DOI: 10.1002/chem.200901204

Insights into the Geometrical Features Underlying β-*O*-GlcNAc Glycosylation: Water Pockets Drastically Modulate the Interactions between the Carbohydrate and the Peptide Backbone

Alberto Fernández-Tejada,[a] Francisco Corzana,*[a] Jesús H. Busto,[a] Gonzalo Jiménez-Osés,[b] Jesús Jiménez-Barbero,[c] Alberto Avenoza,[a] and Jesús M. Peregrina*[a]

There is an increasing interest in clarifying the role of β-*N*-acetyl-D-glucosamine (β-GlcNAc) since the discovery that it modifies the chemical, biochemical, and biomedical behavior of an increasing number of cytoplasmic and nuclear proteins, such as transcription factors, nuclear pore proteins, oncogene products, tumor suppressors, and cytoskeletal proteins.[1] This post-translational glycosylation is highly dynamic[2] and draws comparisons with protein phosphorylation as a biological control mechanism. It has been implicated in gene transcription, nuclear trafficking, protein translation,[3] signal transduction,[1a] regulation of protein–protein interactions,[1] and the sensing of nutritional levels within the cell.[4] Furthermore, there is clear evidence that the aberrant *O*-GlcNAc modification of proteins is correlated with diabetes, tumorgenesis, and even with Alzheimer's disease.[1,5]

From a structural point of view, *O*-GlcNAc glycosylation sites do not show obvious consensus sequences. Additionally, in contrast to 'classical' protein glycosylation, *O*-GlcNAc is not elongated or further modified with a complex array of glycans. However, in spite of the importance of *O*-GlcNAc glycosylation, sparse information has been reported to date concerning the geometry and dynamics of β-*O*-GlcNAc-Ser and β-*O*-GlcNAc-Thr motifs.

On the contrary, significant progress has been made with regard to the understanding of the structural properties of the β-*N*-GlcNAc-Asn fragment.[1e] In fact, while numerous crystal structures containing this fragment have been reported to date,[6a–g] only one crystal structure with a serine residue glycosylated with a β-*O*-GlcNAc has been deposited[6h] in the Protein Data Bank. This crystal structure shows the complex between a β-*O*-GlcNAc glycopeptide with the sequence FAPSNYPAL (named K3G) and the MHC Class 1 H-2D^b antibody. The authors suggested the occurrence of two conformations for the *O*-GlcNAc-Ser glycosidic linkage, each displaying similar occupancy. This experimental finding clearly suggests a significant degree of mobility for this moiety. One of these conformers[6] is shown in Figure 1.

On the other hand, the conformational study in aqueous solution of glycopeptides containing the β-*O*-GlcNAc-Ser/

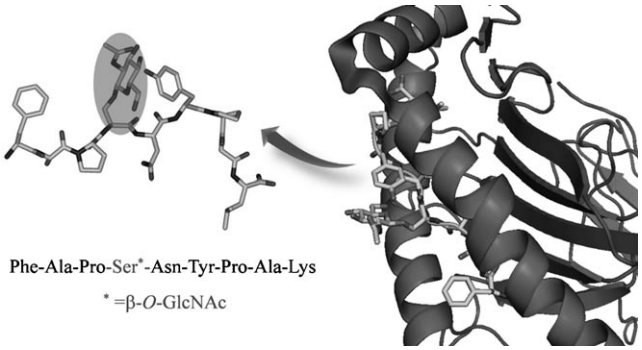


Figure 1. Unique crystal structure found in the Protein Data Bank (1QLF) containing a glycopeptide which incorporates the β-*O*-GlcNAc residue. The structure shows the H-2D^b/glycopeptide complex.[6h]

[a] A. Fernández-Tejada, Dr. F. Corzana, Dr. J. H. Busto, Prof. A. Avenoza, Dr. J. M. Peregrina
Departamento de Química
Universidad de La Rioja
UA-CSIC. Madre de Dios 51
26006 Logroño, La Rioja (Spain)
Fax: (+34) 941 299 621
E-mail: francisco.corzana@unirioja.es
jesusmanuel.peregrina@unirioja.es

[b] Dr. G. Jiménez-Osés
Departamento de Química Orgánica
ICMA-CSIC-Universidad de Zaragoza
Pedro Cerbuna 12, 50009 Zaragoza (Spain)

