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Observation of a unique pattern of bifurcated hydrogen bonds in the crystal structures of the *N*-glycoprotein linkage region models

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Elucidation of the intra- and intermolecular carbohydrate–protein interactions would greatly contribute toward obtaining a better understanding of the structure–function correlations of the protein-linked glycans. The weak interactions involving C–H...O have recently been attracting immense attention in the domain of biomolecular recognition. However, there has been no report so far on the occurrence of C–H...O hydrogen bonds in the crystal structures of models and analogs of *N*-glycoproteins. We present herein an analysis of C–H...O interactions in the crystal structures of all *N*-glycoprotein linkage region models and analogs. The study reveals a cooperative network of bifurcated hydrogen bonds consisting of N–H...O and C–H...O interactions seen uniquely for the models. The cooperative network consists of two antiparallel chains of bifurcated hydrogen bonds, one involving N1–H, C2′–H and O1′ of the aglycon moiety and the other involving N2–H, C1–H and O1″ of the sugar. Such bifurcated hydrogen bonds between the core glycan and protein are likely to play an important role in the folding and stabilization of proteins.

Key words: carbohydrates/C–H...O interactions/H bonding/*N*-glycoprotein models and analogs/X-ray diffraction

Introduction

The oligosaccharide components of glycoproteins play key roles in many extracellular as well as intracellular processes as recognition determinants and modulators of intrinsic properties, including folding and stability, of proteins (Varki *et al*., 1999). Owing to their structural complexity, microheterogeneity, flexibility, and non-availability in sufficient amounts, understanding the structure–function correlations of the protein-linked glycans is indeed a challenging problem in glycobiology. The linkage region constituents, GlcNAc and Asn, are conserved in the *N*-glycoproteins of all eukaryotes, and interestingly Gln, a single carbon homolog of Asn, has not been found to be glycosylated in nature (Spiro, 2002). Elucidation of the conformation of the *N*-glycoprotein linkage region and the molecular basis of intramolecular glycan–protein interactions is of fundamental importance considering that (a) motion of the GlcNAc–Asn linkage can profoundly influence the presentation of the glycan chains on the cell surface and (b) the protein folding and quality-control mechanisms depend on the precise location of the *N*-glycosylation sites.

The crystallization of glycoproteins remains a formidable task and even among the crystal structures of glycoproteins reported, most often part or all of the glycan chain is not observed in the high-resolution electron density map (Imberty and Perez, 1995; Petrescu *et al*., 2004). The statistical analysis of *N*-glycosidic linkages in 26 glycoproteins revealed that the rotomer distribution of the Asn side chains conformed to that observed on non-glycosylated structures (Imberty and Perez, 1995). The protein–glycan interactions were characterized by some hydrogen bonds. In particular, the *N*-linked GlcNAc was observed to interact through O6 and N2, both acting as donors, with the side chains of amino acids surrounding the linkage region. Recent analysis of *N*-glycosylation sites in 506 glycoprotein crystal structures showed that glycosylation altered the Asn side chain torsion angle distribution and reduced its flexibility (Petrescu *et al*., 2004). In globular proteins, the *N*-linked GlcNAc residue was found to be in contact with the protein surface. In many cases, the Asn and core glycan residue(s) were seen to fill a groove and to make extensive contacts with the protein surface.

