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Glycosylation May Reduce Protein Thermodynamic Stability by Inducing a Conformational Distortion

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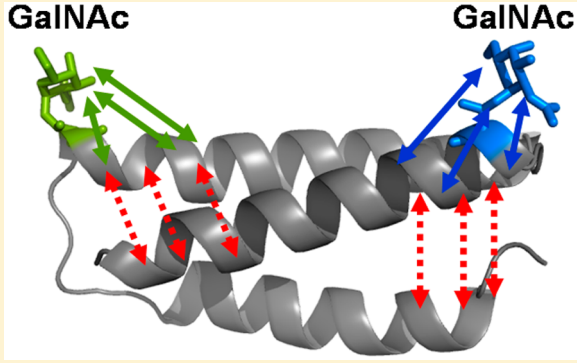
Supporting Information

ABSTRACT: Glycosylation plays not only a functional role but can also modify the biophysical properties of the modified protein. Usually, natural glycosylation results in protein stabilization; however, in vitro and in silico studies showed that sometimes glycosylation results in thermodynamic destabilization. Here, we applied coarse-grained and all-atom molecular dynamics simulations to understand the mechanism underlying the loss of stability of the MM1 protein by glycosylation. We show that the origin of the destabilization is a conformational distortion of the protein caused by the interaction of the monosaccharide with the protein surface. Though glycosylation creates new short-range glycan−protein interactions that stabilize the conjugated protein, it breaks long-range protein−protein interactions. This has a destabilizing effect because the probability of long- and short-range interactions forming differs between the folded and unfolded states. The destabilization originates not from simple loss of interactions but due to a trade-off between the short- and long-range interactions.



Glycosylation is one of the most common modifications occurring in the cell (more than 50% of all proteins are glycosylated) and occurs either co- or post-translationally. Glycans are very diverse in structure and composition because of the availability of a large variety of monosaccharide building blocks that can polymerize to linear or branched glycan structures. It is not only the molecular properties of the glycans that govern their function as recognition markers, but also the number of glycans and the properties of the sites of attachment on the surface. It is thus the structural heterogeneity of glycans, the variety in their molecular composition, and the degree of glycosylation that determine the ability of glycans to control various cellular processes. However, the molecular details of this relationship must still be deciphered to understand the "glycosylation code".[1,2] The carbohydrates can, in principle, modify the biophysical properties of the proteins by modulating, for example, their solubility, aggregation, enzyme resistance, thermal and kinetic stability, structure, and folding.[1,3−5] These effects should be considered as part of the "glycosylation code" as they may also govern the function of glycoproteins.

The linkage between glycosylation and its effects on protein biophysical characteristics is not clear. Nonetheless, several in vitro studies have reported an effect of glycosylation on thermodynamic stability.[5−7] In many cases, glycosylation results in thermal stabilization of the protein (for either N- or O-linked glycosylation[8,9]), although the origin of this effect may differ from case to case.[10] Experimental studies on naturally glycosylated proteins and computational studies showed that the stabilizing effect can arise from stabilization of the native state and destabilization of the unfolded state.[1,6,8] Though the former is due to enthalpic stabilization to the folded state due to glycan-protein interface,[7,11−14] the latter is due to entropic stabilization of the unfolded state.[1] For different systems, it was found that increasing the degree of glycosylation increases the stabilization effect.[15−17] Indeed, stabilization can be considered quite a common effect of glycosylation. However, in our previous computational studies, we showed that the effect of glycosylating the SH3 and Pin WW domains depends on the position of the glycans and may result in a loss of stability. A destabilizing effect of N-or O-linked glycosylation was also observed by other in silico and in vitro studies.[4,6,18−24] Using a native topology-based model to simulate the SH3 domain, we found a large difference in the effect of glycosylation as a function of its attachment site. A negative correlation was found between the thermodynamic stability of the protein and the number of native contacts made by the residue that constitutes the glycosylation site. Glycosylation at more-structured regions (those that have more native contacts) resulted in destabilization, whereas at more-disordered regions, glycosylation resulted in stabilization.[1] However, a destabilization effect was also observed for the in vitro glycosylation of the loop region of the Pin WW protein,[6] reflecting the complex effect of glycosylation on protein thermodynamics. The effect of glycans on thermal stability can also be indirect via the solvent, for example, by disrupting protein−water hydrogen bonds or by changing the entropy of the water in the hydration layers.[8,25]