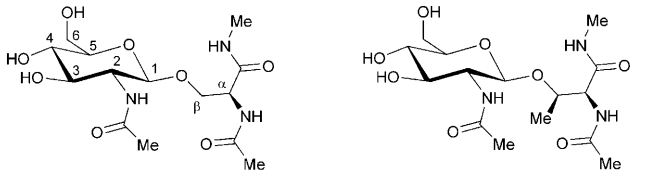
[c] Prof. J. Jiménez-Barbero
Centro de Investigaciones Biológicas (CSIC)
Ramiro Maeztu 9, 28040 Madrid (Spain)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200901204.

Thr moiety has been limited to very few studies, which suggest that the glycosylation of Thr or Ser with β-*O*-GlcNAc promotes turnlike structures.[7] However, in these cases, there is not a clear hypothesis on the mechanism that could support the key influence of the sugar moiety on the peptide backbone.

On chemical grounds, one of the key steps to understand the bioactivity of β-*O*-GlcNAc-Ser and β-*O*-GlcNAc-Thr motifs would be to know their conformational preferences, as well as their dynamics and their interactions (if any) with the peptide backbone. The presentation mode of the GlcNAc moiety could also be of paramount importance for establishing interactions with the corresponding molecular entities.

On this basis, we report herein the conformational analysis in aqueous solution, by NMR spectroscopy and molecular modeling of the simplest model glycopeptides derived from serine and threonine glycosylated with β-*O*-GlcNAc (Scheme 1). The synthesis of the glycopeptides was achieved by following the procedure described by Schmidt and coworkers (see the Supporting Information).[8]



Scheme 1. Model glycopeptides synthesized and studied in this work.

Once the assignment of the NMR spectra was completed (see Experimental Section), selective 1D-NOESY experiments in D_2O (25 °C, pH 5.2) and 2D-NOESY experiments in H_2O/D_2O (9/1) (25 °C, pH 5.2) were then carried out for glycopeptides **1** and **2** (Figure 2). From a quantitative point of view, NOE buildup curves were measured and used to extract the corresponding proton–proton distances. Distances involving the key NH protons were semiquantitatively determined from the integrated volumes of the corresponding cross-peaks. In addition, ^3J coupling constants were measured from the splitting of the resonance signals in the 1D spectra (Figure 2).

The conformational behavior of compounds **1** and **2** was examined by using MD simulations with time-averaged experimental restraints (MD-tar) using the protocol recently described by our group.[9] The results obtained from the MD-tar simulations,[10] for both compounds, showed a close agreement, in numerical terms, between the distances found in the refined models and the experimental NMR data, when using a 16 ns MD-tar simulation in explicit water. The peptide backbone of both derivatives adopted mainly the PPII-like conformation (47% and 43% for **1** and **2**, respectively). Furthermore, around 20% of conformers showed ϕ/ψ dihedral values (backbone) corresponding to helix-like structures (Figure 3a). This result is in good agreement with the weak-medium NOE observed between the NH protons

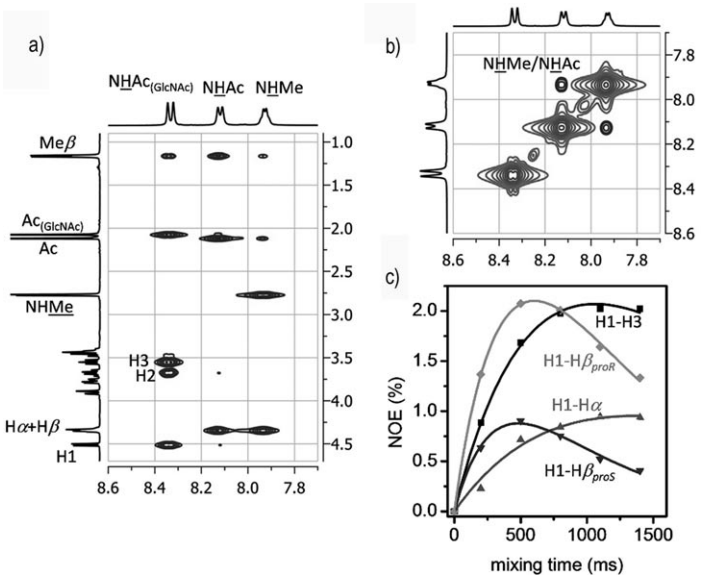


Figure 2. a, b) Sections of the 800 ms 2D NOESY spectrum (400 MHz) in H_2O/D_2O (9:1) at 25 °C of compound **2**, showing the amide cross-peaks. Diagonal peaks and exchange cross-peaks connecting NH protons and water are negative. The NOE contacts are represented as positive cross-peaks. c) NOE buildup curves for H1 of compound **1**.

