ARTICLE

OBC
www.rsc.org/obc

A surface exposed *O*-linked galactose residue destabilises the structure of a folded helix–loop–helix dimer †

Sarojini Vijayalekshmi,[a] Shaji K. George,[c] Linda K. Andersson,[b] Jan Kihlberg[c] and Lars Baltzer*[a]

[a] *Department of Chemistry, Linköping University, 581 83 Linköping, Sweden*
[b] *Department of Chemistry, Organic Chemistry, Göteborg University, 412 96 Göteborg, Sweden*
[c] *Department of Chemistry, Umeå University, 412 527 Umeå, Sweden*

Received 20th March 2003, Accepted 30th May 2003
First published as an Advance Article on the web 11th June 2003

A 42-residue glycopeptide Tn-15 and the corresponding reference polypeptide Thr-15 were designed and synthesized to provide a model system for the study of how glycosylation affects the stability of a molten globule-like protein. Tn-15 and Thr-15 fold into hairpin helix–loop–helix motifs that dimerise to form four-helix bundles and the only difference between the sequences is that Tn-15 carries an *O*-linked *N*-acetylgalactosamine residue at the side chain of threonine-15 whereas the sequence Thr-15 is unglycosylated. An analysis of the mean residue ellipticities at 222 nm of the two polypeptides and of the α-H chemical shift deviations from random coil values showed that glycosylation reduced the helical content of the polypeptides and increased the dissociation constant of the helix–loop–helix dimer to form monomers. The pH dependencies of the helical content of Tn-15 and Thr-15 differed as that of Thr-15 was largely unaffected by pH in the range from pH 4 to pH 10, whereas Tn-15 lost almost half of the helical content at pH 4 upon raising the pH to 10. No single amino acid residue was found to ionize in a way that could explain the observed pH dependence of Tn-15. The temperature dependence of the mean residue ellipticity of Tn-15 revealed a surprising decrease in helicity at 278 K in comparison with that at 293 K, reminiscent of cold denaturation, that was not observed for the reference four-helix bundle Thr-15.

Introduction

Carbohydrate residues in proteins play important roles by directing protein folding pathways, enhancing or reducing conformational stability and by increasing resistance to proteolytic degradation.[1–3] Membrane-bound glycoproteins are rich in information and are often involved in cell–cell recognition and communication. Aberrant glycosylation of proteins is associated with many diseases. Despite the important role played by glycoproteins in human health and disease, our understanding of the relationship between structure and function in natural glycoproteins remains incomplete, the major hurdle being the heterogeneity and complexity of glycosylation. Designed glycoproteins can help us understand the effects of glycosylation on the structure and function of proteins, and pave the way for the design of glycoproteins with improved structure and function as well as provide a platform for the study of protein–carbohydrate interactions. We have shown previously that the structure of a molten globule like folded helix–loop–helix dimer can be stabilized by the introduction of a carbohydrate derivative at the side chain of a lysine residue.[4,5] The effect of glycosylation on the conformational preferences of 15- and 24-residue peptides, and of a coiled coil dimer have been reported by others,[6–8] but access to a folded protein with molten globule like properties made it possible to determine the effect of glycosylation on a model system that mimicked intermediate states of biological maturation of a four-helix bundle protein. The carbohydrate derivatives were linked to the side chain of a solvent exposed Lys-15 in a position that is denoted as a *g* position in the pattern of the heptad repeat, Fig. 1. While the incorporation of the sugar derivatives stabilised the fold of the four-helix bundle the linkage was an unnatural one and left the covalently bound sugar in a position relatively far from the protein surface. In the present study we have chosen to examine the effects of introducing a naturally linked glycosylated amino acid on the structure of the molten globule like molecule. The model protein is the same as that in the previous study, with only one change in the amino acid sequence, Lys-15 was replaced by Thr 15, to allow a comparison not only between

Tn-15

1 11 15 19
N-A-A-D-Nle-E-A-A-I-K-H-L-A-E-**Tn**-Nle-A-A-K

20 23
G-P-V-D

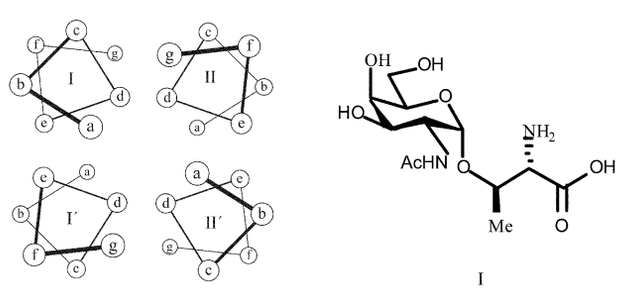
42 34 24
G-A-R-A-F-A-E-F-Orn-K-A-L-Q-E-A-Nle-Q-A-A

Thr-15

1 11 15 19
N-A-A-D-Nle-E-A-A-I-K-H-L-A-E-**T**-Nle-A-A-K

21 23
G-P-V-D

42 34 24
G-A-R-A-F-A-E-F-Orn-K-A-L-Q-E-A-Nle-Q-A-A



I

Fig. 1 Polypeptide sequences of Tn-15 and Thr-15 using the one letter code for amino acids. Tn is the glycosylated amino acid **I**. A helical wheel representation is used to demonstrate the position in the folded structure of **I** relative to the hydrophobic core and solvent exposed positions according to the pattern of the heptad repeat. The incorporation of Tn in a *g* position puts it close to the interface between helices and thus within binding distance of the residues in the hydrophobic core.

