Glycoproteins: glycan presentation and protein-fold stability

Mark R Wormald* and Raymond A Dwek

Glycosylation of proteins has been shown to play a role in a variety of cellular events. Thanks to recent advances in obtaining conformational constraints across glycosidic linkages, structural characterisation of glycoproteins has improved considerably. It is now becoming apparent that N-glycosylation of a folded protein can have a significant stabilising effect on large regions of the backbone structure.

Address: Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

*Corresponding author.
E-mail: mark@glycob.ox.ac.uk

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Introduction

The term 'glycobiology', introduced in 1988, focused attention on the role of oligosaccharides in the context of the proteins to which they were attached. Inspection of the protein databases suggests that as many as 70% of proteins have potential N-glycosylation sites (Asn-X-Ser, where X is not proline). Glycans can affect the physical properties of the proteins to which they are attached and provide lectin-recognition sites. Thus, roles for glycosylation have been shown in protein folding in the endoplasmic reticulum, transport and secretion, anchoring of proteins and protease protection. It is now becoming apparent that glycans can also have a direct structural role, affecting the tertiary or quaternary fold of the protein. This review focuses on the structural properties and roles of N-linked glycans.

Glycan structures

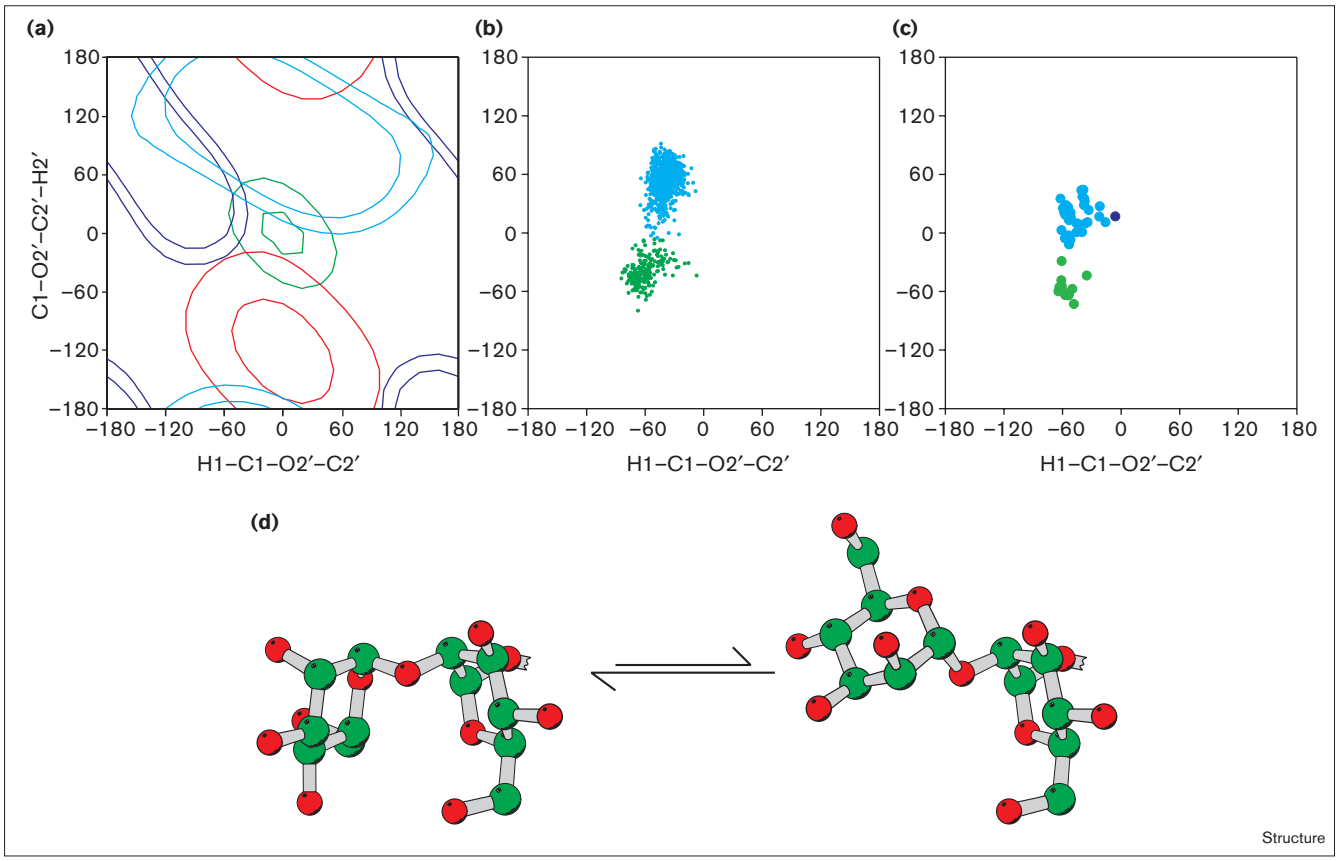
Oligosaccharide structures are determined by characterising the glycosidic linkages between the rigid monosaccharide units. In practice this has been difficult to do. Very few oligosaccharides or glycoproteins crystallise or give resolved electron density for the entire glycan, because of glycan heterogeneity and flexibility. Nuclear magnetic resonance (NMR) spectroscopy has frequently given too few constraints across glycosidic linkages for full characterisation, whilst linkage flexibility leads to average constraints being determined. Molecular modelling studies depend on the development of suitable forcefields.

