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RESEARCH PAPER

O-Linked Glycosylation Leads to Decreased Thermal Stability of Interferon Alpha 2b as Measured by Two Orthogonal Techniques

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ABSTRACT

Purpose Thermal stability is considered an indication of protein fold and conformational stability. We investigate the influence of glycosylation on the thermal stability of interferon alpha 2b (IFN α-2b).

Methods Far ultraviolet light circular dichroism spectroscopy (UV CD) and differential scanning calorimetry (DSC) were used to assess the thermal stability of the European Directorate for the Quality of Medicines IFN α-2b reference standards as well as an O-linked glycosylated IFN α-2b produced in human embryonic kidney cells.

Results Assessment of thermal stability of IFN α-2b and glycosylated IFN α-2b by DSC revealed that non-glycosylated interferon (Tm = 65.7 +/− 0.2°C, $n = 3$) was more thermally stable than the glycosylated variant (Tm = 63.8 C +/− 0.4°C, $n = 3$). These observations were confirmed with far UV CD (Tm IFN α-2b = 65.3 +/− 0.4°C, Tm glycosylated IFN α-2b = 63.6 +/− 0.2°C, $n = 3$). Enzymatic deglycosylation of IFN α-2b resulted in improved thermally stability when assessed with far UV CD and DSC.

Conclusion We demonstrate that O-linked glycosylation decreases the thermal stability of IFN α-2b compared to a non-glycosylated variant of the protein.

KEY WORDS circular dichroism · differential scanning calorimetry · glycosylation · interferon

ABBREVIATIONS

CD	circular dichroism
EDQM	European Directorate for the Quality of Medicines
IFN	interferon
Tm	melting temperature

INTRODUCTION

Stability for protein biologics (protein-based therapeutics) is critical for both efficacy and safety. Numerous approaches to increase the stability and biological activity of biologics have been employed, including encapsulation in nanoparticles (1), genetic modification of the amino acid sequence (2) and surface modification through the use of polymers (3) or sugars/carbohydrates (4). For example, a number of therapeutic products with polyethylene glycol (PEG) surface modification are in development or have been approved for clinical use, such as PEGylated erythropoietin (5) and PEGylated Interferon α-2b (6). In addition to using PEG to modify a biologic's surface, research has also been conducted in surface modification through the use of simple sugars or more complex carbohydrates. For example, the hyperglycosylation of a recombinant

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human erythropoietin (Darbepoetin alfa) has demonstrated improved circulation half-life and enhanced *in vivo* activity (5). Indeed, some have suggested glycoengineering as a general strategy to improve the therapeutic properties and behaviour of protein biologics (7) .

Recently, Loignon and coworkers developed a high-yield process for the production of an O-linked glycosylated human recombinant IFN α-2b in the human embryonic kidney cell line HEK293 (8). This platform offers advantages, since naturally occurring IFN α-2 is O-glycosylated, whereas recombinant IFN α-2b produced in a bacterial expression system is not (9). In the research presented here, we examine the thermal stability, through two orthogonal techniques (far U/V circular dichroism spectropolarimetry and differential scanning calorimetry), of the O-glycosylated human IFN α-2b produced in HEK293 cells, an enzymatically deglycosylated variant of this glycosylated IFN and a non-glycosylated IFN α-2b reference standard produced in *E.coli* and sourced from the European Directorate for the Quality of Medicines (EDQM).

MATERIALS AND METHODS

Materials

Interferon alpha 2b (IFN α-2b) was obtained from the EDQM (Strasbourg, France). O-linked glycosylated IFN α-2b produced in a human kidney (HEK293) cell expression system was produced as already described (8). Protein concentrations were measured using a BCA assay kit from Sigma (St. Louis, USA). SYPRO Ruby protein stain was obtained from Invitrogen (Burlington, Canada), and Ready Gel Tris-HCI Precast Gels were supplied by Bio-Rad (Mississauga, Canada). All other chemicals and materials were supplied by Sigma (St. Louis, USA).