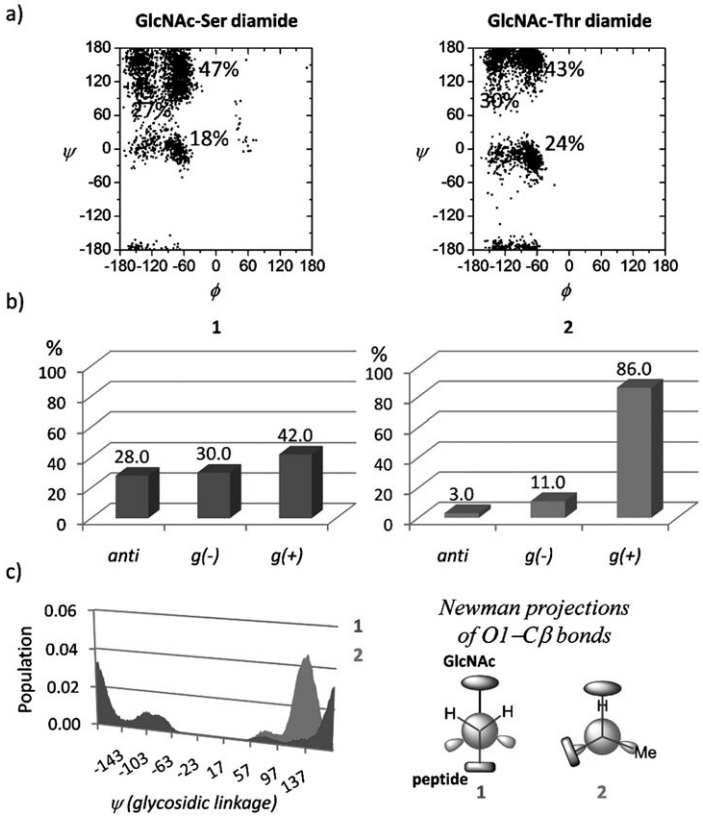


Figure 3. a) ϕ/ψ distributions (backbone) obtained from the MD-tar simulations for compounds **1** (left) and **2** (right). b) χ^1 distributions obtained from the MD-tar simulations for compounds **1** (left) and **2** (right). c) ψ distribution (glycosidic linkage) obtained from the MD-tar simulations for compounds **1** and **2**, showing the Newman projection of the Cβ–O1 bond.

of the backbone (Figure 2b and Supporting Information). As additional evidence of the goodness of the obtained conformational distribution, the experimental and theoretical $^3J(NH_{GlcNAc},H2)$ coupling constants were also in good agreement. The high value of ^3J (9.4 Hz for both compounds) suggests that the orientation of the *N*-acetyl group relative to the sugar framework seems essentially fixed. These results are in agreement with those reported by other authors for β-*O*-GlcNAc carbohydrate[11] or β-*O*-GalNAc glycopeptide derivatives.[12]

As far as the lateral chain (χ^1 torsion angle) is concerned, the simulations suggest that the rotation around χ^1 in the Thr derivative is to some extent restricted, with values for this torsion angle close to 60° (Figure 3b). Although we could not experimentally measure the $^3J_{H\alpha,H\beta}$ value for **2** due to overlapping signals in the spectrum, a $^3J_{H\alpha,H\beta}$ value of 1.8 Hz has been measured for the β-*O*-GlcNAc-Thr motif in a glycopeptide derived from the RNA polymerase II.[7b] In sharp contrast, the lateral chain of the Ser-containing glycopeptide is rather flexible, showing a significant population of each staggered conformation for this torsion angle. This result is in good agreement with the medium $^3J_{H\alpha,H\beta}$ values (6.6 and 5.2 Hz) experimentally observed for derivative **1**.