However, the combination of these three techniques, together with improvements in methodology, is now allowing a more accurate picture of the conformational behaviour of individual linkages to be obtained. Recently, we compiled a database of all the crystallographic information on the glycans attached to proteins and bound to lectins, providing average structures for glycosidic linkages [1]. Advances in NMR techniques, particularly the measurement of heteronuclear coupling constants [2] and the ability to measure weaker nuclear Overhauser effects (NOEs), now provide considerably more conformational constraints. Optimised forcefields for oligosaccharides (recently reviewed in [3]) have improved the quality of the calculated results so that unrestrained calculations can be performed with some confidence. Figure 1 shows a typical set of results obtained for a terminal Manα1–2Manα (where Man is mannose) linkage in the oligomannose oligosaccharide, $Man_9GlcNAc_2$ (where GlcNAc is N-acetylglucosamine). NMR data (Figure 1a) can be used to show that there is not a unique conformation and provide considerable constraints on the range of conformations that are allowed. The molecular dynamics results (Figure 1b) indicate that the linkage adopts two flexible conformations in equilibrium, the combination of which is consistent with the NMR data. The X-ray data (Figure 1c) show that both linkage conformations predicted by molecular dynamics are actually observed in crystal structures.

The number of stable conformers and degree of flexibility varies with the linkage and monosaccharide sequence. For instance, the Glcα1–2Glcα1–3Glcα (where Glc is glucose) trisaccharide forms a rigid unit [4], probably due to hydrogen bonding between the first and third residues. Another example is the branched Lexis X epitope (Galβ1–4(Fucα1–3)GlcNAc; where Gal is galactose and Fuc is fucose) where hydrophobic stacking between the Fuc and Gal rings leads to a relatively rigid system [5]. Longer-range steric interactions may also reduce linkage flexibility, as is observed in multiantennary glycans [6].

One of the questions that still has to be answered is whether the whole structure of an oligosaccharide can be characterised by the conformation and flexibilities of the isolated individual linkages present in the structure. Interestingly, molecular dynamics simulations of $Man_9GlcNAc_2$ [7] suggest that, because of correlated motions of the linkages, the overall topology of the molecule (Figure 2a) is better defined than expected from the high degree of individual linkage flexibility. This may be a result of solvent-mediated hydrogen bonds between the antennae of the structure providing significant conformational constraints (Figure 2b). Perhaps more speculatively, this may arise from dampening of large-scale motions by the solvent shell.

