Effect of *N*-linked glycosylation on glycopeptide and glycoprotein structure

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Asparagine-linked glycosylation is an enzyme-catalyzed, co-translational protein modification reaction that has the capacity to influence either the protein folding process or the stability of the native glycoprotein conjugate. Advances in both glycoconjugate chemical synthesis and glycoprotein expression methods have increased the availability of these once elusive biopolymers. The application of spectroscopic methods to these proteins has begun to illuminate the various ways in which the saccharide affects the structure, function and stability of the proteins.

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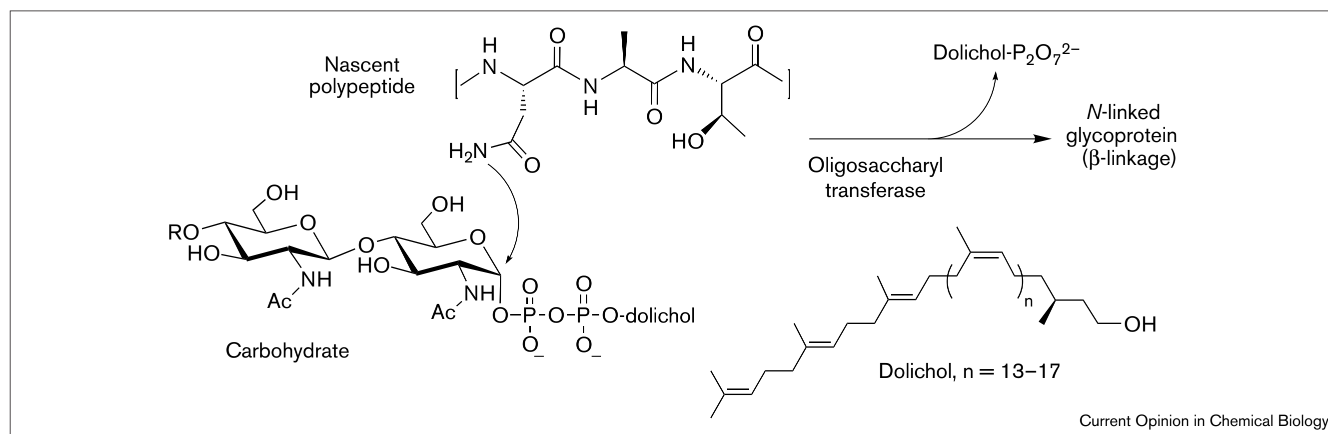
Abbreviations
Glc glucose
$GlcNAc_2$ *N*-acetyl glucosamine
Man mannose
NOE nuclear Overhausen enhancement
RNAse ribonuclease
T_m melting temperature

Introduction

The addition of large glycan structures to the protein backbone can dramatically alter the structure, and consequently the function, of the polypeptide architecture to which they are attached. In *N*-linked glycosylation [1•,2,3], a carbohydrate is covalently linked to an asparagine residue in the consensus sequence Asn–X–Thr/Ser, where X is any amino acid except proline (Figure 1). The transferred carbohydrate comprises 14 saccharide units arranged in a branched triantennary structure ($GlcNAc_2Man_9Glc_3$, where Glc is glucose, $GlcNAc_2$ is *N*-acetyl glucosamine and Man is mannose; Figure 2). After co-translational transfer, the glycan is processed through the action of glycosyl hydrolase and glycosyl transferase enzymes to high-mannose, complex, or hybrid-type oligosaccharide structures.