† Electronic supplementary information (ESI) available: Further NMR details. See http://www.rsc.org/suppdata/ob/b3/b302847j/

DOI: 10.1039/b302847j

glycosylated and non-glycosylated forms but also between natural and non-natural glycosylation linkers. The distance between the protein surface and the sugar is shorter than in the case of the unnatural linker and the carbohydrate–protein interactions were expected to be more pronounced. In addition, the comparison between the threonine containing sequence and that which carries a galactose *O*-linked to a threonine residue avoids the complication of comparing a charged residue with an uncharged one. The stability of the fold of the synthesised glycoprotein has been extensively and carefully analysed by spectroscopic techniques and the synthesis of a four-helix bundle protein that carries two *O*-linked galactose residues demonstrates the strenghth of organic synthesis in addressing problems in glycobiology.

Results

Peptide design

A 42-residue glycopeptide Tn-15 and the corresponding reference peptide Thr-15, Fig.1, were designed and synthesized. The design of the peptides was based on the design of RA-42 and on that of LA-42b, a sequence derived from RA-42 by replacing four Aib residues by Ala, and by replacing an ornithine residue by a lysine.[4,8] RA-42 is a 42-residue polypeptide, designed to adopt a helix–loop–helix structure and dimerise in solution to form a four-helix bundle.[9] The structures of RA-42 and LA-42b were previously studied extensively by NMR and CD spectroscopy and were shown to fold into the designed motifs. The sequence of Tn-15 differed from that of LA-42b only at position 15 in which α-D-GalNAc (1 ⟶ O) Thr [I] replaced the lysine residue of La-42b and was thus expected to fold into the same solution structure. Tn-15 was designed in complete analogy with the parent peptides to fold into two amphiphilic helices each made up of 19 amino acid residues connected by a four-residue loop. In the design of LA-42b, RA-42 and Tn-15 the helix propensities of the constituent amino acids were taken into consideration and residues were incorporated that were capable of salt bridge formation and helix dipole stabilization. In the folded state hydrophobic residues were designed to form shape complementary hydrophobic interfaces and drive the formation of the folded motif. The amino acid sequence of Tn-15 is conveniently described in terms of the heptad repeat pattern $(abcdefg)_n$, in which the *a* and the *d* positions are occupied by hydrophobic residues, Fig. 1. Polar and charged residues were uniformly distributed on the surface of the helices in *c*, *g* and *f* positions and the carbohydrate-linked amino acid residue was introduced in a *g* position. The *b* and *e* positions were used to control the mode of dimerisation. An illustration of the structure of the glycopeptide is shown in Fig. 2. A reference peptide, Thr-15, in which threonine replaced the glycosylated amino acid at position 15, but all other residues remained the same, was also synthesized in order to allow a comparison between the glycosylated and non-glycosylated sequences. In view of the homology between Tn-15 and Thr-15 it was expected that differences in solution behaviour would reflect the effect of glycosylation.

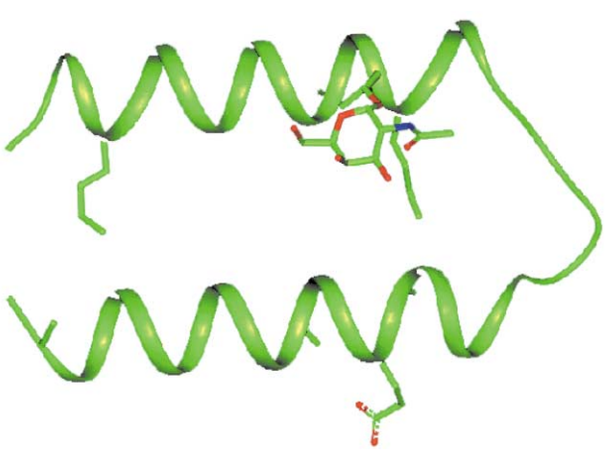


Fig. 2 An illustration of the structure of the glycopeptide describing the position and molecular dimensions of the galactose derivative. Only the monomer is shown for reasons of clarity.