Figure 1



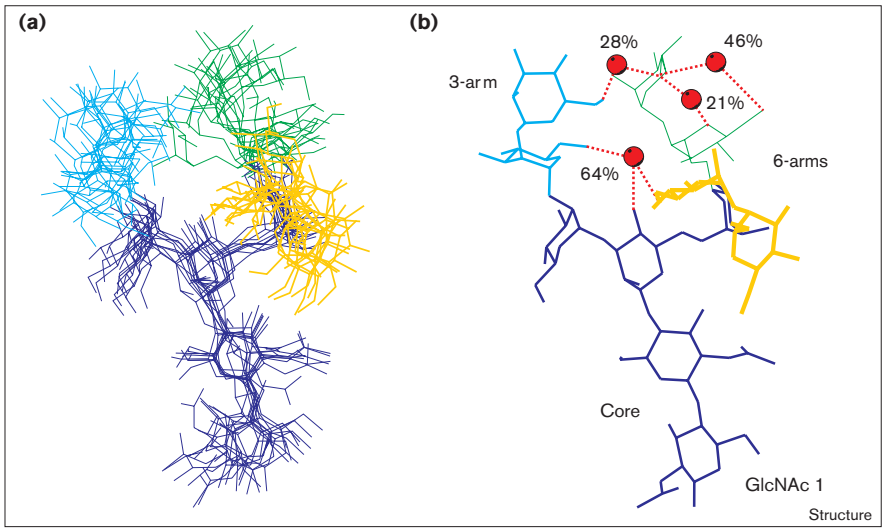
The combination of conformational data obtained from NMR, molecular modelling and X-ray crystallography enable individual glycosidic linkages to be characterised. (a) to (c) show ϕ (H1–C1–O2′–C2′ torsion angle) versus ψ (C1–O2′–C2′–H2′ torsion angle) plots for a Manα1–2Manα glycosidic linkage. **(a)** Distance constraints obtained from NOE data using the two-spin approximation for the D1–C linkage of $Man_9GlcNAc_2$ [7]. Four interproton distance constraints can be obtained from the solution NMR data, which would be sufficient to define completely a glycosidic linkage that adopted a single conformation. Each set of contours on the ϕ/ψ plot (colour-coded) shows the region of conformational space consistent with a single observed NOE. There is no region that is consistent with all four observed NOEs. Thus, the linkage must either be highly flexible or adopt more than one conformation. A region at (–50°, +50°) fits three out of the four NOEs. The NMR results are consistent with a ϕ angle of –50° and a range of ψ angles between –60° to +60°, with more constraints consistent with the higher values of ψ. **(b)** Results for the D1–C linkage of an unrestrained molecular dynamics simulation of $Man_9GlcNAc_2$ [7]. Each dot represents a snapshot of the structure at 1 ps intervals. The linkage conformations clearly fall into two distinct groups with an average ϕ angle of –40° and a ψ angle of –40° (green) or +70° (blue), the latter being more populated. The linkage spends a long time (hundreds of pico seconds) in each conformation before swapping rapidly to the other. **(c)** All the crystallographic structures available for Manα1–2Manα linkages [1]. The structures fall into two distinct groups (coloured blue and green) with an average ϕ angle of –50° and a ψ angle of –55° or +20°, the latter being more common. The dark blue dot is the crystal structure of the Manα1–2Manα–OMe disaccharide. **(d)** Molecular models of the two distinct conformations for the Manα1–2Manα linkage, left (–50°, +50°), right (–50°, –50°). These are in dynamic equilibrium, the equilibrium lying slightly more to the left. Each distinct conformer is not static but shows limited oscillations for both ϕ and ψ.

Conformation of the linkage between protein and N-glycan

The conformational behaviour of the linkage between an N-glycan and its asparagine sidechain has been well characterised by NMR studies of glycopeptides in solution [8,9] and by a statistical survey of crystallographic data [10]. These show the asparagine linkage to be relatively rigid and planar, with a tendency to extend the first glycan residue away from the peptide backbone and into the solvent.

There have also been many studies of the conformational effects of N-glycosylation on small peptides [8,9,11–13]. The general conclusions from these studies are that N-glycosylation does not induce any permanent secondary structure in unstructured peptides but that it does alter the conformational preferences of the peptide backbone in the vicinity of the glycosylation site, leading to a higher probability of more compact conformations. These effects seem to involve only the first few residues of the glycan and are probably mediated by steric and hydrophobic or hydrophilic interactions between the core glycan residues and the neighbouring amino acid sidechains. (Similar results have been obtained in studies of O-linked

Figure 2



Molecular dynamics results on $Man_9GlcNAc_2$ [7]. **(a)** Overlay of individual snapshots taken at 100 ps intervals. The glycan arms are colour-coded (dark blue, $Man_3GlcNAc_2$ core; light blue, 3-arm; green and yellow, two 6-arms). The overall topology of the molecule is well preserved, given the high degree of flexibility for each individual linkage (example in Figure 1). **(b)** Single snapshot of $Man_9GlcNAc_2$ showing the positions of water molecules (red spheres) involved in interarm hydrogen bonding, together with their occupancies based on the molecular dynamics results.

glycopeptides [14] and particularly of heavily O-glycosylated mucins [15]).

Glycans attached to proteins

There are relatively few examples of glycoproteins where extensive structural data on both the peptide and glycan components are available. The main examples are discussed below and illustrate how local environment can lead to different orientations of an N-glycan with respect to its protein. Possibly the most important point to emerge from the examples below is the long-range stabilisation of the tertiary or quaternary fold of a protein by addition of a glycan.