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In this study, we focus on the effect of attaching a glycan at either or both of two specific sites on the MM1 protein. The MM1 protein is a miniaturized model of Gc-MAF, a serum factor that stimulates the phagocytic activity of macrophages.[26] MM1 was designed by transferring the glycosylated loop of Gc-MAF onto α3W (a 3-helix bundle used as a scaffold (Figure 1)[27]) and exhibits the native-like activity of Gc-MAF on macrophages.[28] We selected the MM1 protein for this study because it was shown experimentally that MM1 is destabilized upon glycosylation with α-*N*-acetylgalactosamine (GalNAc) (Figure 1). The effect of double glycosylation at two sites (Thr27 or Thr52) was additive, with each glycosylation event destabilizing the protein by about 1 kcal/mol.[18]

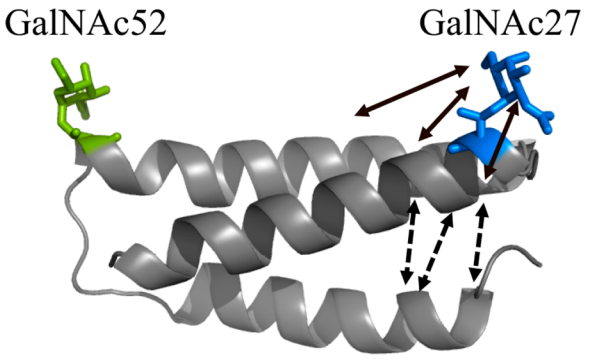

Figure 1. Glycosylated MM1 protein. The MM1 protein (PDB code 1LQ7) glycosylated by *N*-acetylgalactosamine (GalNAc) at Thr27 and Thr52. Potential new interfacial glycan−protein interactions formed following glycosylation are illustrated by solid arrows, whereas lost protein−protein interactions are illustrated by dashed arrows.

In order to reveal the possible mechanisms for the loss of stability, we performed coarse-grained and all-atom molecular dynamic (MD) simulations. Though the former can shed light on the thermodynamic stability of the glycosylated protein, the latter are useful to describe the detailed conformational preferences of the glycosylated protein and of the unique glycan−protein interfaces. Utilizing a combination of these two computational approaches may reveal how glycosylation at certain sites can affect the stability of the native state of the MM1 protein.

The experimentally observed thermodynamic destabilization of the glycosylated MM1 protein may be related to the excluded volume effects of the glycans, which may restrict the entropy of the protein, particularly in the unfolded state. An earlier study of glycosylated Pin WW protein showed that, in most cases, coarse-grained simulations successfully predict the thermodynamic effect of glycosylation on stability.[6] In order to examine this possibility, we studied the folding of the MM1 protein using a coarse-grained native-topology-based model.[6,29] In this model, non-native interactions cannot be formed and the sugars are modeled as excluded volume molecules that are exposed to the solvent and cannot form any contacts with the protein. Following the experimental study, we constructed the glycosylated MM1 protein in silico and placed GalNAc on sites T27 and T52, and the amino acids were represented either by a single bead centered at the Cα of each residue or by all-heavy atoms.