The syntheses of the peptides were carried out on the solid phase using a Pioneer automated peptide synthesizer from Applied Biosystems. Due to the complexity of the synthesis of **I** it was incorporated using a special procedure, in which the resin was removed from the column of the automated peptide synthesizer to allow coupling of the sugar amino acid at a lower concentration than was used in the standard protocol, see Experimental section. After the carefully monitored coupling of **I** the resin was again transferred to the column of the synthesizer and the polypeptide synthesis was completed using the standard procedure.

Solution structure

The CD spectra of Tn-15 and Thr-15 in 50 mm acetate buffer at pH 5 and a peptide concentration of 1 mM are shown in Fig. 3. The spectra in both cases showed the two minima, one at 208 nm and the other at 222 nm, that are characteristic of α-helical proteins. The mean residue ellipticity at 222 nm of Tn-15 was -18000 deg cm^2 dmol^{-1} and that of Thr-15 was -20500 deg cm^2 dmol^{-1}. These values are largely in agreement with the reported values for similar sized helix–loop–helix peptides. While both structures are well developed, the helical content of Tn-15 is significantly less than that of Thr-15, suggesting that the incorporation of the sugar destabilised the four-helix bundle structure.

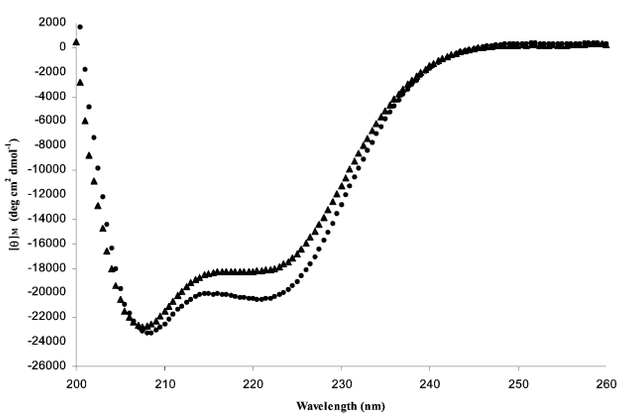


Fig. 3 The CD spectra of Tn-15 (▲) and Thr-15 (●) recorded in 50 mM sodium acetate buffer at pH 5, ambient temperature and a peptide concentration of 1 mM. The mean residue ellipticities at 222 nm are measures of the helical content.

The ^{1}H NMR spectra of the peptides were recorded and assigned using standard procedures from NOESY and TOCSY spectra recorded in H_2O : D_2O (90 : 10 v/v) with 6 volume percent of TFE-d_3 added. For ^{1}H NMR spectra and chemical shift assignments see the Electronic supplementary information (ESI). † The addition of small amounts of TFE sharpens the resonances and decreases the chemical shift dispersion of molten globule like peptides without affecting the overall fold.[10] The decrease in linewidth competes favourably with the decrease in dispersion and the overall effect is to increase resolution in the spectrum as described previously. The assignment strategy follows that of SA-42, which was carried out in similar solvent mixtures.[10] In the spectra of both Tn-15 and Thr-15 $N_iH \leftrightarrow N_{i+1}H$ NOEs were used in the sequential assignments of the amino acid sequences. Upfield αH chemical shifts relative to random coil values were observed for both peptides and were used to identify helical conformations, Fig. 4. Helical segments were identified in the sequence of Tn-15 from residue 4 to resi-

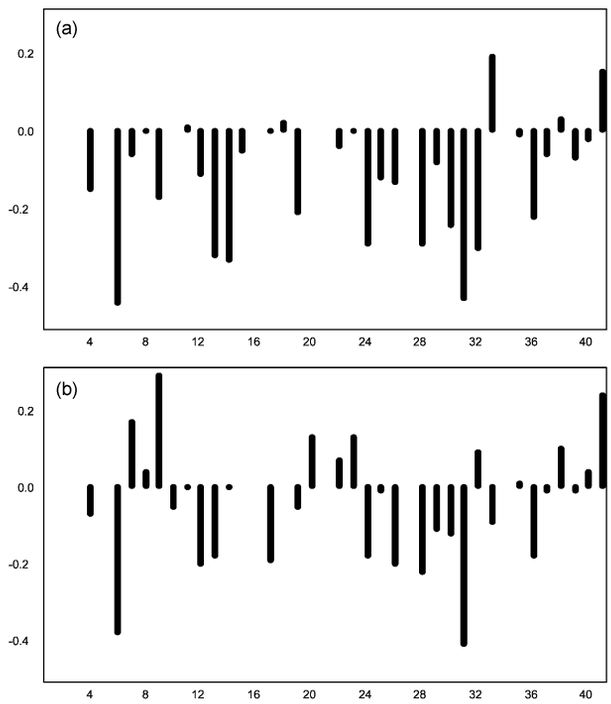


Fig. 4 The α-H chemical shift deviations from random coil values of the residues in Thr-15 (a) and Tn-15 (b).

