Effects of Glycosylation on Peptide Backbone Conformation

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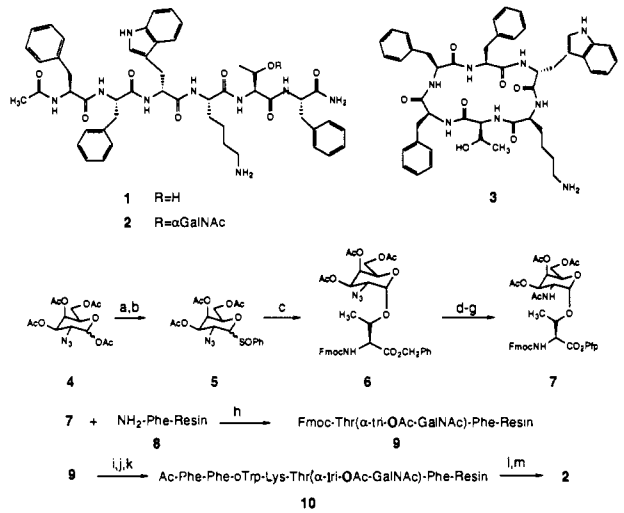
There is biochemical evidence that glycosylation influences peptide and protein structure, plays a role in glycoprotein folding, and increases the stability of some proteins.[1] It has been suggested that these effects are due to a direct interaction of the attached sugars with the peptide (e.g., a hydrogen bond). Glycosylated di- and tripeptides have been used as model systems to look for interactions between attached sugars and the peptide.[2] The results have been inconclusive. We have been studying larger glycosylated peptides to determine how glycosylation affects peptide structure. Our focus is the conformation of the peptide backbone rather than interactions between the sugars and the peptide. Below we present results showing that glycosylation with a single monosaccharide has a profound effect on the backbone conformation of a linear hexapeptide in DMSO, a solvent that, like water, does not promote intramolecular hydrogen-bond formation.[3] Glycosylation limits the conformational space available to this peptide and appears to favor conformations in which the backbone bends away from the sugar.[4] The implications of this result are discussed.

The hexapeptide sequence we are studying is Phe-Phe-D-Trp-Lys-Thr-Phe. The sequence contains a threonine, which is a potential glycosylation site. Veber and Hirschmann[5] designed a constrained (cyclic) analogue of this hexapeptide and showed that it adopts a type II' β-turn around Phe-D-Trp-Lys-Thr. To assess the effects of glycosylation on peptide conformation, we have compared the average backbone conformation of a monoglycosylated linear hexapeptide of this sequence with the linear nonglycosylated hexapeptide and with the constrained peptide.[6]

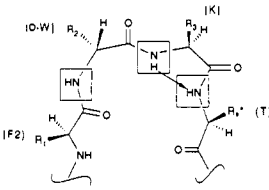
Hexapeptides **1** and **2** were synthesized on Rapp TentaGel resin by solid-phase methods (Scheme I).[7] Cyclic peptide **3** was synthesized as described.[8] ROESY experiments were carried out on all three hexapeptides in DMSO at 30 °C.[9] Since small linear peptides are flexible, the NMR data represent an ensemble average conformation rather than a single conformation. Nevertheless, relative comparisons between ROESY crosspeak intensities (NOEs) provide insight into the ensemble average structure.[10] For flexible peptides, which may not show longer range NOEs, sequential amide-amide NOEs provide useful information about backbone conformation.[11] Histograms of the relative intensities of the amide-amide ROESY crosspeaks for peptides **1**, **2**, and **3** are shown in Figure 1.

For the nonglycosylated hexapeptide **1**, the sequential amide-amide ROESY crosspeaks for Phe-D-Trp-Lys-Thr are approx-

Scheme I[a]



[a] (a) HSPh, BF_3Et_2, CH_2Cl_2, 75%; (b) mCPBA, CH_2Cl_2, -78 °C, 75%; (c) *N*-Fmoc-L-threonine benzyl ester, Tf_2O, 2,6-di-*tert*-butyl-4-methylpyridine, toluene/CH_2Cl_2 (1:1), -78 °C, 70% 1:1 α:β, anomers are separated on silica gel; (d) 5% rhodium on alumina powder, H_2, ethyl acetate/methanol (1:1), 50%; (e) Ac_2O, pyridine, CH_2Cl_2, 95%; (f) Pearlman's catalyst, H_2, 75%; (g) pentafluorophenol, DCC, ethyl acetate, 75%; (h) HOBt, CH_2Cl_2/DMF (1:1); (i) 55% piperidine/DMF; (j) (1) Fmoc-Lys-OPfp, HOBt, CH_2Cl_2/DMF (1:1) (2) 55% piperidine/DMF; (k) identical coupling and deprotection conditions for Fmoc-D-Trp-OPfp, Fmoc-Phe-OPfp, Fmoc-Phe-OPfp, and pentafluorophenyl acetate; (l) 80% trifluoroacetic acid, 15% CH_2Cl_2, 5% thioanisole, 2 h; (m) 0.1 M NaOH, 1 h.



