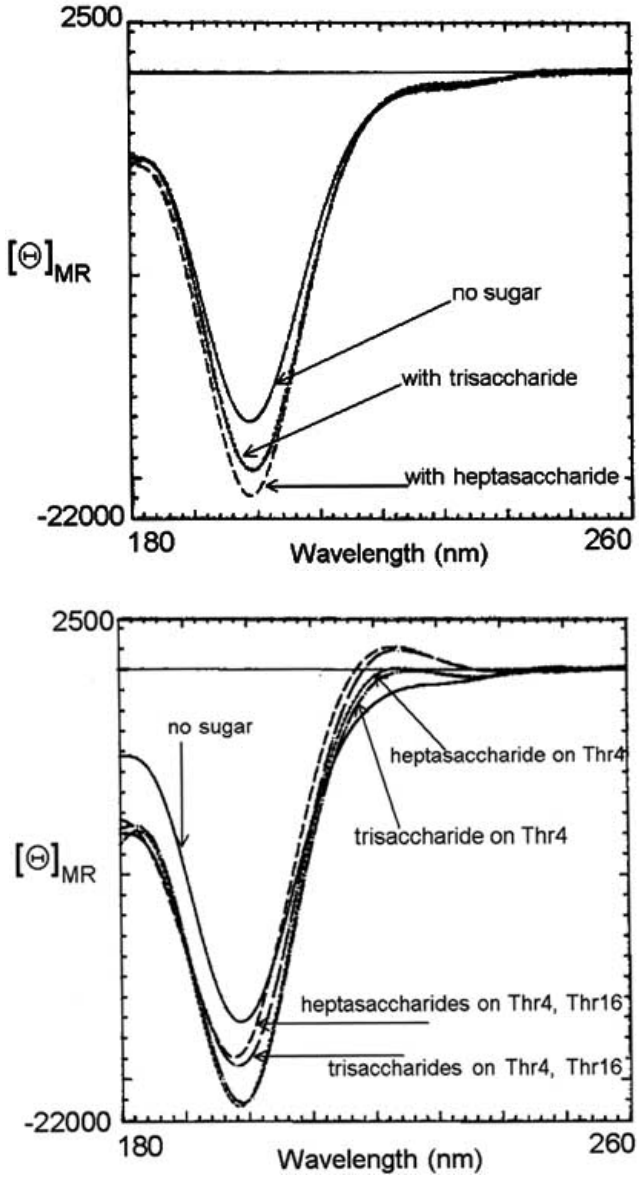

Fig. (2). CD spectra of non-glycosylated and glycosylated mucin peptides in water. The spectra were collected on a Jasco J720 instrument at room temperature. The peptide concentration was 0.2 mg/mL as determined by HPLC. Panel A shows Ser-linked glycopeptides; Panel B depicts the Thr-glycosylated analogs.

Indeed, non-glycosylated and glycosylated versions of a 25-residue mucin 1-repeat peptide exhibit CD spectra very similar to ours, and the secondary structure is assigned to left-handed polyproline II helix [84]. Just like our longer carbohydrates, addition of GalNAc moieties increases the intensity of the near 220 nm positive CD band compared to the non-glycosylated analog, and the authors concluded that this increase indicates a stabilization of the polyproline II helix upon glycosylation [84]. Earlier NMR studies suggest local ordering in the PDTR segment [85,86] in shorter mucin peptide fragments. It needs to be mentioned that the lack of intramolecular hydrogen bonding renders the polyproline II conformation of free peptides in solution indistinguishable from an irregular backbone structure by ^{1}H-NMR spectroscopy [87].

The conformation of a GlcNAc-conjugated mucin-1 peptide when bound to the SM3 monoclonal antibody that recognizes the PDTRP segment was investigated by NMR spectroscopy [88]. Unglycosylated and the glycosylated antibody-bound peptides exhibited similar structure. However, the transfer-NOE build-up rates were different, the glycopeptide having a uniform correlation time. The reasoning was made as to a backbone conformational stabilization by the GlcNAc moiety and/or by its contribution to the binding. The validity of our model GalNAc-maltooligosaccharide carbohydrate compositions is supported by NMR studies on mucin 1 peptides carrying clustered mono-, di-, tri- and hexasaccharide glycodomains [89]. Clustering the GlcNAc-based glycans *via* an α-linkage induces a remarkably stable and extended structure of the

peptide backbone. This effect appears to be independent of the nature of the carbohydrates beyond the innermost N-acetamido-hexose moiety. Interestingly, a β-linked analog shows increased number of peptide-sugar NOE contacts and appears to exhibit an amplified level of β-sheet conformers. For the GVTSAP region, minor but distinct conformer clusters formed by 15-mer unglycosylated or Thr-glycosylated fragments [90]. More abundant and stronger NOE contacts were found for the peptidic part of the glycopeptide in addition to two sugar-peptide NOEs. This indicates a stabilized structure of the glycopeptide. Long-range connections were observed between the sugar moiety on Ser and a Pro side-chain atom 4 residues downstream, once again in addition to sugar-backbone interactions [91].

CONCLUSIONS

The conclusion of the last study is that the non-globular nature of mucin 1 protein is due to both the protein core sequence and the presence of the carbohydrates. The interaction of the carbohydrate and some amino acid residues C-terminal to it agrees with our observations on the secondary structure of O-linked antibacterial glycopeptides [15], and appears to be a general feature of the conformation-modifying effects of Ser/Thr-linked glycosylation in glycoproteins. In spite of many positive data, added sugars may or may not influence the secondary structure of peptides and protein fragments. The strength and type of conformational changes upon sugar incorporation depend upon the starting structure of the peptide, the close neighborhood of the sugar-bearing amino acid residue and the length of the carbohydrate moiety. Taken all reports together, continued and rigorous examination of the structural characteristics of glycopeptides is essential and will provide an exciting research subject for many years to come.

ABBREVIATIONS

Arg	=	L-arginine
Asn	=	L-asparagine
CD	=	Circular dichroism spectroscopy
Cys	=	L-cysteine
DMSO	=	Dimethyl sulfoxide
Fuc	=	D-fucose
Gal	=	D-galactose
GalNAc	=	N-acetyl-D-galactosamine
Glc	=	D-glucose
GlcNAc	=	N-acetyl-D-glucosamine
Gly	=	Glycine
His	=	L-histidine
HIV	=	Human immunodeficiency virus
IgG	=	Immunoglobulin
IR	=	Infrared spectroscopy
Lys	=	L-lysine
Man	=	D-mannose
MHC	=	Major histocompatibility complex protein
MUC	=	Mucin
NMR	=	Nuclear magnetic resonance spectroscopy
NOE	=	Nuclear Overhauser enhancement
Pro	=	Proline
PrP	=	Prion protein
Ser	=	L-serine
Thr	=	L-threonine
Tyr	=	L-tyrosine

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