due 19, and from residue 24 to residue 39. Similar ranges were observed in the sequence of Thr-15. The αH chemical shift deviations were, however, not as pronounced for these peptides as for previously reported sequences, and the shift pattern for Thr-15 was more pronounced than that of Tn-15. In addition some medium range NOEs of the type α,N (i, i + 3) which are characteristic of helical proteins were observed between residues 9 and 12, 25 and 28 (Tn-15 only), 35 and 38, 37 and 40. Spectral overlap prevented a more complete analysis. The aromatic side chains of F-35 and F-38 showed interhelical NOE contacts with the side chain protons of Nle-5, L-12 and I-9, indicating that the sequence folded into a hairpin motif. Furthermore, the protons of the phenyl rings of F-35 and F-38 showed NOE contacts with the side chains of Nle-16 and Nle-27 in agreement with a model in which the peptides dimerise in an antiparallel fashion.

Due to spectral overlap all of the resonances from the galactose moiety could not be assigned, but a set of NOE connectivities have been identified that show interactions between polypeptide and galactose residue protons. Particularly important were the NOE connectivities between the anomeric proton at 5.11 ppm, and the methyl groups from aliphatic side chains of residues in the hydrophobic core at 0.97 ppm that showed interactions between the galactose residue and the hydrophobic groups of the four-helix bundle. NOE contacts were also observed between the anomeric proton and the amide protons of Glu-14 at 8.07 ppm and Nle-16 at 7.96 ppm, in addition to the intra-residue contact between the amide proton at 8.37 ppm and the anomeric proton. Due to the dynamic nature of the molten globule like helical bundles mixing times of 400 ms were used when recording NOESY spectra. Long mixing times may give rise to spin diffusion phenomena but the relatively small number of observable NOEs suggest that this was not the case in the present investigation.

The effect of glycosylation on structure and dynamics of the folded motif

The mean residue ellipticity of the glycopeptide Tn-15 at 222 nm has a less negative value than that of the reference peptide Thr-15 by approximately 2500 deg cm² dmol^{-1}, Fig. 3. The less negative value of the mean residue ellipticity of the glycopeptide as compared to that of the reference peptide shows in a qualitative way that the helical content is smaller. The effect of glycosylation on the helical content is also evident from the αH chemical shift deviations from random coil values, Fig. 4, where the shifts to lower frequencies are less pronounced for Tn-15 than for Thr-15. The conformational stabilities of the folded polypeptides were therefore further investigated using CD and NMR spectroscopy by determining the effect of concentration, pH and temperature on dimer formation.

The negative values of the mean residue ellipticities at 222 nm of Tn-15 and Thr-15 in 50 mM sodium acetate solution at ambient temperature and pH 5.25 became less negative with decreasing peptide concentration, Fig. 5(a). The observed dependence on concentration was due to dissociation of dimers to form monomers and provided a qualitative measure of the stability of the four-helix bundle structure. The more pronounced loss of helicity in Tn-15 in comparison with that of Thr-15 showed that the glycosylated dimers were less stable than the non-glycosylated ones, Fig. 5(a). The decrease in helicity is, however, not easily fitted to a physical model as the variation in mean residue ellipticity cannot be fitted to a function describing the dissociation of dimers to form monomers. The dependence of $[\theta]_{222}$ on pH for the two peptides is shown in Fig. 5(b). The glycopeptide is more sensitive to changes in pH than Thr-15, with a large helical content observed only between pH 4 and pH 5. Above and below these pH values the glycopeptide looses helicity to a large degree. In contrast, the reference peptide was found to be highly helical in the pH range from 4 to 10. The pH dependence of the glycopeptide was difficult to explain and in order to investigate whether this