Structural investigation using well-defined model compounds of *N*-glycoproteins is a valuable approach to obtain the finer details of atomic architecture and molecular recognition and also to understand the effect of structural variation on the linkage region conformation. A major program of our research based on this approach is focused on X-ray crystallographic investigation of *N*-glycoprotein linkage region models and analogs. Our recent crystallographic examination (Lakshmanan *et al*., 2003) of several β-1-*N*-acylamidoglycopyranose derivatives demonstrated, for the first time, the effect of structural variation in both the linkage region sugar and its aglycon moiety on the *N*-glycosidic torsion. As the structure of saccharide or aglycon moiety is varied, the torsion angle ϕ_N (O5-C1-N1-C1′) sweeps a range of values deviating from that of the reference model GlcNAcβNHAc.1H_2O (**1**) (Figure 1), by as much as 31.9°. In an effort to rationalize the differences in the torsion values and to obtain a better understanding of the structural significance of the linkage region constituents, we have now undertaken a comprehensive analysis of glycan assembly in the crystal structures of all *N*-glycoprotein models and analogs reported in literature (Delbaere, 1974; Ohenassien *et al*., 1980; Bush *et al*., 1982; Sriram *et al*., 1997; Sriram D, Lakshmanan T *et al*., 1998; Sriram D, Srinivasan S *et al*., 1998; Lakshmanan and Loganathan, 2001; Aich *et al*.,

Dedicated to late Prof. George Alan Jeffrey

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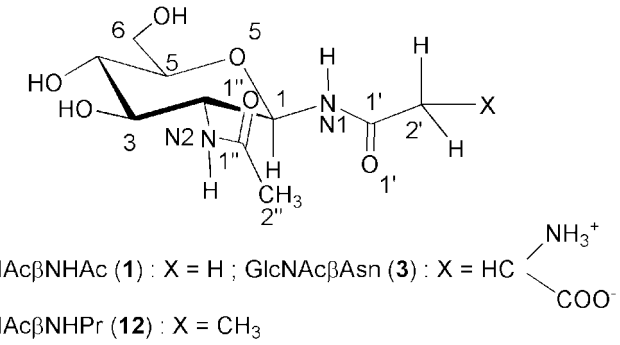

Fig. 1. Structures of *N*-glycoprotein linkage region models **1** and **3** and an analog **12**.

2003; Lakshmanan *et al*., 2003). We disclose herein that GlcNAcβNHAc.1H_2O (**1**) exhibits unique antiparallel chains of hydrogen bonds involving N–H...O and C–H...O interactions, a feature shared *only* by GlcNAcβAsn (**3**) (Delbaere, 1974; Ohenassien *et al*., 1980) and not by any analog including GlcNAcβNHPr.1H_2O (**12**).

The weak interactions involving C–H...O have been attracting immense attention in the domain of biomolecular recognition (Jeffrey and Saenger, 1991; Desiraju and Steiner, 1999). These unconventional hydrogen bonds occurring in the crystal structures of biological macromolecules are suggested to play an important role in their stabilization and function (Wahl and Sundaralingam, 1997). There has, however, been no report so far on the occurrence of C–H...O hydrogen bonds in the crystal structures of models and analogs of *N*-glycoproteins. In the present study, analysis of C–H...X (where X = O / N / Cl) interactions present in the crystal structures of 14 compounds (Figure 2) has been performed.

Results and Discussion

Among the compounds examined, GlcNAcβNHAc.1H_2O (**1**) (Sriram D, Lakshmanan T *et al*., 1998) represents the simplest model of the highly conserved GlcNAc–Asn linkage region. The benzamido analog of **1**, namely GlcNAcβNHBz.1H_2O (**2**) (Sriram D, Lakshmanan T *et al*., 1998), serves to evaluate the competition between π–π stacking and C–H...O hydrogen bonding on the molecular assembly. GlcβNHAc (**5**) (Sriram *et al*., 1997) and RhaβNHAc (**6**) (Lakshmanan *et al*., 2003) are models of GlcβAsn and RhaβAsn, respectively, of the unusual *N*-glycosidic linkages found in *Halobacter halobium* S layer glycoprotein and surface layer glycoprotein of *Bacillus stearothermophillus*, while LacβNHAc.2H_2O (**7**) (Lakshmanan and Loganathan, 2001) is a disaccharide analog of **5**. Compounds **8**, **9** and **10** are β-1-*N*-acetamido derivatives (Lakshmanan *et al*., 2003) of Gal, Man, and Xyl, respectively, and these sugar residues are typically linked to Ser/Thr in O-glycoproteins. The propionamido derivatives, GlcβNHPr (**11**) (Lakshmanan *et al*., 2003), and GlcNAcβNHPr (**12**) (Lakshmanan *et al*., 2003) are analogs of the respective sugar conjugates of Gln, hitherto unknown in nature. The influence of chlorine in place of the methyl group present in the propionamido analog (**11**) on the molecular assembly was sought to be probed by examining the crystal structures of the β-1-*N*-chloroacetamido derivatives (Aich *et al*., 2003) of Glc (**13**) and Gal (**14**). A comparative analysis of hydrogen bond networks present in these 12 crystal structures reported from our laboratory with those of GlcNAcβAsn [**3a**, trihydrate (Delbaere, 1974); **3b**, polyhydrate (Ohenassien *et al*., 1980)] and GlcβAsn **14**, monohydrate (Delbaere, 1974), reported by others, was carried out.