Concerning the glycosidic linkage, the ϕ (O5-C1-O1-Cβ) dihedral angle was found to be rather rigid for both compounds, exhibiting values around −60°, in perfect agreement with the *exo*-anomeric effect.[13] However, it is important to note that the Ser and Thr derivatives showed markedly different behavior in terms of the ψ (C1-O1-Cβ-Cα) dihedral angle (Figure 3c). In fact, for derivative **2**, this angle showed well-defined values around 120°–140°, resulting in an eclipsed conformation for the Hβ−Cβ and O1−C1 bonds. This conformer avoids the steric repulsions between the methyl group at Cβ and the anomeric H1 proton, which would be present when this ψ angle is close to 180°.

Notably, this conformation has also been observed in the glycopeptide derived from the RNA polymerase II, supported by the observed strong NOE between H1 and Hβ protons, exclusive for this eclipsed conformation.[7b] In contrast to the facts for the Thr-containing molecule (**2**), ψ was found to be more flexible for the Ser-analogue (**1**), with a major *anti*-type arrangement for the GlcNAc residue and the peptide moiety (Figure 3c). The different conformers found in these two molecules for the glycosidic linkage were in good agreement with the adiabatic maps calculated by using a systematic approach with the AMBER force field[14] (see the Supporting Information). Curiously, although the anomeric linkage and the chemical nature of the sugar is completely different, it has to be mentioned that an eclipsed ψ conformation has been previously reported by our group for glycopeptides containing the α-*O*-GalNAc-Thr.[15] This similar behavior could indicate that the conformational tendencies for ψ in *O*-glycopeptides are mainly determined by the presence (or not) of the β-methyl group in the β-hydroxy-α-amino acid residue (Thr or Ser, respectively), irrespective of the chemical nature of the carbohydrate moiety and of the configuration at the anomeric center.

Figure 4a shows the MD-tar ensembles that quantitatively reproduced the experimental NMR data. These drastically different ψ values for compounds **1** and **2** are responsible

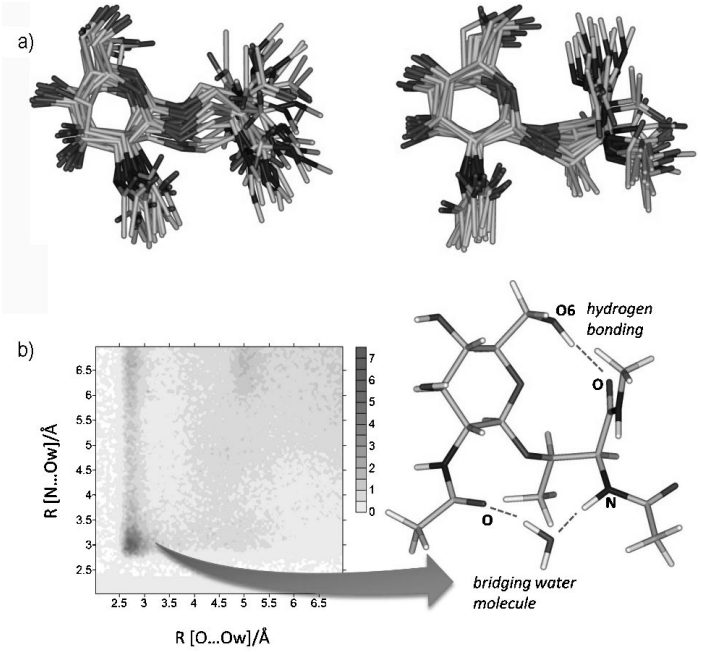


Figure 4. a) Calculated ensembles obtained from the MD-tar simulations for **1** (left) and **2** (right). b) Water pocket between the carbohydrate and the peptide moieties found for compound **2**.

for the existence of a completely different relative orientation between the carbohydrate and the peptide moieties, which affects the presentation mode of the GlcNAc residue. In fact, whereas for Ser-derivative **1**, the acetyl group of the GlcNAc unit is close to the peptide backbone, for its Thr-analogue **2**, the hydroxymethyl group of the carbohydrate fragment is closer to the peptide, allowing the formation of a hydrogen bond between the hydroxymethyl group of the carbohydrate moiety and the carbonyl group of Thr (Figure 4b). This hydrogen bonding was present about 45% of the total trajectory time. In contrast, no hydrogen bond was found above 5% of the total 16 ns trajectory time for compound **1**.