CD2

CD2 is a cell-surface glycoprotein involved in cell–cell recognition. The extracellular part of the molecule consists of two glycosylated immunoglobulin domains. The crystal structure of soluble CD2 has been solved [16], but only after enzymatically removing the glycans to leave a single GlcNAc residue prior to crystallisation. Solution structures of domain 1 have been determined by NMR for the unglycosylated domain [17] and for both the peptide [18] and glycan [19] components of the glycosylated domain. There are no significant differences in the polypeptide conformation of domain 1 among the three structures. Thus, as expected, the presence of the glycan does not alter the basic immunoglobulin fold.

Interestingly, the presence of the protein does not seem to alter significantly the average glycan structure, although it does reduce the flexibility of the core residues, probably due to the interactions with the protein surface [20]. Direct peptide–glycan contacts are seen between the first two GlcNAc residues of the glycan and seven amino acid sidechains (two asparagine, aspartate, two lysine, phenylalanine and threonine) of the peptide [20]. The glycosylated asparagine residue is on the surface of the protein, and so these interactions result in the whole glycan lying parallel to the peptide surface (Figure 3a).

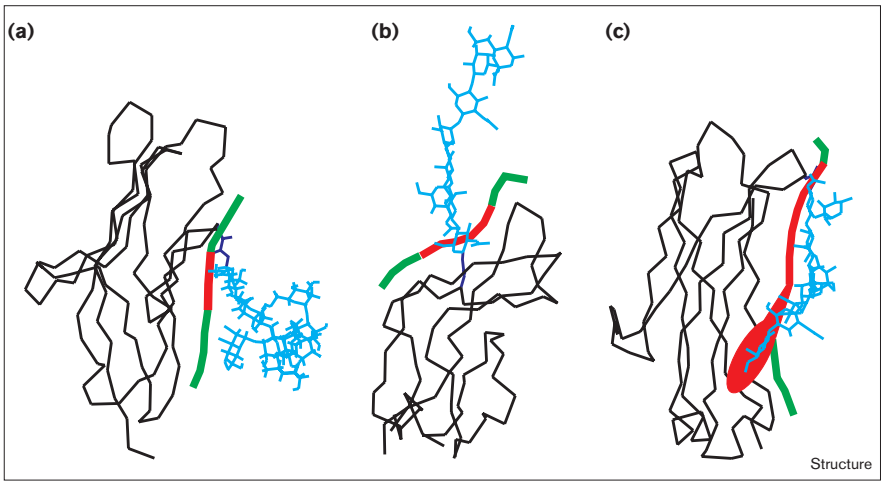
Ribonuclease B

Bovine pancreatic ribonuclease is a mixture of two forms with identical peptide sequence, the aglycosyl ribonuclease A and the monoglucosylated ribonuclease B. In contrast to CD2, ribonuclease B was crystallised with intact glycans. A direct comparison of the X-ray structures of ribonuclease A and B shows no statistically significant differences in the peptide [21].

In the ribonuclease B crystal structure, electron density can be seen for the glycan extending away from the peptide surface into the solvent. This density is discontinuous and cannot be interpreted in terms of a single glycan structure, suggesting that the glycans are either disordered or mobile within the crystal [21]. The NMR parameters for the glycan component of ribonuclease B are virtually identical to those of the free glycan. Thus, again the peptide does not seem to be affecting the average glycan conformation in solution.

However, the situation is very different if the dynamic properties of the peptide and glycan components are investigated. The presence of the glycan reduces solvent access to many regions of the peptide backbone both close to and remote from the glycosylation site (Figure 4), suggesting that it reduces fluctuations of the backbone [22]. This is confirmed by circular dichroism (CD) studies that indicate that the glycan has a small stabilising effect on the peptide fold [22]. ^1H relaxation-rate measurements suggest that the glycan component is not freely moving with respect to the peptide component but that some of the glycan residues

Figure 3



The position of the asparagine sidechain (dark blue) and the interactions between the glycan (light blue) and the local surface of the peptide determine the presentation of the glycan. The coloured bars show the surface of the protein local to the glycosylation site. The glycans interact with the red areas but not with the green areas. **(a)** One of the NMR structures of glycosylated domain 1 of CD2 [20]. The glycosylated asparagine is on the peptide surface. However, interactions of the glycan core with the peptide orient the entire glycan parallel to the surface. **(b)** Part of the hCG crystal structure [24] with a modelled glycan at Asn78. The glycosylated asparagine is in a pocket on the protein surface. Although there are extensive interactions of the glycan core with the peptide, the entire glycan is oriented away from the peptide surface. The glycan was built using the database of average crystallographic glycosidic linkages [1] and added to the crystal structure by overlaying the first GlcNAc residue. **(c)** The crystal structure of the C_H2 region of IgG Fc [33]. The glycosylated asparagine is on a loop pointing away from the peptide surface. However, extensive interactions of the whole of the glycan core and 6-arm with the peptide result in the glycan lying along the surface.

