Probing the Effect of the Outer Saccharide Residues of *N*-Linked Glycans on Peptide Conformation

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Asparagine-linked glycosylation, a co-translational protein modification reaction, has the capacity to influence the protein folding process.[1] Within the extracellular domain of the α-subunit of the nicotinic acetylcholine receptor (nAChR), an *N*-linked glycosylation site at Asn141 has been demonstrated to be essential for the folding and assembly of the multimeric nAChR complex.[2] The disulfide bond between residues Cys128 and Cys142 that closes this conserved extracellular loop is also critical for the structural integrity of the receptor complex; mutation of the cysteine residues[3] or expression of the protein in the presence of reducing agents[4] severely impacts the oligomerization of the protein complex. In addition, this loop includes a conserved proline residue at position 136 that is required for nAChR folding and assembly.[5]

In previous studies of this extracellular loop peptide (peptide **1**),[6] a simple chitobiosyl derivative, representing a truncated analogue of the saccharide attached in vivo, at Asn141 was found to influence the free energy of disulfide bond formation and the *cis/trans* isomerization of the proline peptide bond. Since these processes represent slow steps in the protein-folding process,[7] this observation may provide insight into why *N*-linked glycosylation is required for the folding and oligomerization of the nAChR. The purpose of this report is to examine the structural effects that larger, mature oligosaccharides exert on the structure of **1**. Although it is known that outer saccharide residues play critical roles in glycoprotein function,[8] studies to establish how larger saccharides impact peptide and protein conformation have been limited due to the difficulties associated with the preparation of sufficient quantities of homogeneous samples of complex glycopeptides to allow detailed spectroscopic analysis. Since disulfide bond formation and proline isomerization can be assessed quantitatively, it was envisioned that these aspects of peptide structure could be used to accurately measure the conformational effects of the outer saccharides.

Glycosylated analogues of peptide **1** derivatized with a monosaccharide (**2**), and with a disaccharide (**3**), were synthesized using previously published methods.[9,10] Glycopeptide **4**, an analogue derivatized with a complex-type, biantennary saccharide at Asn141, was prepared via a powerful chemoenzymatic method.[11] Specifically, glycopeptide **2** was subjected to treatment with Endo-M, an endoglycosidase enzyme that catalyzes a transglycosylation reaction between the synthetic glycopeptide and a proteolytic fragment of the glycoprotein transferrin to produce glycopeptide **4** in good yield. Additionally, the outer sialic acid and galactose residues were enzymatically cleaved from **4** to generate glycopeptides **5** and **6** (see Figure 1), respectively.[12]

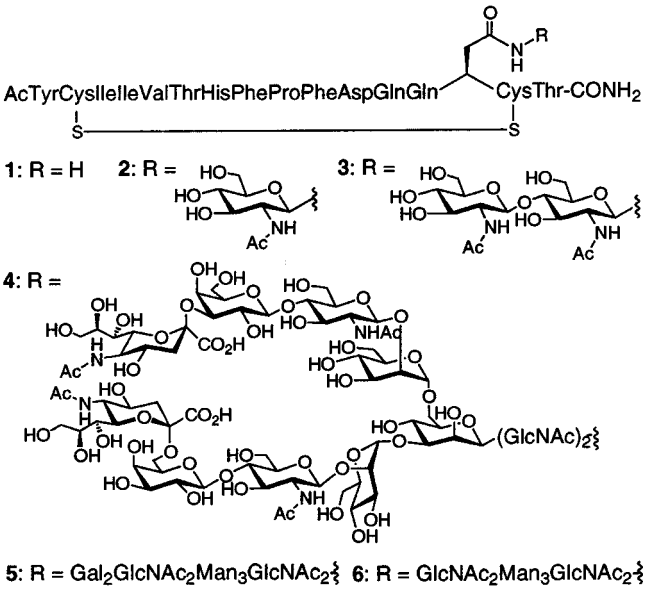

Figure 1. Peptide **1** and glycopeptides **2**, **3**, **4**, **5**, and **6**.