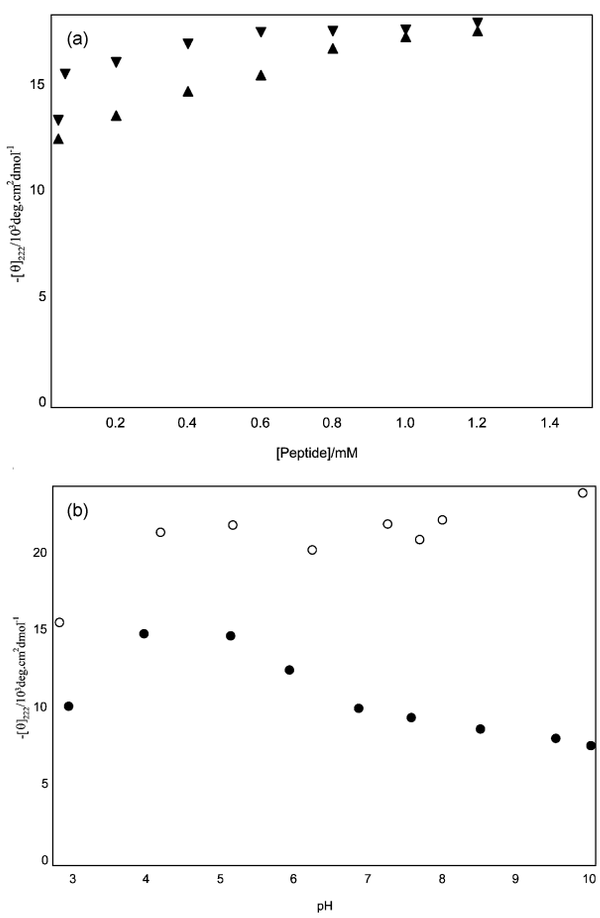


Fig. 5 (a) The concentration dependent mean residue ellipticity at 222 nm of Tn-15 (▲) and Thr-15(▼) recorded in 50 mM sodium acetate buffer at pH 5.2 and ambient temperature.(b) The pH dependent mean residue ellipticity at 222 nm of Tn-15 (●) and Thr-15 (○) recorded in aqueous solution at ambient temperature.

unusual behaviour was due to the differential ionization of the histidine residue, the pK_a of His-11 in Tn-15 was determined by NMR spectroscopy and compared to that of His-11 in Thr-15. In both peptides His-11 had similar pK_a values, 7.1 for Tn-15 and 6.8 for Thr-15, which ruled out the possibility that a differential ionization of His-11 contributed to the observed pH dependence of the glycopeptide. The determination of the pK_a of each Glu residue by a similar strategy was not possible due to spectral overlap. The effect of temperature on the helical content of the two peptides was determined by CD spectroscopy, Fig. 6. Both peptides showed a gradual loss of helicity with temperature at temperatures above 293 K, a behaviour which is typical of molten globular folds. At 353 K the peptides retained approximately 50% of their maximum helicity and no distinct melting point could be observed. The denaturation was found to be reversible and the helical content was recovered after decreasing the temperature. The glycopeptide showed an unusual behaviour for designed folded structures in that it also lost helicity at a temperature below 293 K, a feature reminiscent of the cold denaturation of proteins. One-dimensional ^{1}H NMR spectroscopy is an excellent qualitative measure of folding characteristics of polypeptides and proteins, because of the relationship between chemical shift dispersion, linewidth and the NMR time scale. The expansions of the amide and methyl regions of the ^{1}H NMR spectra of Thr-15 and Tn-15 recorded at different temperatures, Fig. 7, show significant line broadening that varies with temperature. The broad lines observed at low temperature show that both peptides exist as a mixture of several different conformations that are in fast exchange on the NMR time scale, a behaviour that is typical of molten globules. The fact that chemical shift dispersion is lost and linewidth is decreased with temperature shows that both polypeptides are in fast exchange on the NMR timescale. The resolution and NH intensity appears to be lost to a larger degree in Tn-15 in agreement with a model where the glycopeptide is denatured to a larger degree than Thr-15 at the same temperature.

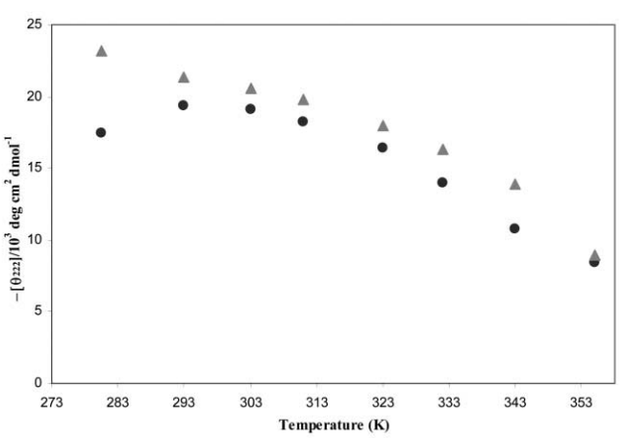


Fig. 6 The temperature dependent mean residue ellipticity at 222 nm of Tn-15 (●) and Thr-15 (▲). The spectra were recorded in sodium acetate buffer at pH 5.2. The peptide concentration was 1 mM.