The CG simulations, at both Cα and all-heavy atom representation, show protein stabilization following the glycosylation. The change in the folding temperatures (T_F, the temperature at which the folded and unfolded states have the same free energy) between the diglycosylated and nonglycosylated forms of MM1 (ΔT_F) is 0.6−3.3%, depending if the MM1 is represented using the Cα or all-heavy atoms (Figure S1 in Supporting Information). This change in T_F may correspond to a shift of about 1−4 °C in the melting temperature and is similar in magnitude to the stabilization measured computationally and experimentally for different proteins.[1] However, the stabilization effect of glycosylation as detected by the coarse-grained simulations contradicts the experimental destabilization reported for the MM1 protein, which suggests that the excluded volume representation of the glycan is not sufficient to capture the effect of glycosylation on this protein.

Following the failure of the CG model to capture the experimentally observed destabilization effect of glycosylation on MM1, we concluded that the GalNAc groups attached to MM1 should not be modeled as excluded volumes and hypothesized that they can potentially interact with the protein

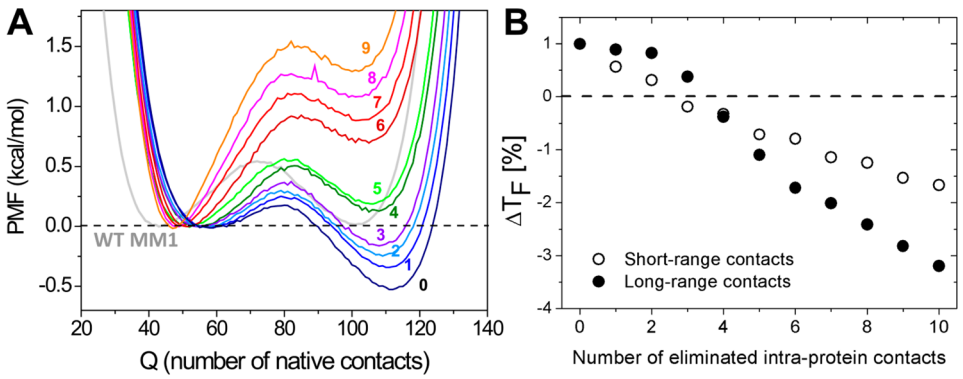

Figure 2. Stability of the MM1 protein following glycosylation based on coarse-grained simulations. (A) Potential of mean force (PMF) of coarse-grained model of MM1 in which different number of long-range protein contacts is eliminated (indicated by the number next to each plot) while 10 glycan-protein contacts are added. The PMF plots are calculated at the folding temperature of the unglycosylated MM1 protein (shown in gray). (B) Change in stability (ΔT_F, relative to the unglycosylated MM1 protein (dashed line) is reported for models of MM1 with different ratios between the number of newly formed glycan−protein interactions and the number of lost intramolecular interactions (illustrated by the solid and dashed arrows, respectively, in Figure 1). The number of added contacts was set to 10 and the number of eliminated contacts (short-range (white) or longer range (black); see Supporting Information for definition of contacts) was scanned between 0 and 10.

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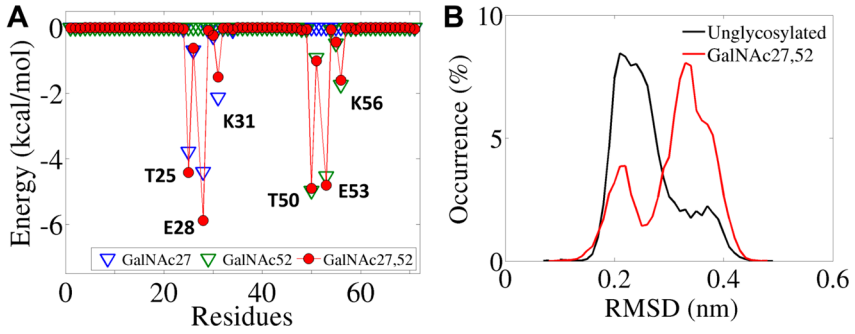