The crystal packing of all the 14 compounds was explored by analyzing their X-ray crystallographic data using the program Mercury 1.3. Each of the short C–H...X (X = O/N/Cl) contacts was ascertained using four different geometrical parameters viz., the distance C...X representing the donor–acceptor distance (*D*); the distance H...X (*d*), the hydrogen bond distance; the C–H...X angle (θ); and the H...X-C angle (φ). Only those interactions for which the *D* values are shorter than 3.6Å and θ values greater than 110° are considered as significant, and these values for compounds **1**, **3**, and **12** are listed in Table I. The values of *D*, ranging from 3.179 to 3.452Å, and θ, varying from 111 to 153°, are in good agreement with those reported (Jeffrey and Saenger, 1991; Desiraju and Steiner, 1999) for C–H...O interactions in small molecules. All these three compounds display a C–H...O hydrogen bond involving the anomeric hydrogen, whereas the one involving C2′–H of the aglycon moiety is observed only in **1** and **3** and not in the propionamide analog, **12**. A combined examination of these C–H...O interactions along with the N–H...O hydrogen bonds reveals the unique cooperative network (Figures 3 and 4). In the crystal structures of **1** and **3**, each of the amido oxygen atoms O1′ and O1″ accepts the donation of two hydrogens from N1–H and C2′–H and N2–H and C1–H, respectively. This results in antiparallel chains of bifurcated hydrogen bonds extending infinitely to serve as double-reinforced pillars that stabilize the molecular architecture (Figures 3 and 4).

In sharp contrast, this unique stabilization motif is absent in **12**, which exhibits only a *single* chain of bifurcated hydrogen bonds consisting of a C–H...O interaction involving the anomeric hydrogen (C1–H) (Figure 5). This striking difference is noteworthy considering that compound **12** is an analog of the hitherto unknown GlcNAcβ-Gln linkage. The N–H...O and C–H...O hydrogen bonds observed for the benzamido analog, GlcNAcβNHBz.1H_2O (**2**), are identical to those of the propionamide derivative **12** (Table II). The molecular assembly of the former is further stabilized by the complementary π–π interactions between the phenyl rings with a close contact of distance of 3.29Å as reported earlier (Sriram D, Lakshmanan T *et al*., 1998). Furthermore, all the other compounds **4**–**11** and **13**–**14** do not display any bifurcated hydrogen bonds involving C2′–H...O1' and N1–H...O1' interactions (Table II). Nevertheless, the monosaccharides **4**, **5**, **6**, **11**, and **13** derived from Glc and Rha, the residues that are known to be attached to Asn in certain bacteria, do consistently show one or more C–H...O interactions involving C2′–H of the aglycon moiety (Figures 6 and 7), revealing the propensity of the Asn side chain to engage in C–H...O interactions. Taken together, the above findings bring out the hallmark feature of the *N*-glycoprotein linkage region constituents GlcNAc and Asn—the double-reinforced pillars consisting of bifurcated hydrogen bonds consisting of C2′–H...O1′ and N1–H...O1′ and C1–H...O1″ and N2–H...O1″ interactions. Being adjacent