On the other hand, important new insights into the role of water in organizing polysaccharide structure, in protein folding, and in folding of small nucleic acids are emerging from different studies,[11,16] which point to a new way toward visualizing the effects of water on these structures. However, few examples have been published concerning the role of water in the modulation of the interactions between the carbohydrate and peptide backbone in glycopeptides,[9,15] which could modify the orientation and/or the flexibility features of the carbohydrate moieties and thus their presentation ability to interact with their biological targets. Therefore, we decided to investigate the anisotropy hydration of both compounds **1** and **2** following our previously described proto-

col.[9] Normalized two-dimensional radial pair distributions were calculated for all possible share water density sites.[17] These calculations lead to water densities higher than the bulk water density, in regions where structural or semistructural water molecules are present.

Figure 4b shows the 2D radial pair distribution obtained for the oxygen atom of the acetyl group of GlcNAc and the Thr-nitrogen of derivative **2**. The shared water density found in the first hydration shell of both atoms revealed the existence of bridging water molecules between them. The density of this shared water site was 7.0 times the bulk density. Importantly, a water pocket involving the *N*-acetyl group has been previously found by ab initio calculations in GlcNAc carbohydrate.[11] On the other hand, although the water pocket observed in glycopeptide **2** was also present in compound **1**, in this case, the density of the shared water site was only about twice that of the bulk. This difference is likely due to both the distinct flexibility of the lateral chains and the different orientation of the aglyconic linkages. Markedly, for both compounds, this water pocket was more persistent (about 1.5 times) when the helix-like structures were present. In addition, both the hydrogen bond and a bridging water molecule were present at the same time about 20% of the trajectory time.

Therefore, we propose a novel and simple model for explaining the different relative orientation of the peptide backbone and presentation of β-*O*-GlcNAc-Thr and Ser moieties. The sugar–peptide interactions are modulated not only by specific hydrogen bonds[18] but also by the existence of water pockets at key sites. It is likely that the existence of these solvent pockets could also have important biological implications, providing the required presentation of the GlcNAc moieties to interact with their biological receptors.

Experimental Section

Characterization of compounds 1 and 2: Compound **1**: M.p. 235–237 °C; $[\alpha]^{25}_{D} = -22.7$ ($c = 0.95$, H_2O); ^{1}H NMR (300 MHz, D_2O): δ = 1.93–2.10 (m, 6H, 2COCH$_3$), 2.70 (s, 3H, NHCH_3), 3.38–3.56 (m, 3H, H_4, H_5, H_3), 3.60–3.77 (m, 2H, H_2, H_6), 3.81–3.94 (m, 2H, H_β, H_6), 4.01 (dd, $^3J_{H,H}$ = 10.8, 6.7 Hz, 1H, H_β), 4.40 ('t', $^3J_{H,H}$ = 5.8 Hz, 1H, H_α), 4.49 ppm (d, $^3J_{H,H}$ = 8.4 Hz, 1H, H_1); ^{1}H NMR (400 MHz, H_2O/D_2O, 9:1): δ = 7.87–7.94 (m, 1H, N*H*Me), 8.10 (d, $^3J_{H,H}$ = 9.4 Hz, 1H, N*H*Ac$_{GlcNAc}$), 8.14 ppm (d, $^3J_{H,H}$ = 6.8 Hz, 1H, N*H*Ac); ^{13}C NMR (100 MHz, D_2O): δ = 21.8 (*C*H$_3$CO), 22.1 (*C*H$_3$CO), 25.9 (NHCH$_3$), 53.8 (C_α), 55.3 (C_2), 60.6 (C_6), 68.1 (C_β), 69.8 (C_4), 73.6 (C_3), 75.8 (C_5), 100.8 (C_1), 171.6, 174.3, 174.5 ppm (3CO); elemental analysis calcd (%) for $C_{14}H_{25}N_3O_8$: C 46.28, H 6.93, N 11.56; found: C 46.38, H 6.90, N 11.61.