Figure 3. Effect of glycosylation on the MM1 protein from atomistic simulations. (A) Energy of interaction between the sugars and each residue of the MM1 protein. The average interaction energies from three 500 ns MD simulations are shown for the monoglycosylated (GalNAc27 or GalNAc52) and diglycosylated (GalNAc27, 52) MM1 protein. (B) Distribution of the RMSD values for the unmodified MM1 protein (black) and the diglycosylated protein (red).

surface. The formation of a new glycan−protein interface may, in principle, increase the thermodynamic stability of the protein unless the new interface leads to a conformational change in the proteins[30,31] and consequently to a weakening or even loss of internal protein interactions then destabilization may result. According to our hypothesis, the balance between the gain of new interfacial glycan−protein interactions (illustrated by solid arrows in Figure 1) and the loss of protein−protein interactions due to the conformational change induced by the glycosylation (illustrated by dashed arrows in Figure 1) will dictate whether the protein is stabilized or destabilized by the modification.

To test this hypothesis, we constructed a CG model, on the basis of the native-topology based model, in which the glycan interacts with residues in its vicinity at the expense of breaking some interhelical interactions. To quantify the outcome of these opposing effects on protein stability, we fixed the number of newly formed glycan−protein interactions at 10 and examined how tuning the number of lost protein−protein contacts (in the range of 0−10) affects protein stability. We also tested how the short- or long-range nature of the eliminated protein−protein contacts affects protein stability. Figure 2 shows a summary of the coarse-grained simulations in which 10 interfacial glycan−protein contacts can be formed and the number of eliminated protein−protein interactions varies between the simulations. Not surprisingly, the addition of interfacial contacts increases protein stability when internal interactions remain intact ($\Delta T_F \sim 1\%$), but stability drops as internal interactions are eliminated. For the case in which 10 interfacial contacts are added but 10 internal protein−protein contacts are lost, destabilization is observed, with the effect being more substantial when the eliminated contacts are longer range ($\Delta T_F \sim -1.7\%$ and -3.2% for short- and long-range contacts, respectively). A loss of about four internal contacts is sufficient to achieve destabilization although the glycan contributes 10 interactions of the same strength. The reason for this asymmetry between the stabilizing and destabilizing contributions of the newly formed and broken contacts, respectively, to thermodynamic stability is their intrinsically different probability of formation in the unfolded and folded state (Figures S2 and S3 in Suppporting Information). The short-range nature of the glycan−protein interactions allows them to be constantly formed and thus their influence on stability is smaller (because they similarly contribute to both the folded and unfolded states), whereas protein−protein intramolecular interactions are mostly formed in and contribute to the folded state. We examine this mechanism of conformational change, which is induced by the glycosylation by adding 10 fixed contacts to the coarse-grained model, but the results are valid for other sizes of the glycan-protein interface.

To support our hypothesis, we needed to show two things: the glycans interact with the protein and that these interactions disrupt the native protein conformation in the folded state. To examine the first point, namely, nature of the interactions between GalNAc and the MM1 protein, we studied the dynamics of the native state of the glycosylated and nonglycosylated MM1 protein using all-atom MD. The comparison between the coarse- and fine-grained simulations can be useful to probe and quantify the coupling between the conformational change and the new interface that were speculated in the coarse-grained model to examine the hypothesis. This approach allows us also to follow the energy of the interactions between the attached glycans and the protein and so test whether or not it is justified to simplify the glycan−protein interactions as an excluded volume. The glycan−protein interface was studied by measuring the interaction energy between GalNAc and every residue within the MM1 protein during three 500 ns simulations of each variant. Figure 3a shows that the sugar in both positions (27 and 52) interacts strongly with specific residues located in its vicinity. The glycan attached to Thr27 or to Thr52 interacts mostly with a patch comprised of Thr, Glu, and Lys. Because the glycosylation sites are identical (in terms of their sequence) and were engineered into this protein, the same set of residues interacts with glycans in each site (Figure 3). The similar interactions of GalNAc at positions 27 and 52 with the protein in the mono- or diglycosylated variants of MM1 illustrate, in accordance with the experimental results, that the two glycosylated sites are not coupled but rather their effects are additive.[18] These MD simulations support the first part of our hypothesis: the sugar does interact with the protein.