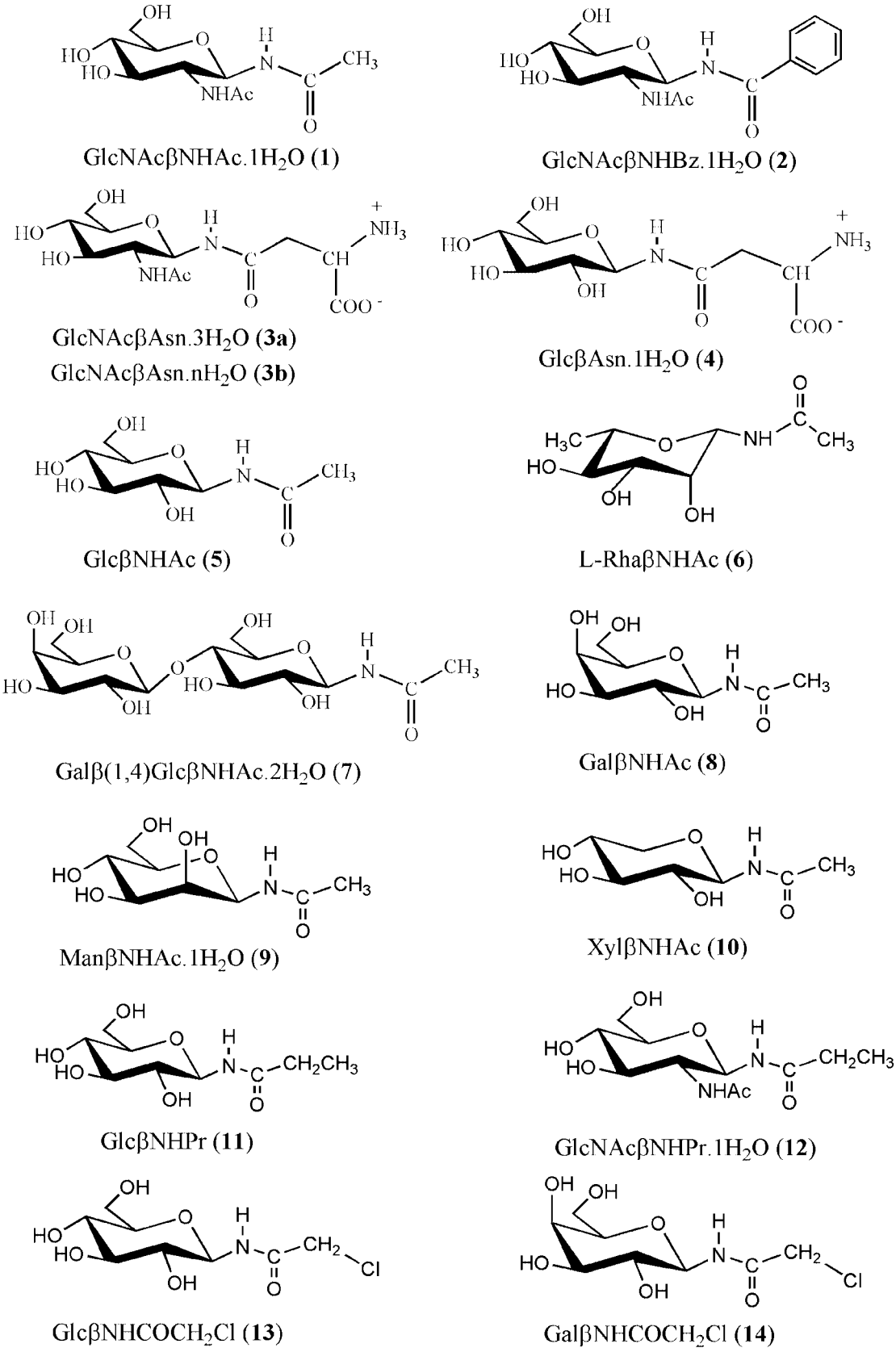

Fig. 2. Chemical structures of all compounds whose X-ray data have been examined.