Compound **2**: M.p. 225–227 °C; $[\alpha]^{25}_{D} = -30.6$ ($c = 1.15$, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 1.16 (d, $^3J_{H,H}$ = 6.2 Hz, 3H, βCH$_3$), 2.02–2.14 (m, 6H, COCH$_3$), 2.76 (s, 3H, NHCH_3), 3.38–3.49 (m, 2H, H_5, H_4), 3.54 ('t', $^3J_{H,H}$ = 9.1 Hz, 1H, H_3), 3.63–3.70 (m, 1H, H_2), 3.76 (dd, $^3J_{H,H}$ = 12.2, 5.2 Hz, 1H, H_{6proR}), 3.89 (dd, $^3J_{H,H}$ = 12.2, 2.1 Hz, 1H, H_{6proS}), 4.30–4.39 (m, 2H, H_β, H_α), 4.50 ppm (d, $^3J_{H,H}$ = 8.4 Hz, 1H, H_1); ^{1}H NMR (400 MHz, H_2O/D_2O, 9:1): δ = 7.89–7.96 (m, 1H, N*H*Me), 8.12 (d, $^3J_{H,H}$ = 9.4 Hz, 1H, N*H*Ac), 8.33 ppm (d, $^3J_{H,H}$ = 7.5 Hz, 1H, N*H*Ac$_{GlcNAc}$); ^{13}C NMR (100 MHz, D_2O): δ = 16.4 (βCH$_3$), 21.8, 22.1 (2*C*H$_3$CO), 25.9 (NHCH$_3$), 55.5 (C_2), 58.1 (C_α), 60.7 (C_6), 69.7 (C_4), 73.6 (C_3), 75.0 (C_β), 75.6 (C_5), 100.0 (C_1), 172.0, 174.8, 174.8 ppm (3CO); elemental analysis calcd (%) for $C_{15}H_{27}N_3O_8$: C 47.74, H 7.21, N 11.13; found: C 47.66, H 7.18, N 11.16.

2D NMR experiments: NMR experiments were recorded on a Bruker Avance 400 spectrometer at 298 K. Magnitude-mode ge-2D COSY spectra were recorded with gradients and using the cosygpqf pulse program with 90 degree pulse width. Phase-sensitive ge-2D HSQC spectra were recorded using z-filter and selection before t1 removing the decoupling during acquisition by use of invigpndph pulse program with CNST2 (JHC) = 145. 2D NOESY experiments were made using phase-sensitive ge-2D NOESY with WATERGATE for H_2O/D_2O (9:1) spectra. Selective ge-1D NOESY experiments were carried out using the 1D-DPFGE NOE pulse sequence. NOEs intensities were normalized with respect to the diagonal peak at zero mixing time. Experimental NOEs were fitted to a double exponential function, $f(t) = p_0(e^{-p1t})(1-e^{-p2t})$ with p_0, p_1, and p_2 being adjustable parameters.[19] The initial slope was determined from the first derivative at time $t = 0$, $f'(0) = p_0p_2$. From the initial slopes, interproton distances were obtained by employing the isolated spin pair approximation. The calculated ^3J values were obtained from the simulations by applying the appropriate Karplus equation[20] to the corresponding torsion angles.

Molecular dynamics simulations: MD-tar simulations were performed with AMBER[21] 6.0 (AMBER94),[22] which was implemented with GLYCAM 04 parameters[23] to accurately simulate the conformational behavior of the sugar moiety. NOE-derived distances were included as time-averaged distance constraints, and scalar coupling constants J as time-averaged coupling constraints. A $<r^{-6}>^{-1/6}$ average was used for the distances and a linear average was used for the coupling constants. Final trajectories were run using an exponential decay constant of 16 ns and a simulation length of 16 ns with explicit water molecules (TIP3P).[24]

Acknowledgements

We thank the Ministerio de Ciencia e Innovación (project CTQ2006-05825/BQU, Ramón y Cajal contract to F.C. and grant to A.F.-T.) and the Universidad de La Rioja (project EGI08/06). We also thank CESGA for computer support.

Keywords: glycopeptides · glycosylation · hydration · molecular dynamics · NMR spectroscopy

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Received: May 6, 2009
Published online: June 19, 2009

