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Biophysical Chemistry 134 (2008) 157–167

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Modulation of protein stability by *O*-glycosylation in a designed Gc-MAF analog

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Received 14 December 2007; received in revised form 28 January 2008; accepted 8 February 2008
Available online 21 February 2008

Abstract

The post-translational modification of proteins by the covalent attachment of carbohydrates to specific side chains, or glycosylation, is emerging as a crucial process in modulating the function of proteins. In particular, the dynamic processing of the oligosaccharide can correlate with a change in function. For example, a potent macrophage-activating factor, Gc-MAF, is obtained from serum vitamin D binding protein (VDBP) by stepwise processing of the oligosaccharide attached to Thr 420 to the core α-GalNAc moiety. In previous work we designed a miniprotein analog of Gc-MAF, MM1, by grafting the glycosylated loop of Gc-MAF on a stable scaffold. GalNAc-MM1 showed native-like activity on macrophages (Bogani 2006, J. Am. Chem. Soc. 128 7142–43). Here, we present data on the thermodynamic stability and conformational dynamics of the mono- and diglycosylated forms. We observed an unusual trend: each glycosylation event destabilized the protein by about 1 kcal/mol. This effect is matched by an increase in the mobility of the glycosylated forms, as evaluated by molecular dynamics simulations. An analysis of the solvent-accessible surface area shows that glycosylation causes the three-helix bundle to adopt conformations in which the hydrophobic residues are more solvent exposed. The number of hydrophobic contacts is also affected. These two factors, which are ultimately explained with a change in occupancy for conformers of specific side chains, may contribute to the observed destabilization.

Keywords: De novo design; GalNAc; Thermodynamic stability; *O*-glycosylation; Molecular dynamics

1. Introduction

Glycoproteins are a class of naturally occurring bioconjugates in which carbohydrates of varied complexity are post-translationally attached to individual amino acids, either asparagine in N-linked glycosylation or serine/threonine in O-linked glycosylation. The carbohydrate component can affect many disparate functions spanning from cell–cell communication events, to modulation of protein-protein interactions, to the emergence of antigenicity in autoimmune and alloimmumune reactions [1,2].

The most prevalent type of O-linked glycosylation is mucin-type glycosylation, which is initiated by the enzymatic attachment of α-*N*-acetylgalactosamine (GalNAc) from UDP-GalNAc to the β-hydroxyl group of either a serine or a threonine in the cytosol. The nascent oligosaccharide is further decorated with the step-wise addition of monomers in the Golgi [1,2]. Although no universal sequence motif for *O*-glycosylation has been identified, the site of attachment is often preceded at the −1 position and/or followed at the +3 position by a proline, increasing the probability of finding the site in a turn position. Glycosylation can affect biophysical properties such as solubility, thermal stability, aggregation, folding, and structural dynamics. For example, natural glycoproteins generally exhibit slightly higher denaturation temperatures and structure dynamics than their deglycosylated counterparts; these effects correlate with the number of glycans bound to the protein surface rather than with their size [3]. Beyond these observations, it can be difficult to dissect the contributions of each glycosylation event in the context of specific structural motifs using natural

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doi:10.1016/j.bpc.2008.02.005

glycoproteins, which are often characterized by differential utilization of multiple glycosylation sites and/or by heterogeneicity of the complex oligosaccharides. Synthetic model proteins can overcome these obstacles, and have been used by several groups to investigate the effect of simple carbohydrate conjugates, which can be either chemically incorporated during solid-phase synthesis [3–5], or biosynthetically using nonsense codon suppression [6].

Dynamic changes in the composition of the oligosaccharide moieties can trigger the loss or acquisition of function in nuclear and cytosolic proteins, in concert with phosphorylation [7,8], and in secreted proteins [9]. Vitamin D binding protein (VDBP, also known as group complement, Gc) is an abundant serum glycoprotein with functions ranging from the transport of vitamin D, to the scavenging of acting monomers from the bloodstream, to the immunomodulation of macrophage activity [10]. The latter function is mediated by the sequential enzymatic cleavage of its trisaccharide moiety to the core α-GalNAc residue linked to Thr 420 [9]. This processing, which involves surface enzymes on B and T lymphocytes, transforms VDBP into a serum factor (Gc-MAF/VDBP-MAF) that stimulates the Fc mediated phagocytic activity of macrophages [11]. Further processing to cleave the GalNAc moiety results in complete loss of activity on macrophages (REF). Gc-MAF has shown promise for the development of therapeutic agents based on its macrophage-activating properties as adjuvant in cancer therapy [10–13].