Discussion

The role of glycosylation in disease and protein folding is well documented but the elucidation of its function is hampered by the complexity and diversity in naturally occurring glycoproteins. The development of the field of *de novo* design of proteins has made it possible to study biological problems of great complexity in model proteins. We and others have previously reported on the role of glycosylation of helical, folded polypeptides and shown by NMR and CD spectroscopy that the incorporation of carbohydrate residues affected the stability of folded helical dimers.[4,7,8] The model protein described by us

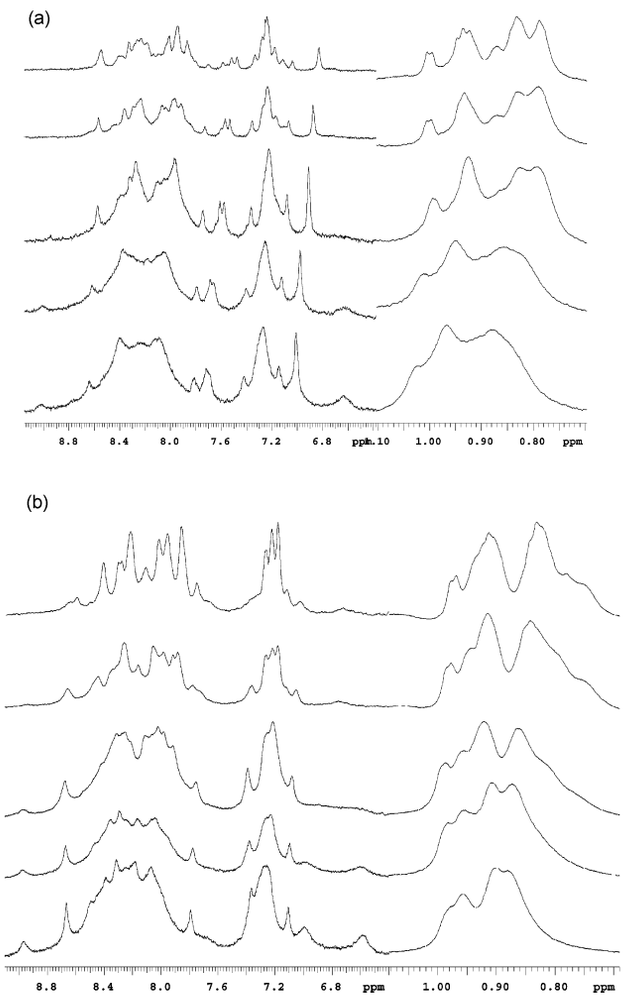


Fig. 7 Parts of the ^{1}H NMR spectra of Thr-15 (a) and Tn-15 (b) in H_2O–D_2O (90 : 10 v/v) as a function of temperature. From the bottom to the top the temperatures are 278, 283, 293, 303 and 313 K respectively. Note that for clarity only expansions of methyl and amide NH chemical shift regions of the spectra are shown.

had the properties of a so called molten globule, in which a large number of conformations are in rapid exchange on the NMR time scale. Glycosylation of proteins may affect solubility, folding and conformational stability at various stages of their life cycles and exert control of the life time it has in circulation. Our understanding at the atomic level of the interplay between structure and function of glycoproteins remains incomplete and an important goal in studying a model glycoprotein is to characterize the effect on structure of the incorporated sugar residue. Molten globule-like structures represent intermediates at various stages of biological maturation and the accessibility to a model system where the effect of glycosylation could be directly observed prompted the interest in its structure and dynamics. In our previous study the linkage of the sugar to the polypeptide was an unnatural one, a galactose and a cellobiose derivative were linked by amide bonds to the side chain of a lysine residue. Although glycosylation was found to stabilize the helix–loop–helix dimer, it was found to be of great interest to determine whether the observed effects were specific for that mode of glycosylation, and whether the naturally linked carbohydrate residue would show a similar behaviour. More specifically the distance between the carbohydrate residue and the polypeptide backbone and side chains is different, with the distance being much smaller in the naturally linked glycopeptide. Also the difference in charge between glycosylated and non-glycosylated polypeptides in the previous report could have introduced effects not directly related to the interaction between the sugar residue and the polypeptide

scaffold. NMR and CD spectroscopy are ideal techniques for determining the relationship between structure and function at the atomic level, and a 42-residue polypeptide was therefore synthesized in which an *N*-acetylgalactosamine residue was *O*-linked to a threonine residue and compared to the corresponding threonine-containing polypeptide.

The synthesis of a 42-residue specifically glycosylated polypeptide chain presents problems in synthesis but mainly opportunities in determining structure–function relationships. It was executed using a sugar amino acid that was incorporated into the sequence using standard solid-phase peptide synthesis protocols. The synthesis of the sugar amino acid **I** has been reported previously.[11] In the 84-residue symmetric dimer formed from the sequence Tn-15 there were two specifically incorporated *O*-linked *N*-acetylgalactosamine residues that allowed the direct observation of stability of the folded protein as a function of glycosylation. The comparison between Tn-15 and Thr-15 was also unaffected by a change in charge upon the introduction of the galactose residue as both residues are neutral in the accessible pH interval. The sugar residue was incorporated in a *g* position of the helix–loop–helix motif according to the pattern of the heptad repeat, Fig. 1. A *g* position is solvent exposed and not directly involved in helix bundle formation, but located at the interface between helical subunits with the possibility to interact with residues in the hydrophobic core, should such interactions arise. The effect of **I** on four-helix bundle stability would therefore not be expected to be due to steric interference with hydrophobic core formation. Differential hydrogen bonding to the helix backbone was instead considered as a viable reason for observed effects on stability. Threonine is not a helix stabilizing residue, due to its capacity for hydrogen bonding to the helix backbone amide groups. An *O*-linked *N*-acetylgalactosamine presents more hydroxyl groups that can participate in hydrogen bonding, and could thus be expected to show less intrinsic helix propensity than Thr, but on the other hand the monosaccharide residue is farther removed from the helical backbone than the hydroxyl group of Thr.