To examine the second half of the hypothesis, we evaluated the conformational preferences of the glycosylated and nonglycosylated proteins by means of root-mean-square deviation (RMSD) measurements. Figure 3b shows the distribution of RMSD values for the non- and doubly glycosylated variants, and reveals a substantial shift to larger RMSD values in the glycosylated protein. The difference between the RMSD distributions indicates the occurrence of a conformational change that is induced by the glycosylation; thus, consistent with our hypothesis, glycosylation disrupts the wild-type protein conformation.

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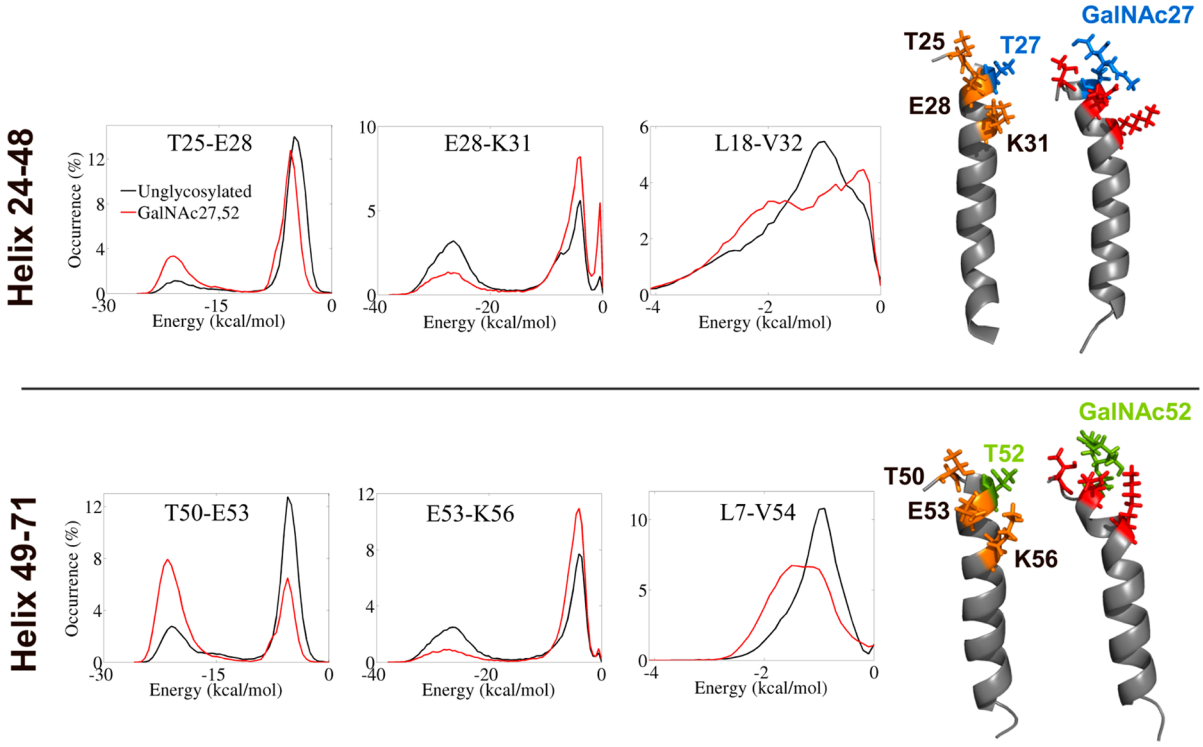

Figure 4. Intraprotein energy interactions in the unmodified MM1 protein and the diglycosylated variant. The interaction energies were calculated between selected residues in the vicinity of the two glycosylation sites: Thr27 (in helix 24−48) and Thr52 (in helix 49−71). In all plots, black and red curves represent calculated energy distributions for the un- and the diglycosylated of MM1 protein (GalNAc27,52), respectively. The snapshots on the right illustrate the conformational changes in the MM1 protein due to glycosylation (at T27 and T52). The conformations of the two helices that include the two glycosylation sites are compared in the unmodified and the diglycosylated variants. The structures illustrate the kink in the helix induced by interactions between GalNAc27(52) and T25(50), E28(53), and K31(56).