Table I. Geometrical parameters for C–H...O hydrogen bonds observed in **1**, **3** and **12**

C–H...O bonds	*d* (H...A) Å	*D* (D...A) Å	<DHA (°)
GlcNAcβNHAc.1H_2O (**1**) [$\mathbf{P2_1}$, monoclinic]			
C2′–H...O1′	2.66	3.397	137
C1–H...O1″	2.56	3.254	131
C2″–H...O5	2.54	3.180	122
GlcNAcβAsn.3H_2O (**3a**) [$\mathbf{P2_1}$, monoclinic]			
C2′–H...O1′	2.62	3.263	130
C1–H...O1″	2.74	3.452	134
C2″–H...O5	2.58	3.316	139
C6–H...O3′	2.65	3.432	146
GlcNAcβAsn.nH_2O (**3b**) [$\mathbf{P2_1}$, monoclinic]			
C2′–H...O1′	2.55	3.233	134
C1–H...O1″	2.63	3.446	131
C6–H...O3′	2.64	3.439	153
GlcNAcβNHPr.1H_2O 12 [$\mathbf{P2_1}$, monoclinic]			
C1–H...O1″	2.53	3.244	129
C2″–H...O5	2.64	3.170	111

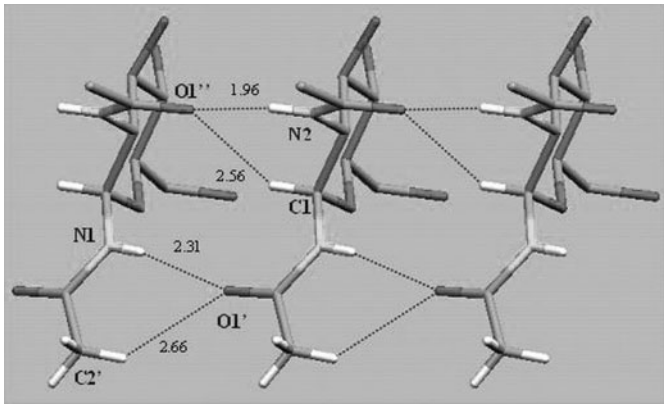

Fig. 3. Packing diagram of GlcNAcβNHAc.1H_2O (**1**) [$\mathbf{P2_1}$, monoclinic]. Shown in dotted lines are the bifurcated hydrogen bonds involving N–H...O and C–H...O interactions forming a pair of antiparallel chains. H atoms attached to O are omitted for clarity.

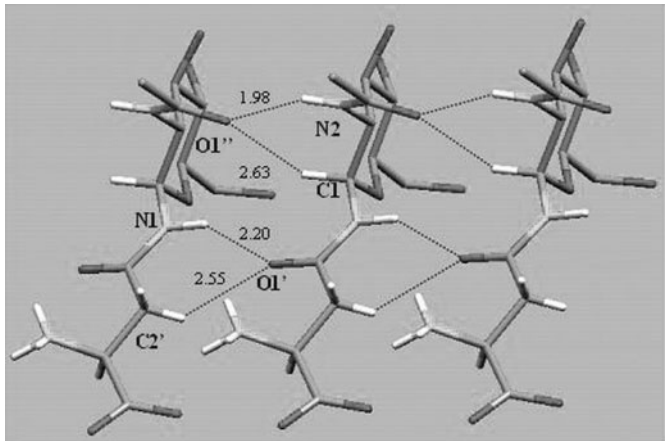

Fig. 4. Packing diagram of GlcNAcβAsn.nH_2O (**3b**) [$\mathbf{P2_1}$, monoclinic]. Shown in dotted lines are the bifurcated hydrogen bonds involving N–H...O and C–H...O interactions forming the same pattern as seen in Fig. 3. H atoms attached to O are omitted for clarity.