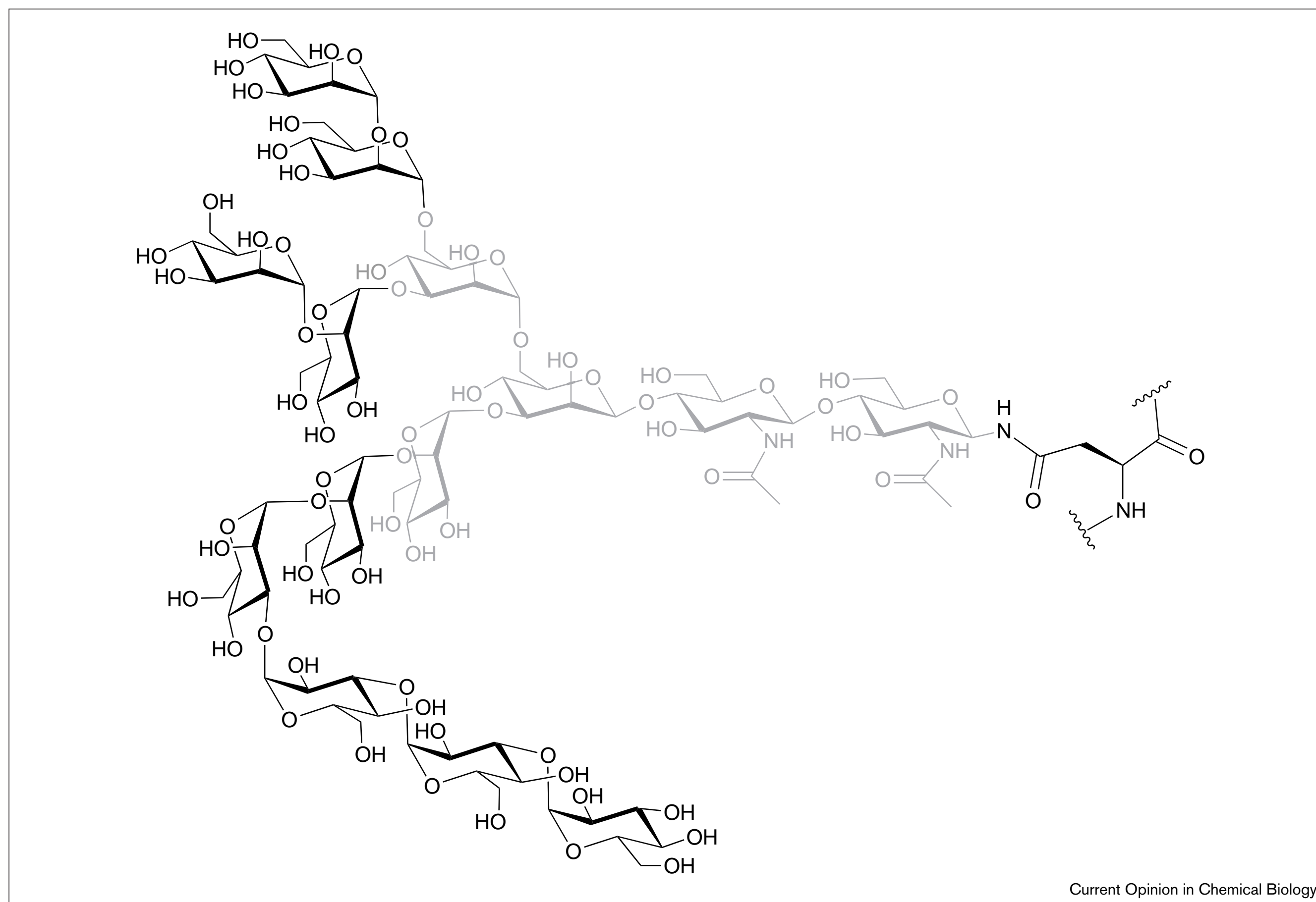
The *N*-linked glycan has the potential to strongly influence the structure of the protein biopolymer to which it is covalently attached [4–6]. *N*-linked oligosaccharides are large, hydrophilic structures with molecular weights ranging from 1884 Da, for a high-mannose carbohydrate ($GlcNAc_2Man_9$), to 2851 Da, for a sialylated triantennary complex saccharide ($GlcNAc_2Man_3GlcNAc_3Gal_3NeuAc_3$, where Gal is galactose and $NeuAc_3$ is *N*-acetylneuramic acid). The respective volumes of these saccharides are 1840 Å^3 and 2542 Å^3, respectively [7]. Sugar models were constructed and energy minimized with the AMBER forcefield using the DISCOVER module of the molecular modeling program Insight. Volumes were calculated from these structures using the program Spartan. A solvation shell is not included in these volumes. These sizes are quite significant in comparison to the protein, as illustrated in Figure 3. Glycosylation is therefore unique from other common protein modifications such as phosphorylation; for example, a phosphate group has a molecular weight of 98 Da and a volume of approximately 84 Å^3, values considerably smaller than even the most diminutive *N*-linked carbohydrate.