To better understand the conformational change that the MM1 protein undergoes upon glycosylation, we focused on the intrahelical interaction in the vicinity of the modified residues. For the wild-type and diglycosylated proteins, Figure 4 shows the energy of the interactions occurring at the glycosylation sites within the stretch between Thr25 and Lys31 (on the helix that spans residues 24−48; upper figures) as well as within the stretch between Thr50 and Lys56 (on the helix that spans residues 49−71; lower figures). In both cases, the interaction becomes stronger upon glycosylation. On the other hand, the interaction between residues Glu28(53) and Lys31(56) becomes weaker (Figure 4). The observed changes in residue−residue interactions are similar for mono- and diglycosylated variants of the MM1 protein. The changes in the strength of the interactions between some residues reflect a structural change imposed by the glycan.

Figure 4 shows the two helices that comprise the glycosylated sites and span residues 24−48 and 49−71. These sites exhibit a conformational distortion (a bend in the helix) that arises from the interface formed between GalNAc27 (GalNAc52) and the patch comprised of Thr25 (Thr50), Glu28 (Glu53), and Lys31 (Lys56). The effect of GalNAc on the helix to which it is attached is observed in the breaking of the helical hydrogen bonds between Thr27(52) and Lys31(56) (Figure S5 in Supporting Information). The structural consequences of glycosylation are not merely local, as can be seen from the interhelical energies between Leu18 and Val32 or between Leu7 and Val54 (Figure 4).

The computational study presented here suggests that the in vitro loss of stability observed upon glycosylation of the MM1 protein may be related to conformational changes to its native state induced by interactions between the glycan and the protein surface. A previous computational study on the MM1 protein suggested that glycosylation destabilizes MM1 by increasing the solvent exposure of hydrophobic residues near the loops connecting the helices that arises from a change in the rotamer population of Thr50.[18] Using extensive atomistic simulations, we found that the attached sugars may strongly interact with the protein surface, which leads to a change in the intraprotein energy interactions. It is likely that the previously observed variation in the rotamer population of Thr50 is related to the conformational distortions reported here.

This study illustrates a nontrivial effect of glycosylation on protein structure and stability. In accordance with our hypothesis, we found that glycans can interact with a protein and that these interactions disrupt protein conformation. However, the origin of the destabilization is not the loss of interactions in the folded state by the distortion as they are compensated by the glycan−protein interactions. The free energy of the folded state is therefore hardly changed. The destabilization originates from changing the enthalpy of the unfolded state by shifting the balance between the creation of short-range glycan−protein interactions and the destruction of long-range intraprotein interactions toward the latter. We show that eliminating only 4 long-range contacts while adding 10 short-range contacts is sufficient to destabilize the protein. The response of the protein to glycan−protein interactions may be protein dependent and is expected to occur more commonly with α-helical than with β-sheet rich proteins as the latter are expected to be more resistant to distortion to protein-glycan

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interactions. Indeed, several crystal structures show that glycosylation does not affect protein structure and a recent MD study of pinWW, which includes two β-hairpins, observed no significant structural deviations upon glycosylation and PEGylation, even when the conjugates strongly interact with the protein.[25] It is suggested that the new interface may not always result in stabilization and this should be considered when selecting protein sites for glycosylation or other modifications that may share similar destabilization mechanism.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.5b01588.

Methods and Supplementary data. (PDF)

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Notes

The authors declare no competing financial interest.

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