Deglycosylation of O-Linked Glycosylated Interferon α-2b

Deglycosylation of O-linked glycosylated IFN α-2b was carried out with the EDGLY deglycosylation kit (Sigma; St. Louis, USA), in 50 mM NaH_2PO_4 pH 7.4, according to manufacturer's instructions. Deglycosylation was confirmed with 1-D SDS-PAGE visualized with SYPRO Ruby protein stain (Invitrogen, Burlington, Canada) and a Bio-Rad Molecular Imager Gel Doc XR+system with Quantity One 1-D analysis software (Bio-Rad, Mississauga, Canada).

Circular Dichroism Spectropolarimetry

Samples were diluted to appropriate concentrations with buffer (5 mM Na H_2PO_4 pH 7.5) in 1 cm quartz cuvettes (Hellma, Müllheim, Germany) and were analyzed on a Jasco 815 spectropolarimeter (Jasco International, Tokyo, Japan) equipped with Peltier thermal control unit. The instrument and thermal control unit were controlled with Spectra Manager Software (JASCO International Co., Ltd., Tokyo, Japan). The spectra for structural analysis represent the average of 5 scans from 260 to 180 nm with a step size of 0.1 nm and a response time of 1 s. The calculation of secondary structure was conducted using Dichroweb (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) with CDSSTR references set optimized for wavelengths between 190 nm and 240 nm (10) with results presented as mean +/− standard deviation of at least three separate experiments. For thermal denaturation studies, circular dichroism (measured in millidegrees) was monitored at 222 nm (11) between 20°C and 90°C in 1°C per minute increments. Data were analyzed with Sigma Plot 9.01 utilizing the method outlined by Greenfield (11) with results presented as mean +/− standard deviation of at least three separate experiments.

Differential Scanning Calorimetry

Samples were diluted to appropriate concentrations with buffer (50 mM Na H_2PO_4 pH 7.4), degassed and analyzed with a Calorimetry Science Corporation N-DSC III controlled with DSCRun™ software (now TA Instruments, New Castle, Delaware, USA). Samples were run under 3 atmospheres of pressure with the temperature increasing between 5°C and 100°C with 2°C per minute increments. Data were analyzed with Calorimetry Science Corporation CP Calc™ software with the transition temperature (Tm) presented as mean +/− standard deviation of at least three separate experiments unless otherwise noted.

Interferon Potency Assay

Samples of IFN α-2b, O-linked glycosylated IFN α-2b and deglycosylated IFN α-2b were prepared as described above and their potency compared using a reporter gene assay as described previously (12). Briefly, this assay utilizes an Interferon Stimulated Response Element (ISRE)-containing promoter coupled to a firefly luciferase gene which is stably transfected in a human kidney cell line (HEK293) (12).

Statistical Analysis

Paired *T*-test analysis for circular dichroism and differential scanning calorimetry studies was carried out utilizing SigmaPlot 11.2.0.5 software. Significance was designated as $P<0.05$.

RESULTS

Far U/V Circular Dichroism Spectropolarimetry of Interferon α-2b Reference Standard and O-Linked Glycosylated Interferon α-2b

Far U/V CD spectropolarimetry was used to assess both secondary structure and thermal stability in the IFN α-2b reference standard (EDQM) and the O-linked glycosylated variant. The spectrum obtained for both proteins was typical of a protein with a high content of alpha-helical secondary structure with strong negative bands at 222 nm and 208 nm and a strong positive band at 190 nm (Fig. 1). Calculation of secondary structure using the CDSSTR deconvolution algorithm showed similar amounts of alpha-helical content in the two proteins, although a slight but insignificant (P=0.243) decrease in beta sheet content was observed for the glycosylated interferon variant (Table I). The secondary structure content is similar to what has been observed in previous studies (13). Thermal stability of IFN α-2b and the O-linked glycosylated IFN α-2b variant was monitored at 222 nm as the temperature of the cell was incrementally raised from 20 to 90°C. These data showed that both IFN α-2b variants lost alpha helical content as the temperature increased; IFN α-2b (Tm=65.3 +/− 0.4°C, Fig. 2, Table I) was significantly (P= 0.003) more thermally stable than O-linked glycosylated IFN α-2b (Tm=63.6 +/− 0.2°C, Table I).