Figure 1



N-linked glycosylation is catalyzed by oligosaccharyl transferase. The amide of the asparagine sidechain attacks the anomeric carbon of the glycosyl donor to form a covalent linkage between the protein and sugar. The glycosyl donor substrate is anchored in the membrane of the endoplasmic reticulum through the dolichol, which is an extremely hydrophobic isoprene. R, $(Man)_9$ $(Glc)_3$.

Figure 2



Initially transferred tetradecasaccharide. The pentameric core, found in all *N*-linked glycans, is highlighted in gray. Wavey lines indicate the polypeptide.

N-linked glycans can affect protein structure in two capacities. First, as *N*-linked glycosylation occurs co-translationally, addition of the carbohydrate to the partially folded, nascent polypeptide can impact on, or facilitate, the protein-folding process. Second, the carbohydrate can stabilize the mature protein. Studies focusing on large glycoproteins may employ site-directed mutagenesis to remove or create glycosylation sites. Alternatively, a variety of glycosidases may be enlisted to fully (PNGase F) or partially (Endo-H) deglycosylate the protein. The modified protein can then be assayed for altered function, or examined spectroscopically for altered structure. It must be noted that enzymatic deglycosylation of mature proteins will not reveal whether the carbohydrate is important for protein folding, although these studies do indicate whether the carbohydrate plays a critical role in stabilizing the native glycoprotein. Studies with short oligopeptides can also provide insight into the effect of glycosylation on structure. Small peptides are flexible, and may resemble the nascent polypeptide that is co-translationally glycosylated *in vivo*. This review summarizes some of the latest findings in these areas and delineates the recent technical advances that are being enlisted to provide insight into the structural and functional consequences of glycosylation.

Glycoconjugate synthesis

Critical for the structural evaluation of glycopeptides and glycoproteins is a readily available supply of material. Chemical and chemo-enzymatic methods for the synthesis of glycoconjugates have advanced significantly [7]. Most notably, an increasing number of glycoconjugates derivatized with large, branched oligosaccharides are being synthesized in substantial (milligram) quantities. Several syntheses of glycopeptides derivatized with the conserved pentameric core ($GlcNAc_2Man_3$; see Figure 2) have been reported [8–11]. Larger *N*-linked glycoconjugates have been successfully prepared using a combination of chemical and enzymatic methods. For example, a peptide derivatized with the heptasaccharide $GlcNAc_2Man_3GlcNAc_2$ was synthesized, and then subjected to galactotransfereases and sialotransferases to generate a sialated complex-type biantennary saccharide [12]. Glycosyl transferases have also been used to derivatize Asn34 of ribonuclease (RNAse) B with the selectin ligand sialyl Lewis X [13]. In addition, commercially