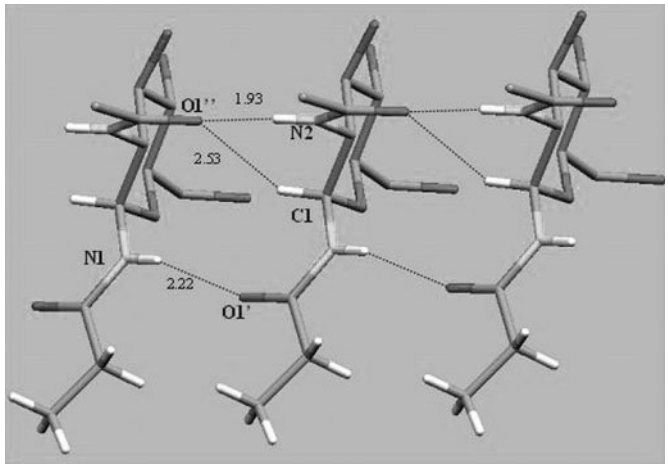

Fig. 5. Packing diagram of GlcNAcβNHPr.1H_2O **12** [$\mathbf{P2_1}$, monoclinic]. Shown in dotted lines are the bifurcated hydrogen bonds involving N–H...O and C–H...O interactions forming only a *single* chain. H atoms attached to O are omitted for clarity.

to the electrophilic carbonyl carbon and hence acidic, C2′–H has the inherent potential to form a strong C–H...O hydrogen bond. The ready donation by the anomeric C1–H is also consistent with the earlier observation (Derewenda *et al*., 1995) that carbons adjacent to N atoms form particularly strong hydrogen bonds. Incidentally, no C–H...N interaction is noticed in any of the 14 compounds nor do the chloroacetamido compounds, **13** and **14**, show any C–H...Cl hydrogen bond in the crystal.

In conclusion, we speculate that the results of the present work have significant implications in the folding and stabilization of proteins and also in the formation of the *N*-glycosidic linkage, the defining event in the biosynthesis of *N*-glycoproteins, catalyzed by the oligosaccharyltransferase. The speculation gains credence from the recent studies based on NMR spectroscopy. First, the measurement of nuclear Overhauser effects (NOEs) for a soluble form of human CD2, a cell-surface glycoprotein on T lymphocytes and natural killer cells, revealed that the protein-proximal GlcNAc–GlcNAc disaccharide was in close contact with amino acid residues of a β-sheet and that proper orientation of this sheet within CD2 was required for folding of the CD58-binding site (Wyss *et al*., 1995). The C–H...O hydrogen bonds are weak individually with energies ranging from 1 to 2 kcal/mol. However, the combination of N–H...O and C–H...O interactions of the type unraveled in the present work can bring about pronounced effects on the folding equilibrium and stabilization of proteins, considering the narrow margins by which tertiary folds of proteins are stabilized over the denatured state. Second, analysis of the chemical shifts and NOE intensities measured for the

Table II. Geometrical parameters for C–H...O hydrogen bonds observed in **2**, **4–11,** and **13–14**