NMR and CD spectroscopic studies revealed that glycosylation destabilized the helix–loop–helix dimers since helicity was reduced and therefore the dimer–monomer equilibrium was driven towards a larger population of the monomer. Although a less negative value of the mean residue ellipticity means that the helical content is decreased, it cannot be strictly interpreted in terms of an increased dissociation constant. We therefore also investigated the concentration dependence of the helicities of Thr-15 and Tn-15 to show unequivocally that the stability of the dimer of Tn-15 was less than that of Thr-15. The glycosylated dimer was found to be less stable than the non-glycosylated one. NOE contacts between hydrophobic core residues and the anomeric proton of **I** suggested that the galactose residue interacts specifically with some core residues but not with others. A common residue to be found in binding sites of galactose transferases and lectins is Trp, and the pyranose ring has partial hydrophobic character. It is therefore not unexpected that an *N*-acetylgalactosamine residue in a *g* position can interact with the hydrophobic residues of the core and thus compete with helix–helix interactions, especially if the interactions are intramolecular. However, hydrogen bonding to backbone amides cannot be excluded and can probably be combined with hydrophobic interactions, although the binding energy of hydrogen bonds in aqueous solution is known to be small. Competition for backbone hydrogen bonding would be expected to reduce the helical content and therefore to decrease the opportunities for core residues in *a* and *d* positions to form shape complementary hydrophobic interfaces, resulting in less efficient dimer formation and thus in increased dissociation.

The pH dependence is very difficult to rationalize since the maximum helicity of Tn-15 coincides with the ionization of aspartic and glutamic acid residues at pH 4–5, and since the pH profile of Thr-15 helicity is very different although the introduced substituent does not ionize and therefore should not affect pK_a values of Asp and Glu residues.

The observation of a behaviour that mimics cold denaturation of proteins is very surprising and warrants further investigation. A model system for the systematic elucidation of the thermodynamic parameters responsible for cold denaturation is to our knowledge not yet available. The further study of this intriguing phenomenon is underway.

Conclusion

The effect of glycosylation of a molten globule like helix–loop–helix dimer has been determined. The incorporation of an *N*-acetylgalactosamine residue on the surface of a folded four-helix bundle motif destabilized the dimer in comparison with the non-glycosylated sequence, and gave rise to a surprising pH profile and to a phenomenon that appears to be related to cold denaturation. The monosaccharide residue interacts with residues in the hydrophobic core and may give rise to the observed destabilization through a combination of hydrogen bonding and hydrophobic effects that destabilize the folded dimer. The observed effect is the opposite to that reported previously where the glycosylation of a lysine residue in the same position as that of the *N*-acetylgalactosamine residue reported here, gave rise to increased stability upon glycosylation. We conclude that glycosylation of a threonine residue in close proximity to the hydrophobic core has a destabilizing effect, whereas removal of charge in the same position has a stabilizing effect.

Experimental

Peptide synthesis

Both peptides were synthesized on a Pioneer automated peptide synthesizer following a standard Fmoc protocol. The peptide chains were assembled on a PEG-PS polymer having a substitution level of 0.17 mmol g^{-1} on a 0.1 mm scale. Amino acid couplings were carried out in DMF solution using TBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) as the activating agent and DIPEA (diisopropyl ethyl amine) as the base. The side chain protecting groups were *tert*-butyl (*t*Bu) for threonine, *tert*-butoxycarbonyl (Boc) for lysines and ornithine, *tert*-butoxy (*t*BuO) for aspartic and glutamic acids, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) for arginine and trityl (Trt) for histidine, glutamine and asparagines and and 4,6-*O*-benzylidene as well as 3-*O*-*tert*-butyldimethylsilyl for the α-D-GalNAc(1 → O)Thr residue. The final peptides were cleaved from the polymer using 10 mL of the TFA cocktail mixture: TFA–thioanisole–ethane-1,2-dithiol–anisole (90 : 5 : 3 : 2 v/v) per gram of polymer. The cleavage mixture was evaporated under nitrogen and the peptides precipitated using cold diethyl ether. The white solid was resuspended in cold diethyl ether, centrifuged thrice, dissolved in distilled water and freeze dried to give the peptides as white fluffy solids. The crude peptides were purified using reversed-phase HPLC on a semi-preparative C-8 Kromasil column using 38% propan-2-ol and 0.1% TFA in water as the eluent at a flow rate of 10 mL per minute. The purified peptides were identified using MALDI-TOF spectrometry and the observed masses were 4510.49 for Tn-15 and 4305.91 for Thr-15, which were close to the calculated masses of 4510.111 and 4306.884. The synthesis of the glycopeptide was carried out in three steps. First the C-terminal fragment from Gly42 to Nle16 was assembled on the Pioneer peptide synthesizer as described above. The Fmoc protecting group on Nle-27 was cleaved manually using 20% piperidine. The polymer was then removed from the column and Fmoc α-D-GalNAc (1 → O)Thr was coupled to the free amino group of Nle16 manually in DMF solution using the same reagents as above. The reason for