The solution structures of peptides were investigated using standard homonuclear 2D NMR techniques. An NOE-restrained simulated annealing protocol revealed that the peptides adopted a turn structure at the central residues of the loop (His134 and Phe135).[13] Although inspection of the NOESY data revealed no major structural changes between the nonglycosylated and glycosylated peptides, the glycosylated peptides exhibited a number of spectroscopic characteristics that together reveal a modest, but detectable effect upon modification with larger saccharide derivatives.

NMR studies reveal several weak NOE signals between the asparagine and proximal *N*-acetylglucosamine in glycopeptide **4**, but these NOE signals were not observed for peptides **2** and **3**, suggesting that the outer saccharides may conformationally stabilize the core N-acetyl glucosamine residues. The absence of these NOE signals could also be due to the lower molecular weight of **3**; however, examination of a series of reference cross-peaks of glycopeptide **3** and **4** revealed that the change in molecular weight did not impact the relative NOE intensities of these peptides. It is noteworthy that neither glycopeptide **5** or **6** exhibited any significant changes in the NOESY spectra relative

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(1) (a) Rudd, P. M.; Dwek, R. A. *Crit. Rev. Biochem. Mol.* **1997**, *32*, 1−100. (b) O'Connor, S. E.; Imperiali, B. *Chem. Bio.* **1996**, *3*, 803−812.
(2) (a) Mishina, M.; Tobimatsu, T.; Imoto, K.; Tanaka, K.-I.; Fujita, Y.; Fukada, K.; Kurasaki, M.; Takahashi, H.; Morimoto, Y.; Hirose, T.; Inayama, S.; Takahashi, T.; Kuno, M.; Numa, S. *Nature* **1985**, *313*, 364−369. (b) Gehle, V. M.; Sumikawa, K. *Mol. Brain Res.* **1991**, *11*, 17−25.
(3) Sumikawa, K.; Gehle, V. M. *J. Biol. Chem.* **1992**, *267*, 6286−6290.
(4) Gelman, M. S.; Prives, J. M. *J. Biol. Chem.* **1996**, *271*, 10709−10714.
(5) Fu, D.-X.; Sine, S. M. *J. Biol. Chem.* **1996**, *271*, 31479−31484.
(6) Rickert, K. W.; Imperiali, B. *Chem. Biol.* **1995**, *2*, 751−759.
(7) (a) Koide, S.; Dyson, J.; Wright, P. E. *Biochemistry* **1993**, *32*, 12299−12310. (b) Scmid, F. X.; Mayr, L. M.; Mucke, M.; Schonbrunner, E. R. *Adv. Protein Fold.* **1993**, *44*, 25−66. (c) Wedemeyer, W. J.; Welker, E.; Narayan, M.; Scheraga, H. A. *Biochemistry* **2000**, *39*, 4207−4242.
(8) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683−720.

(9) ES MS for **1** ($C_{94}H_{133}N_{23}O_{28}S_2$): Calcd 2098.36; Obsd $[MH]^+$ 2099.) 3, 4,6-(*O*-acetyl)-*N*-acetylglucosamine-modified asparagine was synthesized as previously reported (Christiansenbrams, I.; Meldal, M.; Bock, K. *J. Chem. Soc. Perkin Trans. 1* **1993**, 1461−1471). This residue was used directly in peptide synthesis to generate an acetylated derivative of **2**. Deprotection with 10 equiv of NaOMe in MeOH afforded **2**. MALDI MS for peptide **2** ($C_{102}H_{146}N_{24}O_{33}S_2$): Calcd 2299.5; Obsd $[MH]^+$ 2300.

(10) ES MS for peptide **3** ($C_{110}H_{159}N_{25}O_{38}S_2$): Calcd 2504.7; Obsd $[MH]^+$ 2505. See Supporting Information.

(11) Haneda, K.; Inazu, T.; Mizuno, M.; Iguchi, R.; Yamamoto, K.; Kumagai, H.; Aimoto, S.; Suzuki, H.; Noda, T. *Bioorg. Med. Chem. Lett.* 1998, *8*, 1303−1306. MALDI MS for **4** ($C_{176}H_{266}N_{28}O_{89}S_2$): Calcd 4303.31; Obsd $[MH]^+$ 4303.

(12) ES MS for Glycopeptide **5** ($C_{156}H_{235}N_{27}O_{73}S_2$): Calcd 3719.49; Obsd $[MH]^+$ 3719.) ES MS for **6** ($C_{144}H_{215}N_{27}O_{63}S_2$): Calcd 3395.54; Obsd $[MH]^+$ 3396.