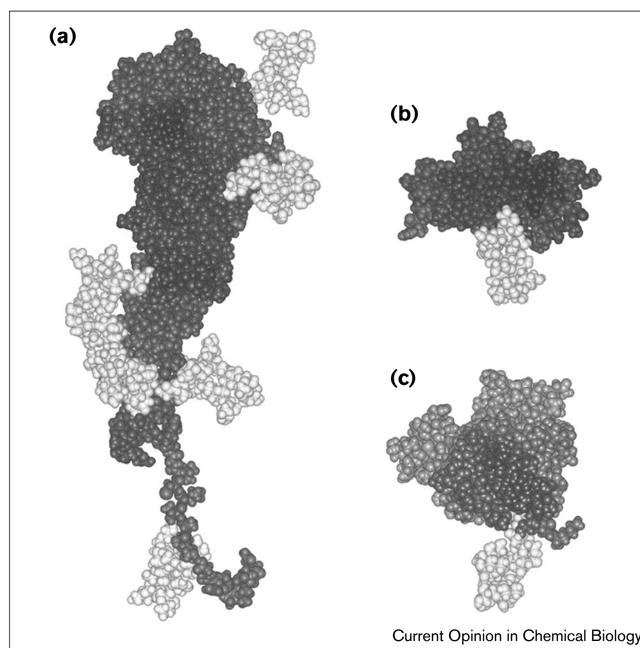
available glycoproteins, such as fetuin and ribonuclease, have been used as a source for complex and high-mannose oligosaccharides, respectively. The *N*-linked saccharides can be released from the protein using hydrazine, purified, and then coupled to asparagine [14•]. Alternatively, endoglycosidases can be used to transfer carbohydrates derived from proteolytic digests of commercially available glycoproteins to synthetic glycopeptides containing an Asn–GlcNAc residue [15••]. The enzyme Endo-M has been used in such transglycosylation reactions to synthesize both complex and high-mannose *N*-linked glycopeptides. Additionally, a high-mannose glycopeptide containing the non-natural GlcNAc–Glc–Asn linkage (the natural linkage is GlcNAc–GlcNAc–Asn) was generated via a transglycosylation reaction using the endoglycosidase Endo-A and a synthetic glycopeptide acceptor containing an Asn–Glc residue [16•].

The production of glycoproteins in heterologous expression systems has also been the subject of recent research [17]. It is critical to note that prokaryotes such as *Escherichia coli* cannot biosynthesize *N*-linked glycoproteins; therefore, eukaryotic expression systems must be used in order to produce functional proteins. Although mammalian cells produce high-mannose, complex, and hybrid-type glycans, these cell lines may be difficult and expensive to work with. In contrast, expression in fungal systems is inexpensive and growth in minimal media enables isotopic labeling for NMR studies at relatively low cost. For example, an isotopically labeled 83-residue fragment of thrombomodulin has been produced in the yeast *Pichia pastoris* using a fermentation protocol that generates 90 mg of the desired protein per liter of cell culture [18•]. Yeast can only produce high-mannose-type glycan structures, however, and has a tendency to hyper-mannosylate the saccharides [19]. Recently introduced expression in baculovirus-infected insect cells is useful for the production of large quantities of glycoprotein [20•]. This system produces high-mannose-type glycans as well, although the hyper-mannosylation found in yeast is not observed. The co-expression of galactosyl [21,22] or *N*-acetylglucosylamine [23] transferases along with the desired protein results in production of complex-type oligosaccharides. The *in vivo* application of glycosyltransferases in various expression systems represents a promising approach to engineering the desired glycan moiety of glycoproteins.

Carbohydrates increase global protein stability

In general, glycoproteins are more stable than their corresponding unglycosylated counterparts, even when there are no major structural changes associated with glycosylation. A variety of techniques have been employed to investigate this phenomenon, including calorimetry, denaturation studies, NMR hydrogen/deuterium exchange measurements, and proteolytic digestion analysis. In most cases, it is concluded that glycoprotein stabilization results from largely entropic, rather than enthalpic effects.