have similar mobilities to the amino acid sidechains, whereas others have considerably more dynamic freedom [23]. Importantly, the glycan has a significant effect on the peptide dynamics, whereas the peptide seems to have a smaller and more localised effect on the glycan dynamics.

Human chorionic gonadotrophin

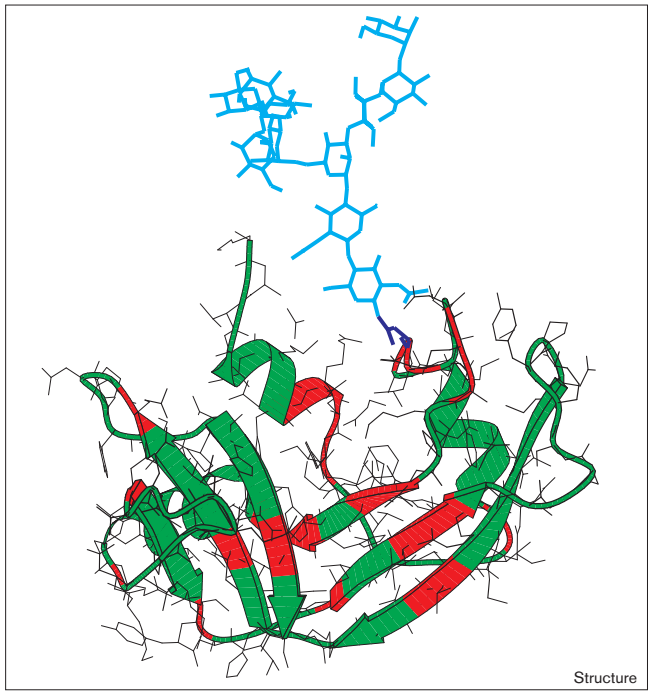
The hormone human chorionic gonadotropin (hCG) is a heterodimer with both the α and β chains glycosylated. A crystal structure is available for hCG [24] but partial deglycosylation (using hydrofluoric acid) was required before crystallisation. NMR studies on the free α subunit show that deglycosylation does not notably affect the peptide conformation [25]. However, deglycosylation does reduce the thermal stability of the α subunit [26].

NMR studies on isotopically ^{13}C,^{15}N-enriched α chain in the intact hCG dimer have shown that the glycans seem to extend into the solvent, having similar conformational properties to the free oligosaccharides [27]. No NOEs were observed between the peptide and the glycans [27]. In contrast, NMR studies on the free α subunit have shown extensive contacts between the first two GlcNAc residues of the glycan at Asn78 and the peptide hydrophobic core [25,28], leading to a reduction in mobility of the core glycan residues. These extensive interactions result from the linkage site being situated in a pocket in the protein surface, with the first two glycan residues making contacts with the sides of the pocket. This orientates the remainder of the glycan out into the solution (Figure 3b).

Immunoglobulin G Fc

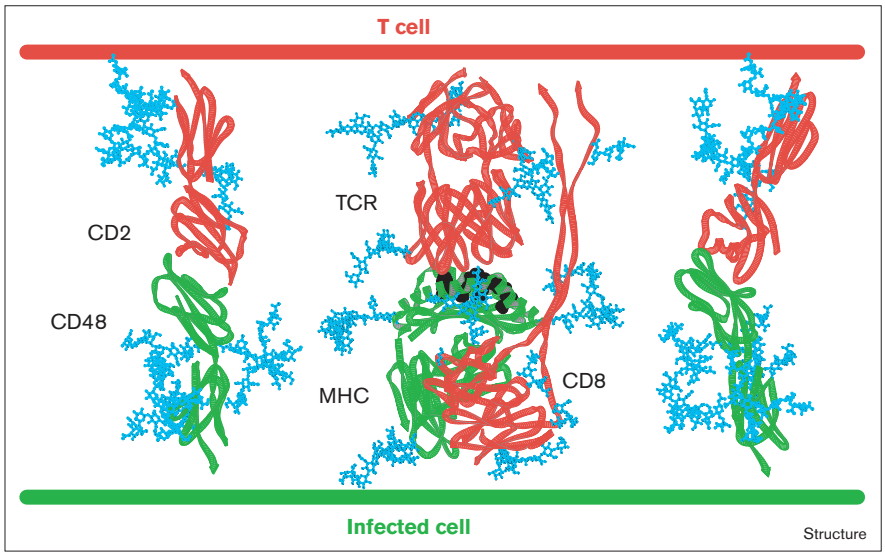
The first glycoprotein to be crystallised was immunoglobulin G Fc (IgG Fc). The structure at 3.5 Å resolution [29] showed that the two glycans occupy the space between the C_H2 domains, with extensive contacts between the