(13) The NMR assignments of the saccharide of peptide **4** have been reported (Lu, J.; van Halbeek, H. *Carbohydr. Res.* **1996**, *296*, 1−21). See Supporting Information for full details.

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Table 1. Percentage of Prolyl−Peptidyl Bond *cis* Isomer at pH 7.7, 65 °C; Rates of *cis/trans* Isomerization at pH 7.7, 65 °C

peptide	% *cis*	$k_{cis-trans}$ (s^{-1})	$k_{trans-cis}$ (s^{-1})
1	39	14.7 ± 0.2	23.0 ± 0.2
2	31	7.26 ± 0.1	16.2 ± 0.1
3	32	7.10 ± 0.2	17.4 ± 0.2
6	26	5.33 ± 0.5	15.2 ± 0.5

to **4**, suggesting that the outermost saccharides do not have a major impact on the solution structure of the peptide. Analysis of the amide variable temperature coefficients for the *trans* conformers indicated that most amide protons were unprotected from solvent exchange. However, His134 exhibited a substantially lowered amide variable temperature coefficient, suggesting an increased conformational stability in this region. Notably, for this residue, the glycosylated derivatives exhibited a lower coefficient than the nonglycosylated derivative **1** (peptide **1**, (−)4.46 ppb/K; glycopeptide **3**, (−)3.09 ppb/K; glycopeptide **4**, (−)3.11 ppb/K), suggesting that glycosylation stabilizes this secondary structure formation. Since the structures of glycopeptides **4**−**6** appeared to be highly similar, further analysis focused on derivative **6**, which exhibited the simplest spectral characteristics.

The free energy of formation of the disulfide bond is an indirect assessment of the conformation of the molecule; preorganization of the free sulfhydryl groups of the cysteine residues will lower the entropic cost of forming the macrocycle, resulting in a net lowered free energy of formation. The equilibrium constant of intramolecular disulfide bond formation can be measured by incubating the peptide with a reference thiol; disulfide exchange between the sulfhydryl groups brings the sample into equilibrium, and the equilibrium constant can then be calculated as previously described.[14] In this study, peptides **1**, **2**, and **6** exhibited small differences in K_{eq} values. Nonglycosylated peptide **1** exhibited the lowest K_{eq} value (20.0 ± 0.2 mM), suggesting that the sulfhydryl moieties are the least favorably positioned to form the macrocycle. Higher K_{eq} values were exhibited by both glycopeptide **2** (24.6 ± 1.7 mM) and **6** (28.9 ± 2.3 mM). These data, along with the previously published value for chitobiose (38 mM),[6] suggest that addition of the first two saccharide residues has an impact on disulfide bond formation, but that the outer saccharides do not appear to impart any significant additional effect.

Investigation of the proline amide isomers also provided an opportunity to investigate the effect of *N*-linked glycosylation on the conformational properties of this peptide. As expected for a proline residue flanked by aromatic phenylalanine residues, peptide **1** exhibits a high content of *cis*-proline isomer.[15] A notable divergence in the amount of *cis* isomer occurred among the peptide derivatives as the temperature was increased to 65 °C. As summarized in Table 1, the percentage of *cis* isomer *decreases* as increasing numbers of saccharide residues are added to the polypeptide backbone.

At temperatures above 37 °C, proline isomerization becomes sufficiently fast to observe chemical exchange peaks between *cis* and *trans* conformers in NOESY spectra (Figure 2). These exchange peaks were used to quantify the rate of *cis/trans* isomerization using eq 1.[16] The rates of isomerization at 37 °C were too low to be accurately quantitated; however, after raising the temperature to 65 °C, the rate of *cis/trans* isomerization for nonglycosylated peptide **1** was measured to be 2-fold faster than