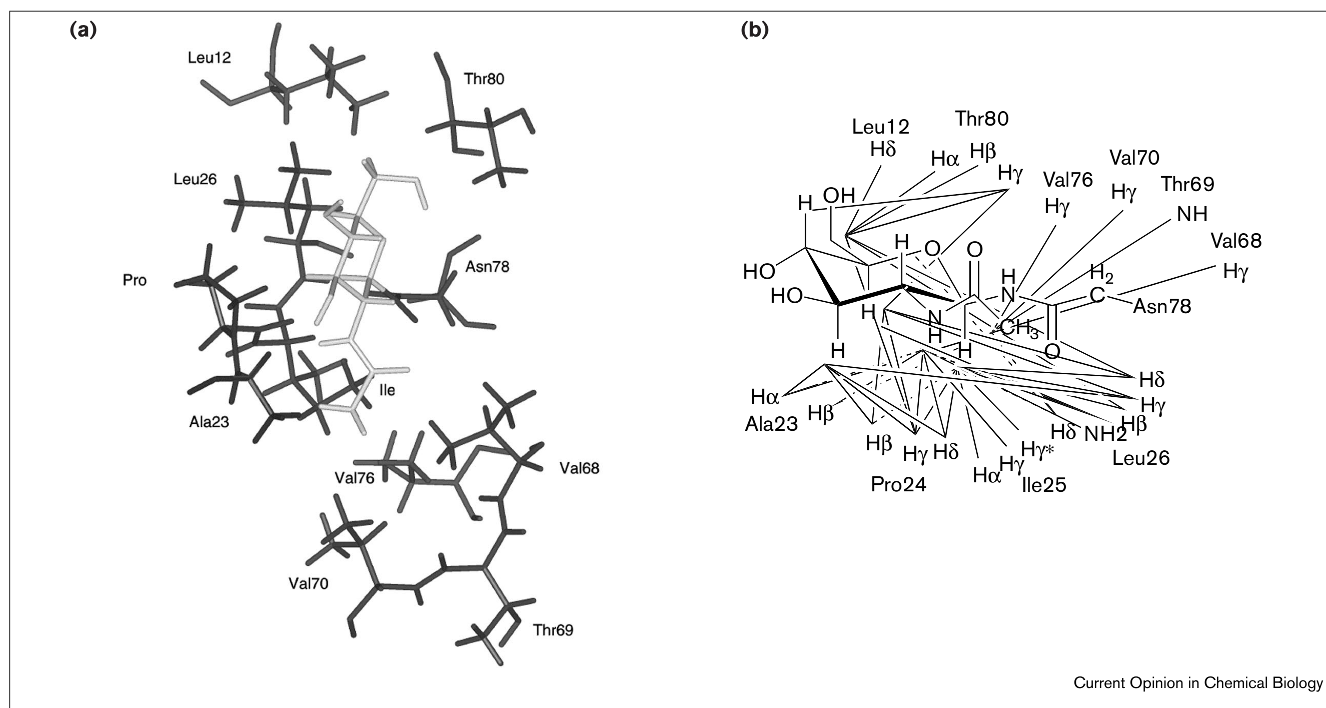
Figure 3



Structures of three typical glycoproteins (protein shown in dark gray; carbohydrate shown in light gray). **(a)** A subunit of hemagglutinin protein from influenza virus. **(b)** CD2 structure. **(c)** RNAse B structure.

The human chorionic gonadotropin hormone (hCG) has been the subject of intensive NMR studies [24••,25]. The 92 residue α-subunit in the αβ heterodimeric protein is glycosylated at Asn52 and Asn78. Mutagenesis studies indicate that, whereas the Asn52 glycosylation site appears to be implicated in signal transduction and heterodimer association [26], the carbohydrate at Asn78 plays a critical role in maintaining the structure of the α-subunit. Furthermore, although the glycan at Asn78 plays no direct role in biological activity, mutants lacking Asn78 are poorly secreted and rapidly degraded *in vivo* [27]. Valuable structural information has been obtained by studying the partially glycosylated α-subunit (derivatized with a single GlcNAc at Asn78 and Asn52). Extensive nuclear Overhausen enhancement (NOE) interactions between the carbohydrate and the protein are noted. Importantly, the majority of these NOEs are observed between hydrophobic residues, such as Leu12, Ile25, Val68 and Val76, and the axial hydrogens H1, H3, and H5 and the *N*-acetyl protons of the β-GlcNAc as illustrated in Figure 4. The apparent effect of the single carbohydrate on the α-subunit structure is to sheild the hydrophobic core in between the β-hairpins (defined by turns including residues 20–23 and 70–74). The observed contacts emphasize an important feature of protein–carbohydrate interactions; these are commonly dominated by hydrophobic interactions. It is also significant that glycosylation beyond the asparagine-linked GlcNAc has a small but significant effect on the thermal stability of the α-subunit.