Figure 4



The presence of the glycan (light blue) on ribonuclease B reduces the amide proton/deuterium exchange rates compared with ribonuclease A for extensive regions of the peptide backbone (shown in red) both local to and remote from the glycosylation site [34]. The glycoprotein structure is based on the crystal structure of ribonuclease B [21] and one of the structures of $Man_9GlcNAc_2$ determined by NMR and molecular dynamics [7].

Figure 5



Schematic representation of part of the interface between a T cell and an infected cell (murine). The T-cell proteins are shown in red, the infected cell proteins in green. Recognition of an infected cell by a T cell requires the presentation of antigenic peptides (black) by major histocompatibility complex (MHC) class I molecules on the surface of the infected cell to the T-cell receptor (TCR). However, to obtain the prolonged cell–cell interaction necessary for elimination of the infected cell requires many other protein–protein interactions to occur [35], such as stabilisation of the TCR–MHC complex by CD8 and binding of multiple copies of CD2 on the T cell to multiple copies of CD48 (or CD58 in humans) on the target cell. All these proteins are highly glycosylated. The glycans do not interfere with the surfaces used for the contacts between proteins on different cells, but will have a considerable effect on the contacts between (organisation of) proteins on the same cell and on interactions between a protein and its own membrane.

peptide and the glycans (Figure 3c). These interactions lead to a distortion of the glycosidic linkage involving the glycan 6-arm terminal galactose residue [1]. ^{13}C [30] and ^{1}H [31] NMR relaxation studies in solution have shown the glycans to have the same dynamic properties as the peptide, thus their immobilisation is not a crystal artefact. Removal of the terminal 6-arm galactose residues results in increased mobility of the entire glycan [31]. The crystal structure of IgG Fc with missing 6-arm galactose residues gives no resolvable density for the glycans [32], also indicating increased glycan mobility. Thus, in contrast to the previous examples, the peptide significantly affects the glycan conformation and the orientation of the glycan with respect to the peptide surface is determined by strong interactions involving the terminal galactose (rather than just the chitobiose core). Comparison of the agalacto X-ray structure with normal IgG Fc reveals no changes to the peptide backbone within the domains, but the lower hinge region is disordered in the agalacto structure [32]. Thus, the glycans seem to alter/stabilise the Fc quaternary structure.

Glycoproteins at the cell surface

The majority of glycoproteins, such as CD2, are found at the cell surface. The interactions between such proteins involved in cell–cell recognition events are frequently weak. Strong cell–cell interactions are obtained from many such interactions occurring simultaneously on two complementary surfaces, providing a high level of specificity. The complementarity of these surfaces will depend not only on their composition but also on the presentation, spatial organisation and diffusion rates of the many cell-surface proteins involved. The glycans on these molecules will affect their orientation and their packing on the cell surface (Figure 5). This means that frequently important biological effects of glycosylation may only be manifest in the *in vivo* situation.

Conclusions

Even though only a limited number of glycoproteins have so far been studied in detail, the following points seem to be emerging. First, the presentation of an N-linked glycan on a glycoprotein is determined, in most cases, by interactions between the surface and the first couple of glycan residues. Second, linkage of a glycan to a protein rarely changes the average structure of the glycan. The glycan residues are usually more mobile than the protein to which they are attached but the mobility of the core residues is reduced. Third, N-glycosylation of a folded protein (in contrast to the glycosylation of peptides) does not affect the average backbone fold. It does, however, seem to have a significant stabilising effect on large regions of the backbone structure remote from the glycan, presumably by reducing the backbone flexibility. The presentation of the glycans on glycoproteins can affect their availability for recognition by lectins but may also play a role in modulating protein organisation on cell surfaces.The most general structural effect of protein glycosylation may turn out to be the alteration of the dynamics of both the peptide and glycan parts of the structure.

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