Assessment of Thermal Stability of Interferon α-2b and O-Linked Glycosylated Interferon α-2b with Differential Scanning Calorimetry

Thermal stability assessed by far U/V CD is solely determined through the thermal decay of alpha helical structure. Although IFN α-2b is an alpha helical bundle protein, it does contain significant quantities of non-helical secondary structure; thus, the thermal stability of each of the two IFN variants was also assessed with differential scanning calorimetry. Calculation of thermal stability of IFN α-2b and the O-linked glycosylated IFN α-2b variant revealed a similar trend as that observed with far U/V CD analysis. IFN α-2b demonstrated a mean Tm, from three experiments, of 65.7 +/− 0.2°C (Fig. 3, Table I), and the glycosylated variant had a statistically different mean Tm (P=0.001) of 63.8°C +/− 0.4 (Table I).

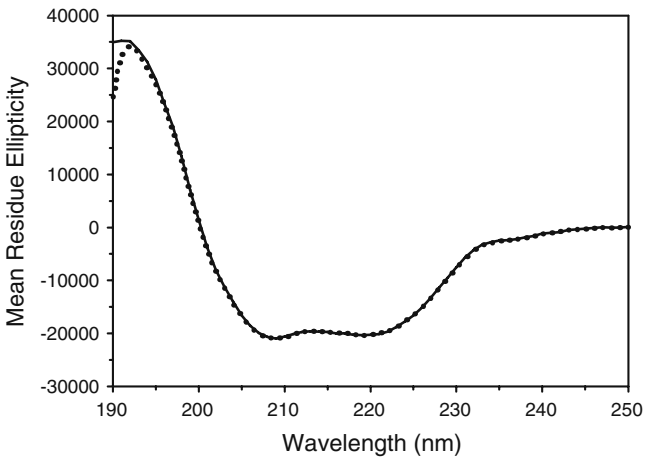

Fig. 1 Far U/V CD spectra of IFN α-2b reference standard (solid line) and O-linked glycosylated IFN α-2b (dotted line) in 5 mM NaH_2PO_4 ph 7.4. Each spectrum represents the mean of at least three separate runs of five accumulations each on a Jasco 810 spectropolarimeter.

Deglycosylation of O-Linked Glycosylated Interferon α-2b

The enzymatic deglycosylation of O-linked glycosylated IFN α-2b was analyzed with SDS-PAGE and SYPRO ruby staining (Fig. 4) and analyzed with Quantity One 1-D analysis software. Analysis showed that IFN α-2b incubated at 37°C for 48 h (Fig. 4 Lane 2) showed a single band with an apparent molecular weight of 16 kDa. No change in apparent molecular weight of IFN α-2b was observed when enzyme was included during the incubation (Fig. 4 Lane 3). When O-linked glycosylated IFN α-2b was incubated for 48 h, a heavy band with an apparent molecular weight of 19 and faint lighter band at 16.6 kDa were observed (Fig. 4 Lane 4). When O-linked glycosylated IFN α-2b was enzymatically deglycosylated, SDS-PAGE analysis showed very faint bands at approximately 18.4 kDa and 16.6 kDa and a strong band with an apparent molecular weight of 16 kDa (Fig. 4, Lane 5). These results suggest that enzymatic deglycosylation was successful with approximately 95% of the glycosylated material being deglycosylated. Upon enzymatic deglycosylation of O-linked glycosylated IFN α-2b, the product demonstrated improved thermal stability relative to the glycosylated variant from which it was derived, with Tm values determined by both far UV CD (Tm=64.8 +/− 0.5°C, Table I) (P=0.248) and DSC (Tm=65.3°C, Table I) now closely approximating those determined for the IFN α-2b reference standard (EDQM).

Potency Assay

A reporter gene assay using a minimal promoter containing Interferon Stimulated Response Element (ISRE) coupled to a firefly luciferase gene, stably transfected in a human kidney cell line (HEK293) (12), was used to assess the potency of the IFN α-2b reference sample, O-linked glycosylated IFN α-2b and deglycosylated IFN α-2b. Results for the assessment are shown in Fig. 5 and demonstrate the O-linked glycosylated IFN has similar potency to deglycosylated IFN α-2b in this assay. Further-

Table I Summary of Thermal Stability as Measured by far U/V Circular Dichorism and Differential Scanning Calorimetry for IFN α-2b, O-linked Glycosylated IFN α-2b and Deglycosylated IFN α-2b