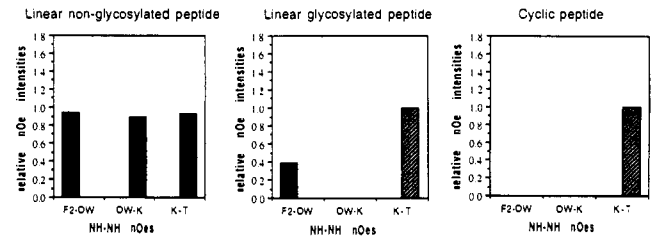


Figure 1. (Top) NOEs indicative of secondary structure in the β-turn. (Bottom) Relative intensities of the amide-amide ROESY crosspeaks for peptides **1**, **2**, and **3**.

(1) (a) Kassenbrock, C. K.; Garcia, P. D.; Walter, P.; Kelly, R. B. *Nature* **1988**, *333*, 90. (b) Kozutsumi, Y.; Segal, M.; Normington, K.; Gething, M. J.; Sambrook, J. *Nature* **1988**, *332*, 462. (c) Ashwell, G.; Morell, A. G. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1974**, *41*, 99.

(2) (a) Ishii, H.; Inoue, Y.; Chujo, R. *Int. J. Peptide Protein Res.* **1984**, *24*, 421. (b) Maeji, N. J.; Inoue, Y.; Chujo, R. *Biopolymers* **1987**, *26*, 1753. (c) Maeji, N. J.; Inoue, Y.; Chujo, R. *Int. J. Pept. Protein Res.* **1987**, *29*, 699. (d) Mimura, Y.; Inoue, Y.; Maeji, N. J.; Chujo, R. *Int. J. Pept. Protein Res.* **1989**, *34*, 363. (e) Hollosi, M.; Perczel, A.; Fasman, G. D. *Biopolymers* **1990**, *29*, 1549.

(3) DMSO was used because the linear nonglycosylated hexapeptide is not water soluble. The peptides do not aggregate in DMSO at the concentrations used (3.7 mM). For some elegant work on the importance of hydrogen bonding in peptide backbone structure, see: Liang, G.-B.; Desper, J. M.; Gellman, S. H. *J. Am. Chem. Soc.* **1993**, *115*, 925.

(4) Kessler et al. have concluded from NMR studies comparing cyclic hexapeptides and the corresponding glycosylated cyclic hexapeptides that there is no change in the backbone conformation of peptides upon glycosylation. However, cyclic peptides are constrained, attenuating any effects of the sugar. Kessler, H.; Matter, H.; Gemmecker, G.; Kottenhahn, M.; Bats, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 4805.

(5) Veber, D. F.; Holly, F. W.; Paleveda, W. J.; Nutt, R. F.; Bergstrand, S. J.; Torchiana, M.; Glitzer, M. S.; Saperstein, R.; Hirschmann, R. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2636.

(6) Carbohydrate moieties found in natural glycopeptides and glycoproteins are very large, but it is thought that most of the structural effects of glycosylation are due to the first one or two carbohydrate units. See, for example: (a) Rose, M. C.; Voter, W. A.; Sage, H.; Brown, C. F.; Kaufman, B. *J. Biol. Chem.* **1984**, *259*, 3167. (b) Matsuura, H.; Takio, K.; Titani, K.; Greene, T.; Levery, S. B.; Salyan, M. E. K.; Hakomori, S. *J. Biol. Chem.* **1988**, *263*, 3314. (c) Matsuura, H.; Greene, T.; Hakomori, S. *J. Biol. Chem.* **1989**, *264*, 10472.

(7) Compound **4** was synthesized according to the procedure of Lemieux, R. U.; Ratcliffe, R. M. *Can. J. Chem.* **1979**, *57*, 1244.

(8) Brady, S. F.; Varga, S. L.; Freidinger, R. M.; Schwenk, D. A.; Mendlowski, M.; Holly, F. W.; Veber, D. F. *J. Org. Chem.* **1979**, *44*, 3101.

(9) Bothner-By, A. A.; Stephens, R. L.; Lee, J.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811.

(10) Although ROESY crosspeak intensities can be influenced by Hartmann-Hahn and off-resonance effects, these factors are not expected to invalidate the relative comparisons of amide-amide crosspeak intensities.