In previous work, we reported that a miniaturized model of Gc-MAF, called MM1, exhibits native-like activity on macrophages [14]. MM1 was designed by transferring the glycosylated loop of Gc-MAF, believed to be responsible for its biological activity, onto α3W, a well characterized 3-helix bundle used as a scaffold [15]. The process was repeated for both loops connecting helix 1 to helix 2 and helix 2 to helix 3; initially, only loop 1 was glycosylated to GalNAc-MM1 on Thr 27, which corresponds to Thr 420 in Gc-MAF. The peptide and its monoglycosylated form showed a high content of α-helical secondary structure and thermodynamic stability consistent with proteins of that size, suggesting that the insertion of the hexogeneous loop from Gc-MAF was well tolerated. However, we noticed that GalNAc-MM1 was destabilized by about 1 kcal/mol compared to MM1 in chemical denaturation experiments. It is possible of that the *O*-glycosylation of threonine could play a role in modulating the local secondary structure of MM1, and by analogy of Gc-MAF.

Given the therapeutic promise of MM1 [14], we decided to further investigate the energetic contribution of *O*-glycosylation. Hereto we prepared the diglycosylated form, $GalNAc_2$-MM1, in which in addition to Thr 27 in the first loop the second loop is glycosylated at Thr 52, also corresponding to Thr 420 in Gc-MAF. In this paper, we compare the thermodynamic properties of the three peptides, showing an unusual destabilizing effect correlated with the number of glycans. We used molecular dynamics simulations to explore the conformational dynamics of the miniaturized protein, to investigate the possible role of N-capping, and to relate the differences in motion to the differences in stability of the glycosylated and unglycosylated constructs in a qualitative manner. Our simulations indicate an increased conformational drift throughout the sequence, and a small increase in the exposed hydrophobic surface for the glycosylated form. These results suggest that the destabilization of the protein upon glycosylation may be caused by a disruption in the packing of the protein and an increase of the solvent exposed hydrophobic surface.

2. Materials and methods

2.1. Protein design, synthesis and purification

The model peptides were designed using Insight software (Biosym Technologies, Inc.) as described earlier [14]. Briefly, the glycosylated loop of VDBP-MAF [16,17] was spliced onto the structure of the scaffold 3-helix bundle α3W [15,18]. The PDB accession code is 1LQ7, and the full sequence is: GSRVKALEEKVKALEEKVKALGGGGRIEELKKK-WEELKKKIEELGGGGEVKKVEEEVKKLEEEIKKL.The process was repeated for the second loop and the model was optimized by energy minimization routines using the Discover module (cvff force field). The final sequence of MM1 is: GSRVKALEEKVKALEEKVKALGNATPTELAKLKKK-WEELKKKIEELGNATPTEVKKVEEEVKKLEEEIKKL. In this work, three alternative versions of the peptide were investigated: the aglycosylated control peptide MM1, the monoglycosylated (GalNAc-MM1), and diglycosylated ($GalNAc_2$-MM1) counterparts, and where either the N-terminal loop or both loops were *O*-glycosylated. All peptides were synthesized by standard solid-phase methods using α-GalNAc-threonine as a building block at the desired site. The peptides were manually cleaved from the resin, precipitated with ethyl ether, and purified by reverse HPLC. The protective acetyl groups in GalNAc-MM1 and $GalNAc_2$-MM1 were removed by treatment with sodium methoxide in methanol. After solvent evaporation the deprotected peptide was desalted and further purified by size exclusion chromatography. The molecular mass of pure peptides was confirmed via matrix-assisted laser desorption mass spectrometry (MALDI-TOF).

2.2. Chemical denaturation studies

Guanidine and urea denaturation profiles were obtained by monitoring the change of ellipticity at 222 nm (θ_{222}) as a function of denaturant concentration on JASCO 710 spectropolarimeter equipped with PTC 424S Peltier temperature controller and ATS-429S Automatic titration system as described previously [14]. A 2 μM solution of peptide in 50 mM potassium phosphate, pH = 7.0 was titrated with a second solution identical to the first but containing either 7.5 M GdnHCl or 9 M urea. Observed ellipticity values (θ_{obs}) were fit using the model previously described [19].

2.3. Parameterization of N-Acetylgalactosamine

Since parameters for *N*-acetylgalactosamine (Fig. 1) were not available in either the CHARMM 22 force field for proteins [20] or the CSFF force field for carbohydrates [21] it was necessary to develop parameters for this sugar. Many of the