	IFN α-2b	O-linked glycosylated IFN α-2b	Deglycosylated IFN α-2b
Tm Far U/V CD °C	65.3 +/− 0.4	63.6 +/− 0.2	64.8 +/− 0.5
Tm DSC °C	65.7 +/− 0.2	63.8 +/− 0.4	65.3[a]
Alpha Helix%	47.1 +/− 1.1	47.7 +/− 1.5	48.0 +/− 1.0
Beta Sheet%	22.4 +/− 2.7	19.7 +/− 2.1	17.7 +/− 0.6
Beta Turn%	10.6 +/− 1.1	10.7 +/− 1.2	11.0 +/− 1.0
Unordered%	19.8 +/− 1.3	23.0 +/− 1.0	23.7 +/− 0.6

[a] A single sample was assayed

more, using this potency assay, interferon produced in HEK293 cells appears to be less potent than the IFN α-2b reference standard regardless of its glycosylation status (Fig. 5).

DISCUSSION

In these studies, we investigated the influence of O-linked glycosylation on the thermal stability of interferon alpha 2b (IFN α-2b) with two orthogonal techniques. When compared to a reference IFN α-2b sourced from the EDQM, we observed a decrease in thermal stability with both far U/V CD and differential scanning calorimetry for O-linked glycosylated IFN α-2b. Additionally, we observed a decrease of about 30–40% in *in vitro* potency with the glycosylated variant.

Conformational stability of a protein as measured by resistance to denaturation by heat or chaotropic agents is often considered a good first indicator of both shelf-life and *in vivo* half-life for therapeutic proteins. Because many of the common degradation pathways of biologics (deamidation, oxidation, proteolysis, etc.) are accelerated when proteins are fully or partially unfolded (14,15), thermal stability measurements are much used in formulation development studies. Likewise, intrinsic conformational stability is expected to provide increased resistance to chemical and proteolytic degradation of therapeutic proteins *in vivo* (16) but is probably just one of several factors that influence drug bioavailability and *in vivo* half-life. Nonetheless, several studies have demonstrated that increasing the conformational stability of therapeutic enzymes, cytokines and antibodies can correlate to increased efficacy and/or half-life *in vivo* (17–20).

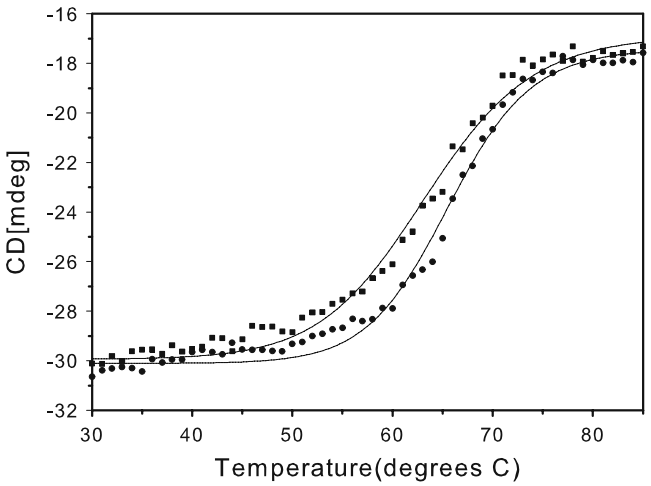

Fig. 2 Representative normalized thermal denaturation of at least three separate experiments for IFN α-2b reference standard (●) and O-linked glycosylated IFN α-2b (■) as measured by far U/V circular dichroism with a Jasco 815 spectropolarimeter.

In this regard, a number of studies have shown the benefits of glycosylation for therapeutic proteins, benefits which include improvements in both stability and circulatory lifetimes (21). For example, Narhi and coworkers demonstrated that erythropoietin with N- and O-linked carbohydrates expressed in mammalian cells demonstrated improved conformational stability with respect to chemical denaturing agents (guanidinium HCl), pH and temperature when compared to erythropoietin expressed in *Escherichia coli* (22). Another example of the utility of glycosylation is provided by Runkel and coworkers through their studies of interferon beta (IFN β). These researchers noted two hydrogen bonds between the carbohydrate and Asn-86 in helix-C and Gln-23 in helix-A and observed a nearly 4.5°C increase in thermal stability of the glycosylated IFN β-1a (Tm=67.3 +/− 0.3°C) over that of the non-glycosylated IFN β-1a (Tm=62.9 +/− 0.3°C) (23). These researchers also suggested that the greater *in vitro* potency they observed for IFN β-1a (Avonex) *versus* IFN β-1b (Betaseron) was due to the improved stability of IFN β-1a afforded by its N-linked carbohydrate structure (23).