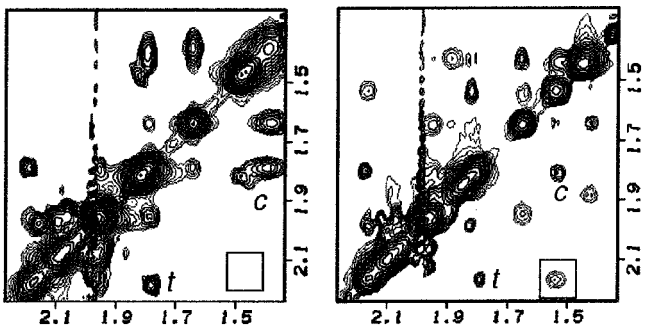

Figure 2. Exchange (NOESY) spectra of peptide **1** 7 °C (left) 65 °C (right).

that of the glycosylated derivatives (Table 1). The rate of isomerization was decreased most dramatically by the addition of the first *N*-acetylglucosamine residue; addition of the outer saccharides appeared to have an additional, albeit modest, effect.

$$k_{trans-cis} = 2\chi_c/\tau_m(\arctan h\,[I_{ct}/\chi_t I_{cc} + \chi_c I_{tt}]) \qquad (1)$$

Adoption of the correct prolyl isomer is often a slow step in the protein-folding process due to the relatively high activation energy required for isomerization (20 kcal/mol).[17] The saccharide could slow the rate of peptide bond isomerization by more effectively solvating the proline residue; desolvation of the proline amide bond has been proposed to favor the neutral transition state of the isomerization process.[18] However, it is unlikely that the smaller saccharides that decorate peptides **2** and **3** would be able to directly interact with the proline peptide bond. Alternatively, the saccharide may decrease the flexibility at the glycosylation site; numerous examples have been described where glycosylation rigidifies or stabilizes the structure of the peptide or protein.[19] A decrease in flexibility of the peptide backbone may result in a decrease in the rate of *cis*−*trans* interconversion of the proline amide bond.

This study suggests that addition of the *N*-linked saccharide affects certain key steps of protein folding, namely disulfide bond formation and *cis/trans* proline isomerization. Herein we directly compare the conformational effect of truncated saccharides with larger oligosaccharide structures found in vivo. Although the first two *N*-acetylglucosamines residue mediate the bulk of the conformational effect, the addition of the outer saccharide residues also appear to influence the peptide structure, indicating that nature may also utilize these outer residues to favorably affect peptide structure. The spectroscopic studies presented suggest that the primary conformational effect of the *N*-linked glycan is derived from the first two saccharide residues; the lack of observed NOE signals between saccharide and peptide suggests that the saccharide rigidifies the local structure of the peptide and does not interact directly with specific residues (such as proline) in the peptide backbone.

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Supporting Information Available: Details of glycopeptide synthesis, NMR studies, determination of K_{eq} values and chemical shift assignments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) (a) Lin, T.-Y.; Kim, P. S. *Biochemistry* **1989**, *28*, 5282−5287. (b) Falcomer, C. M.; Meinwald, Y. C.; Choudhary, I.; Talluri, S.; Milburn, P. J.; Clardy, J.; Scheraga, H. S. *J. Am. Chem. Soc.* **1992**, *114*, 4036−4042.

(15) (a) Grahwohl, C. Wuthrich, K. *Biopolymers* **1976**, *15*, 2043. (b) Wu, W. J.; Raleigh, D. P. *Biopolymers* **1998**, *45*, 381−394.

(16) See Supporting Information for experimental conditions. (a) Keller, M.; Sager, C.; Dunny, P.; Schutkowski, M.; Fisher, G. S.; Mutter, M. *J. Am. Chem. Soc.* **1998**, *120*, 2714−2720. (b) Perrin, C. L.; Dwyer, T. *Chem. Rev.* **1990**, *90*, 935−967. Macura, S.; Farmer, B. T.; Brown, L. R. *J. Magn. Reson.* **1986**, *70*, 493−499.

(17) Fischer, G. *Chem. Soc. Rev.* **2000**, *29*, 119−127.

(18) (a) Eberhardt, E. S.; Loh, S. N.; Hinck, A. P.; Raines, R. T. *J. Am. Chem. Soc.* **1992**, *114*, 5437−5439. (b) Fischer, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1415−1436.

(19) For recent examples, see: (a)Bann, J. G.; Peyton, D. H.; Bachinger, H. P. *FEBS Lett.* **2000**, *473*, 237−240. (b) Live, D. H.; Kumar, R. A.; Beebe, X.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12759−12761.