Figure 4



Interaction of saccharide with human chorionic gonadotropin. **(a)** *N*-acetylglucosamine (light gray) shown with contacting residues (dark gray). **(b)** Observed protein carbohydrate NOEs. Perhaps surprisingly, the NOE data reveal numerous contacts between the saccharide and hydrophobic amino acids.

A thermodynamic study of unfolding of the ovomucoid first domain has been performed [28]. The effect of the two *N*-linked carbohydrates on the folding process of this protein was assessed quantitatively using differential scanning calorimetry. This 68-residue domain, which contains two glycosylation sites at Asn10 and Asn52, was examined in both the glycosylated and non-glycosylated forms. The melting temperature (T_m) of the glycosylated derivative was increased by 4.8°C, corresponding to an increase in the free energy of unfolding (ΔG^o_u) of ~2.1 kJ. As the enthalpy of both proteins was essentially the same, however, the stabilization of glycosylated ovomucoid was attributed to entropic factors. It is proposed that the carbohydrate reduces the disorder of the unfolded protein, thereby destabilizing the unfolded state of the glycosylated variant compared to the non-glycosylated one.

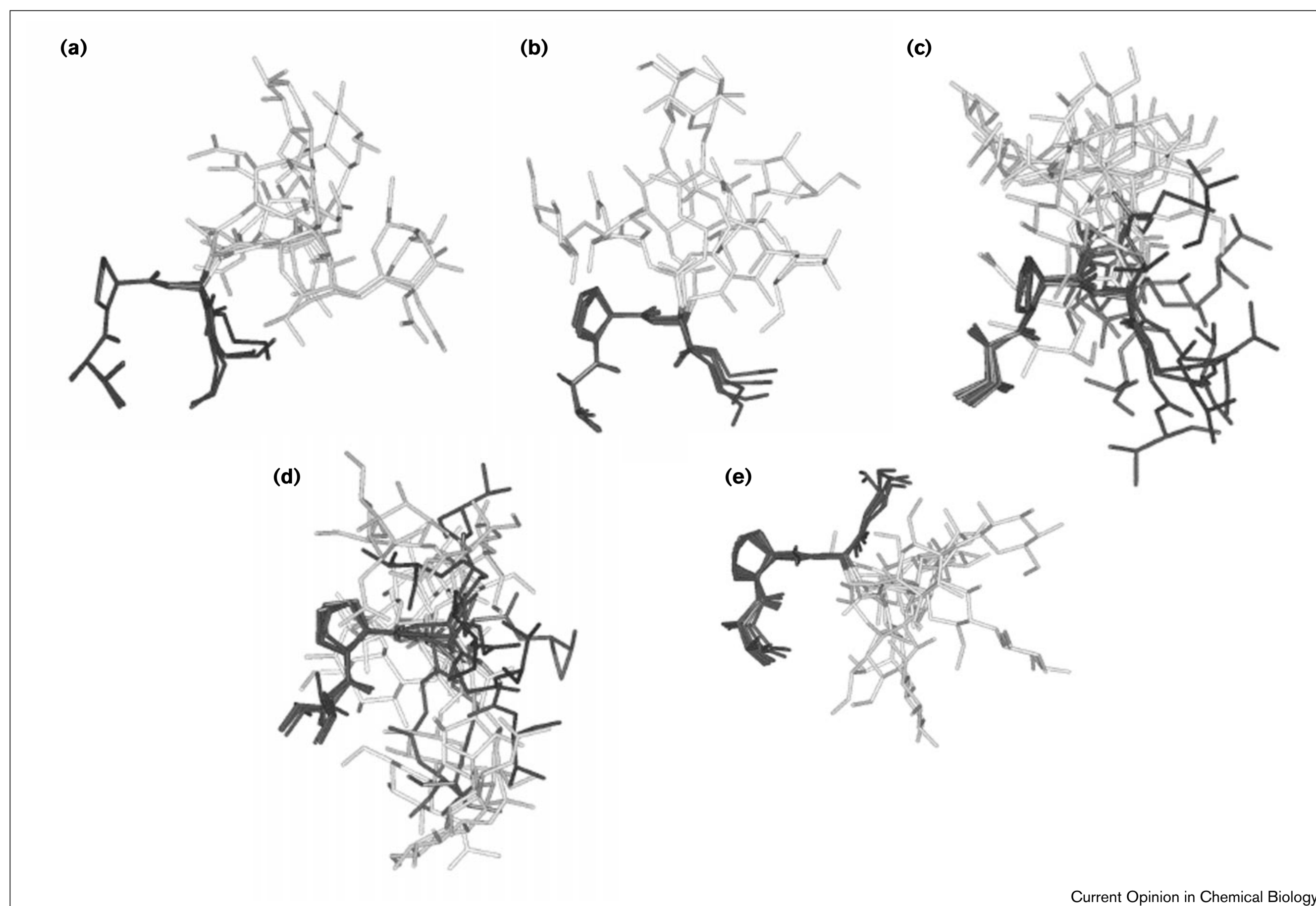
In a recent study [29], five glycoproteins were subjected to enzymatic deglycosylation with various glycosidases to produce either the completely or partially deglycosylated products. Although circular dichroism measurements suggest that secondary structure was not affected by glycosylation, comparison of the unfolding temperatures suggested enhanced stability in the glycosylated derivatives. The T_m values of avidin and ovotransferrin, glycoproteins that each contain one *N*-linked saccharide, were approximately the same for the glycosylated and non-glycosylated forms. In contrast, the more heavily glycosylated proteins invertase (nine *N*-linked sites), fetuin (three *N*-linked sites) and glucoamylase (30 *O*-linked sites) exhibited increases in T_m ranging from 1.4°C to 2.3°C