C–H...O bonds	*d* (H...A) Å	*D* (D...A) Å	<DHA (°)
GlcNAcβNHBz.1H_2O (**2**) [$\mathbf{P_1}$, triclinic]			
C1–H...O1″	2.44	3.130	129
C2″–H...O5	2.53	3.155	117
GlcβAsn.1H_2O (**4**) [$\mathbf{P2_1}$, monoclinic]			
C3′–H...O6	2.68	3.398	140
C2′–H...O4	2.36	3.262	158
GlcβNHAc (**5**) [$\mathbf{P2_12_12_1}$, orthorhombic]			
C2′–H – O4	2.55	3.328	139
GlcβNHPr (**11**) [$\mathbf{P2_12_12_1}$, orthorhombic]			
C2′–H...O6	2.70	3.524	143
C2′–H...O4	2.58	3.362	138
C3′–H...O1′	2.59	3.413	145
GlcβNHCOCH_2Cl (**13**) [$\mathbf{P2_12_12_1}$, orthorhombic]			
C2′–H...O6	2.56	3.411	147
C2′–H...O4	2.44	3.204	135
C2′–H...O1′	2.69	3.616	160
LacβNHAc.2H_2O (**7**) [$\mathbf{P_1}$, triclinic]			
C5A–H...O6A	2.57	3.377	139
C6A–H...O2A	2.69	3.414	135
C3B–H...O4B	2.50	3.297	138
L-RhaβNHAc (**6**) [$\mathbf{P2_1}$, monoclinic]			
C2–H...O3	2.46	3.295	150
C2′–H...O4	2.60	3.465	161
C2′–H...O5	2.63	3.488	138
GalβNHAc (**8**) [$\mathbf{P2_12_12_1}$, orthorhombic]			
C2–H...O5	2.68	3.401	133
C6–H...O1′	2.61	3.345	134
GalβNHCOCH_2Cl (**14**) [$\mathbf{P2_12_12_1}$, orthorhombic]			
C2′–H...O1′	2.41	3.356	175
C6–H...O1′	2.71	3.267	116
ManβNHAc. 1H_2O (**9**) [$\mathbf{P2_12_12_1}$, orthorhombic]			
C2′–H...Ow	2.57	3.530	160
XylβNHAc (**10**) [$\mathbf{P2_12_12_1}$, orthorhombic]			
NIL	NIL	NIL	NIL

amino acid residues close to the glycan chain at Asn78 in the α-subunit of human chorionic gonadotropin (Erbel *et al.*, 2000) has shown that GlcNAc–Asn unit shields the protein surface through interactions with predominantly hydrophobic amino acid residues. Based on the present work, one can

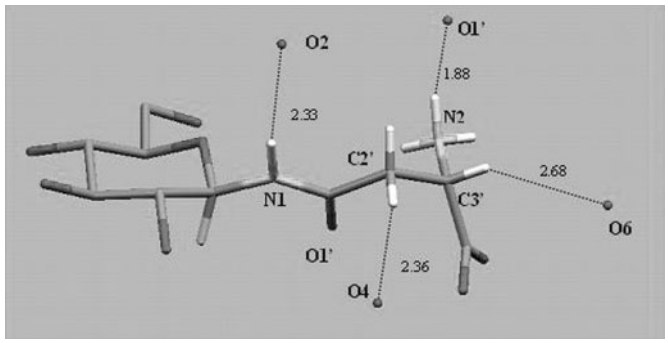

Fig. 6. Packing diagram of GlcβAsn.1H_2O (**4**) [$\boldsymbol{P2_1}$, monoclinic]. Shown in dotted lines are the N–H...O and C–H...O interactions. The bifurcated type of hydrogen bond involving N1–H, C2′–H and O1′ is clearly absent. H atoms attached to O are omitted for clarity.

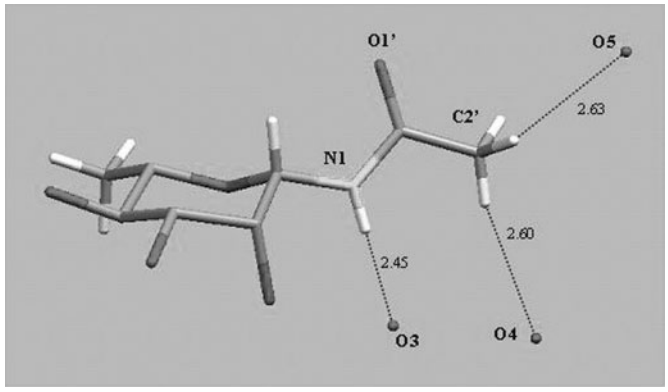

Fig. 7. Packing diagram of L-RhaβNHAc (**6**) [$\boldsymbol{P2_1}$, monoclinic]. Shown in dotted lines are the N–H...O and C–H...O interactions. The bifurcated type of hydrogen bond involving N1–H, C2′–H and O1′ is clearly absent. H atoms attached to O are omitted for clarity.