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imately equal in intensity. Each dipeptide unit is thus able to adopt one or more conformations during the mixing period in which the amide protons are close enough to give rise to a ROESY crosspeak. All the amide–amide crosspeaks are of similar intensity, indicating that peptide **1** is very flexible (barring the highly unlikely alternative explanation that this short linear peptide is helical in DMSO). In contrast, the sequential amide–amide crosspeak intensities for glycosylated peptide **2** have different intensities. The Lys-Thr ROESY crosspeak is particularly large, and the D-Trp-Lys crosspeak cannot be detected. Thus, glycosylation with a single monosaccharide changes the average backbone conformation of a linear peptide dramatically, excluding conformations in which the amide protons of D-Trp and Lys approach closely. At the same time, glycosylation appears to *decrease* the average distance between the Lys and Thr amide protons.

The relative amide–amide crosspeak intensities for D-Trp, Lys, and Thr in the linear glycosylated and cyclic peptides are remarkably similar. In the cyclic peptide, D-Trp, Lys, and Thr are in the $(i + 1)$, $(i + 2)$, and $(i + 3)$ positions, respectively, of a type II′ β-turn. A strong amide–amide NOE between $(i + 2)$ and $(i + 3)$ and a weak or nonexistent amide–amide NOE between $(i + 1)$ and $(i + 2)$ is considered strong supporting evidence for a type II′ β-turn.[11] Although the glycosylated peptide is more flexible than the cyclic peptide, the similarity in the relative intensities of the amide–amide NOEs suggests that glycosylation favors conformations in which the peptide backbone bends *away* from the site of glycosylation.[12] In this regard, it is worth pointing out that sugars are frequently found in turn regions of proteins.[13]

In conclusion, we have found that glycosylation of an internal threonine with a single GalNAc residue dramatically changes the ensemble average backbone conformation of a linear hexapeptide.[4] We see no evidence for a specific hydrogen bond. Moreover, the conformational change extends at least two residues beyond the site of glycosylation. The most likely explanation for the observed changes is that the presence of the sugar *excludes* many conformations for steric reasons. We think that attached sugars may influence protein folding, glycoprotein structure, and thermal stability in a similar manner, by restricting conformational space. In any case, it is clear that glycosylation profoundly alters peptide backbone conformation even in the absence of specific hydrogen bonds between the sugar and the peptide. This result raises the question of whether the identity of the specific sugar matters if the general shape and size are similar. Using flexible peptides as model systems, it should be possible to probe the effects of different types of sugars on the average backbone conformation. It should also be possible to assess the influence of even larger saccharides (e.g., di- or trisaccharides) on the conformation of the peptide backbone.[6] Ultimately, such knowledge should prove useful in glycopeptide design.

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Supplementary Material Available: Spectral data (DQF-COSY, ^{1}H NMR and FAB-MS) and HPLC purification conditions for compound **2**; contour plots of amide–amide crosspeak regions for compounds **1**, **2**, and **3**; contour plots of the amide–CαH crosspeak region for the cyclic peptide and compound **2** (10 pages). Ordering information is given on any current masthead page.

(11) (a) Wagner, G.; Neuhaus, D.; Worgotter, E.; Vasak, M.; Kagi, J. R. H.; Wuethrich, K. *J. Mol. Biol.* **1986**, *187*, 131. (b) Wuethrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons, Inc.: New York, 1986; Chapter 9.

(12) Other indicators of turn conformation include small $^3J_{N_iH_i}$ coupling constants and medium-range NOEs. Small $^3J_{N_iH_i}$ coupling constants are rarely observed in linear systems. Moreover, there are no medium-range $d_{\alpha N}(i, i + 2)$ NOEs even in the constrained cyclic peptide. These facts make it difficult to draw more specific conclusions about the structure of the linear glycosylated peptide. Dyson, H. J.; Rance, M.; Houghten, R. A.; Lerner, R. A.; Wright, P. E. *J. Mol. Biol.* **1988**, *201*, 161. Information obtained from CD experiments on short peptides can also be complicated to interpret. Miick, S. M.; Martinez, G. Y.; Fiori, W. R.; Todd, A. P.; Millhauser, G. L. *Nature* **1992**, *359*, 653.

(13) It has been suggested that attached sugars stabilize secondary structure. Inter alia: (a) Urge, L.; Gorbics, L.; Otvos, L., Jr. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 1125. (b) Paulsen, H.; Busch, R.; Sinnwell, V.; Pollex-Krüger, A. *Carbohydr. Res.* **1991**, *214*, 227. (c) Paulsen, H. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 823. Others have suggested that the enzymes responsible for N-glycosylation may recognize nascent turn structure in Asn-X-Thr(Ser) containing peptides. Inter alia: (a) Bause, E. *Biochem. J.* **1983**, *209*, 331. (b) Imperiati, B.; Shannon, K. L.; Rickert, K. W. *J. Am. Chem. Soc.* **1992**, *114*, 7942.