relative to the degree of deglycosylation of the derivatives. The differences in enthalpy of unfolding did not vary between the glycosylated and non-glycosylated counterparts. Additionally, the carbohydrate enhanced the reversibility of the unfolding process, perhaps by increasing the protein solubility and discouraging the formation of aggregates.

RNAse A and B are small, globular proteins that differ only in the attachment of a high-mannose-type carbohydrate chain to Asn34 of RNAse B. Recent studies on these proteins have attempted to quantify the thermodynamic and kinetic parameters of unfolding RNAse A and B [30]. Using proteolysis, the kinetic stability of RNAse B was determined to be approximately 3 kJ/mol higher than RNAse A at 52.5°C. Contrary to other studies, the authors concluded that the increase in stability was due to enthalpic, not entropic, factors. Thermodynamic stabilities determined using UV spectroscopy to monitor the unfolding process indicated that RNAse B is more stable by about 2.5 kJ/mol, independent of temperature. Previous studies have demonstrated that the carbohydrate enhances the dynamic stability of the protein [31].

Local structure and stability

Glycosylation can affect the local secondary structure of proteins. By facilitating the formation of a key segment of

Figure 5



Ensemble of NMR structures of peptides from hemagglutinin protein derivatized with various saccharides (peptide depicted in dark gray, saccharide shown in light gray). **(a)** GlcNAcGlcNAcAsn. **(b)** GlcGlcNAcAsn. **(c)** GlcNAcAsn. **(d)** GlcNAcGlcAsn. **(e)** GlcGlcAsn.

secondary structure, glycosylation can potentially play a crucial role in directing the protein-folding pathway. Alternatively, glycosylation can rigidify the residues proximal to the glycosylation site. In either case, the modulation of local structure might serve to enhance the overall stability of the protein, or might enable the protein to perform some required function at the affected site, such as bind to a ligand or receptor.