conceive that the carbonyl oxygen atoms of the C-2 acetamido group of each GlcNAc residue of the core disaccharide and also that of the amido aglycon moiety of the *N*-glycoprotein linkage region are the potentially strong acceptor sites in the glycan part for the C–H donation from the hydrophobic amino acid residues of the protein part. On the other hand, C2′–H of the Asn side chain and the anomeric C1–H represent the strong donors for the acceptor oxygen atoms present in the β-sheet or β-turns of the protein part. Last, the proposed *cis-trans* (*E–Z*) isomerism (Peluso *et al.*, 2002) of the amido aglycon moiety during the oligosaccharyltransferase catalyzed *en bloc* attachment of glycan chain onto the amido nitrogen atom of the Asn could be a key structural transformation process, preceding protein folding, wherein the association–dissociation dynamics of C–H...O hydrogen bonds along with N–H...O interactions between the glycan and protein parts are likely to play a important role.

Materials and Methods

Analysis of the X-ray data set

The atomic coordinates of 17 X-ray crystal structures of *N*-glycoprotein models and analogs were obtained from the

Cambridge Structural Database (CSD, November 2004 release). The reference codes of 15 of these structures in the CSD, their crystallographic quality factors (R) and the corresponding compound codes and numbers used in the present work are as follows: CAKFAV, 2.50, GlcNAcβNHAc.1H_2O, **1**; CAKHIF, 3.30, GlcNAcβNHBz.1H_2O, **2**; BEHPIN, 5.40, GlcNAcβAsn.3H_2O, **3a**; ASGPRS, 6.00, GlcNAcβAsn.nH_2O, **3b**; BEHPOT, 7.20, GlcβAsn.1H_2O, **4**; RESJEE, 3.28, GlcβNHAc, **5**; AVUVAO, 2.49, L-RhaβNHAc, **6**; OCATAN, 2.69, LacβNHAc.2H_2O, **7**; AVUVIW, 3.82, GalβNHAc, **8**; AVUVES, 4.02, ManβNHAc.1H_2O, **9**; AVUVOC, 3.86, XylβNHAc, **10**; AVUTOA, 5.65, GlcβNHPr, **11**; AVUTUG, 5.26, GlcNAcβNHPr.1H_2O, **12**; ERESAV, 4.94, GlcβNHCOCH_2Cl, **13**; ERESEZ, 2.64, GalβNHCOCH_2Cl, **14**.

All structures are based on room temperature measurements. Among 34 C–H...O contacts observed, 28 of them are bonded with $d_{H...O} < 2.7$Å, four of them bonded with $d_{H...O} < 2.5$Å, and two of them bonded with $d_{H...O} < 2.4$Å. Among acceptor oxygen atoms involved in C–H...O contacts, 15 of them are carbonyl oxygens, 13 are hydroxyl oxygens, five are ring oxygens (O5) and only one is from water molecule. All the C–H...O contacts observed are of intermolecular hydrogen bond type. The two additional structures available in the CSD with the reference codes of BUBTIB and PUVQUS were not included in the study. The former corresponds to an anhydrous form (Bush *et al.*, 1982) of compound **1** and several H atoms of both the acetamido groups are missing in the reported structure. The latter structure corresponds to the β-1-*N*-benzamido-D-glucopyranose (Sriram D, Srinivasan S *et al.*, 1998) that has oxygen atoms O2 and O6 disordered over two positions.

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