the use of this procedure was that the incorporation of the sugar amino acid, which is obtained only after advanced organic synthesis, must be carefully optimised in a small vessel. The polymer was then returned to the synthesiser column and completed as described above. Synthesis of the protected α-D-GalNAc(1 → O)Thr residue was performed as described previously.[11]

NMR spectroscopy

^{1}H NMR spectra of peptides dissolved in 90% H_2O–10% D_2O containing 6 vol% TFE-d_3 at pH 5–5.5 were recorded on a Varian Inova 600 spectrometer operating at 303 K. The peptide concentration was 1 mM in a sample volume of 550 μl. The one-dimensional spectra and the TOCSY and NOESY spectra were recorded with preirradiation of the water resonance. Typical 90° pulses were 6.5 μs for 1D, TOCSY and NOESY spectra and the spin lock pulse for the TOCSY experiment was 23.1 μs with a window function of 33.8 μs. The acquisition times were 1.8, 0.2 and 0.2 s respectively for the 1D, TOCSY and NOESY experiments. The mixing times were 400 ms for the NOESY experiment and 60 ms for the TOCSY experiment. 2 × 256 Increments were recorded for the 2D experiments and the data were processed using linear prediction algorithms. The pK_a values of His-11 was determined from ^{1}H NMR spectra recorded in 99.8% D_2O at 1 mM peptide concentrations. The pH of the peptide solutions was adjusted to the desired values by the addition of dilute NaOD and DCl and measured directly in the NMR tube before and after recording the spectra. A function describing the dissociation of a monoprotonic acid was fitted to the experimental results using the Igor Pro software.

CD spectroscopy

CD spectra were recorded on a JOBIN YVON CD6 spectropolarimeter routinely calibrated with d-camphor sulfonic acid in 0.1, 0.5 or 1 mm cuvettes in the interval from 200–260 nm. Peptide stock solutions were prepared by weight in water or buffer solution, as desired, assuming a water content of 25% for the lyophilized peptides. For the concentration dependent study, peptide stock solutions were prepared in sodium acetate buffer (50 mm, pH 5.25) and diluted to the desired concentrations by pipetting. For the pH dependent studies, peptide solutions (0.3 mm) were prepared in water and the pH was adjusted to the desired values by the addition of NaOH or HCl.

References

1 R. A. Dwek, *Chem. Rev.*, 1996, **96**, 683–719.
2 H. Lis and N. Sharon, *Eur. J. Biochem.*, 1993, **218**, 1–27.
3 A. Varki, *Glycobiology*, 1993, **3**, 97–130.
4 L. K. Andersson, G. Stenhagen and L. Baltzer, *J. Org. Chem.*, 1998, **63**, 1366–67.
5 L. K. Andersson, G. T. Dolphin, J. Kihlberg and L. Baltzer, *J. Chem. Soc., Perkin Trans. 2*, 2000, 459–64.
6 X. Huang, J. J. Barchi Jr., F. D. T. Lung, P. P. Roller, P. L. Nara, J. Muschik and R. R. Garrity, *Biochemistry*, 1997, **36**, 10846–10856.
7 L. Kirnarsky, O. Prakash, S. M. Vogen, M. Nomoto, M. A. Hollingsworth and S. Sherman, *Biochemistry*, 2000, **39**, 12076–12082.
8 S. Mehta, M. Meldal, V. Ferro, J. O. Duus and K. Bock, *J. Chem. Soc., Perkin Trans. 1*, 1997, 1365–1374.
9 L. Baltzer, A-C. Lundh, K. Broo, S. Olofsson and P. Ahlberg, *J. Chem. Soc., Perkin Trans. 2*, 1996, 1671–76.
10 S. Olofsson and L. Baltzer, *Folding and Design*, 1995, **1**, 347–356.
11 S. K. George, T. Schwientek, B. Holm, C. A. Reis, H. Clausen and J. Kihlberg, *J. Am. Chem. Soc.*, 2001, **123**, 11117–11125.