The structure of glycosylated human CD2 has been solved by NMR [32]. Recent work has quantified the extent of mobility of both the protein backbone and *N*-acetyl groups of the first two GlcNAc residues using ^{15}N NMR relaxation measurements [33]. The motion of the amide nitrogen of the glycosylation site is highly restricted. Additionally, the residues proximal to the glycosylation site also show substantial rigidity. The *N*-acetyl group of GlcNAc1 is highly restricted, comparable to the mobility of many amide nitrogens in the rigid polypeptide backbone. These results suggest that the carbohydrate is required to stabilize the region surrounding the glycosylation site. Digestion with Endo-H reveals that a single GlcNAc is sufficient to stabilize the protein.

Neo-glycoconjugates, including non-natural carbohydrate moieties, were used to probe the role that the sugar plays in stabilizing a β-turn conformation found at the *N*-linked glycosylation site at Asn285 of the hemagglutinin protein of influenza virus [34•,35]. Derivatization of a short peptide taken from this region with the saccharides GlcNAcGlcNAc, GlcGlc, GlcGlcNAc, GlcNAcGlc and GlcNAc reveal that the *N*-acetyl group of the proximal sugar is critical for maintaining a β-turn conformation (Figure 5). As no NOEs were observed between the polypeptide and carbohydrate, it can only be inferred that the *N*-acetyl group is acting to stabilize the polypeptide backbone without making specific contacts. The carbohydrate might be acting in a steric role, serving to limit the conformational space available for the peptide to sample. Alternatively, the *N*-acetyl group might possess unique solvation properties that are critical for maintaining a more compact peptide conformation. Computational studies performed on this peptide system confirm that the carbohydrate increases the propensity for the peptide to adopt a β-turn conformation [36]. The study concluded that the addition of the carbohydrate contributes only entropic terms, whereas the hydrophobic contacts made among the

peptide residues contribute enthalpic terms. It is proposed that as the nascent polypeptide folds, the increased entropy of the highly flexible carbohydrate moiety that is attached to the polypeptide compensates for the reduced entropy of the newly folded peptide.

Assembly and orientation of proteins

In addition to affecting the structure and stability of the protein itself, *N*-linked glycosylation can play a more global role, such as assisting the assembly of oligomeric complexes, and enabling cell-surface glycoproteins to orient themselves on the surface of a cell.

Human erythrocyte CD59 is a cell-surface glycoprotein that binds to CD8 or CD9 and is present on a wide variety of cell types [37]. This protein contains one conserved *N*-linked glycosylation site, as well as a glycosyl-phosphatidylinositol (GPI) anchor and several *O*-linked glycans. The *N*-linked carbohydrate is highly heterogeneous, consisting of more than one hundred complex-type glycoforms. Although the glycan does not appear to play a major role in affecting CD59 structure or binding ability, it is proposed that the glycan affects the packing of the protein on the cell surface, as well as preventing aggregation of the protein on the cell surface. Additionally, the hydrophilic glycan is proposed to limit interactions with the lipid bilayer and thereby facilitate diffusion of the protein along the cell membrane.

Conclusions

Research of the past several years has established that carbohydrates play a key role in protein structure and stability. Frequently, however, it is not clear how the saccharide exerts its structural influence over the protein biopolymer. Combined with modern spectroscopic techniques, the advances that have come in carbohydrate synthesis and chemo-enzymatic methods for glycopeptide and glycoprotein production are beginning to illuminate the hitherto elusive molecular detail of the carbohydrate–protein interaction.

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