Our results, although unexpected, are not unprecedented; previous studies have shown that the presence of carbohydrate does not always improve the stability of the protein. Studies by Yesilyurt and coworkers investigated the stability of homologous protein units of β-hemocyanin from *Helix pomatia*. These researchers found that for this complex respiratory protein from the roman snail there was no linear correlation between the level of glycosylation and the unfolding temperature of its functional units, which share 50% sequence homology (24). Furthermore, Spiriti and coworkers studied the effect of O-linked glycosylation on a miniprotein analog of the macrophage-activating factor Gc-MAF (25). They concluded that the presence of an O-linked

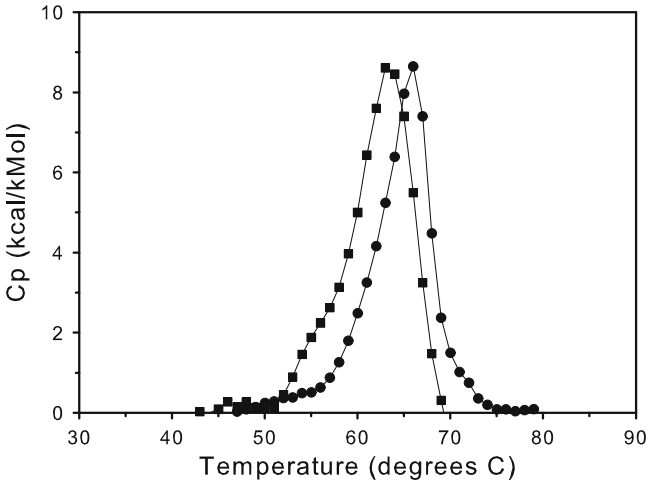

Fig. 3 Representative normalized thermograms of at least three separate experiments for IFN α-2b reference standard (●) and O-linked glycosylated IFN α-2b (■) as measured by differential scanning calorimetry.

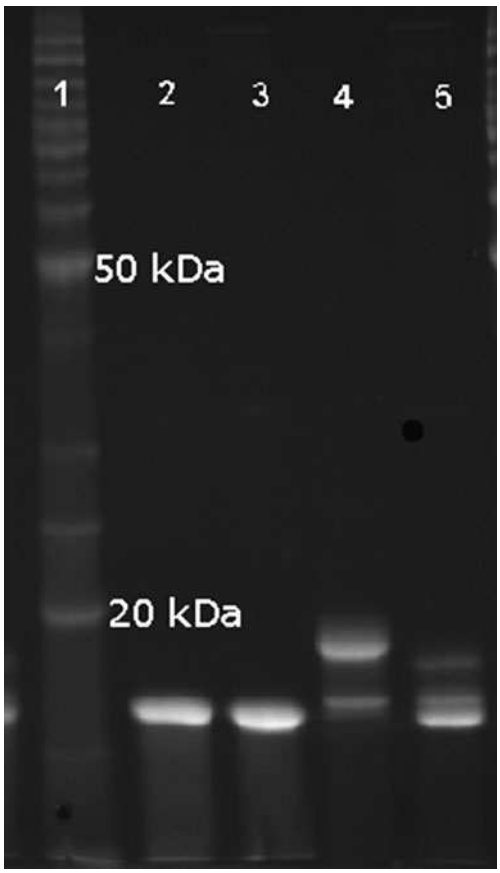

Fig. 4 12% SDS-PAGE of IFN α-2b, O-linked glycosylated IFN α-2b and enzymatically deglycosylated IFN α-2b. Lane 1, BenchMark™ molecular weight markers. Lane 2, IFN α-2b incubated at 37°C for 48 h. Lane 3, IFN α-2b incubated with deglycosylation enzymes at 37°C for 48 h. Lane 4, O-linked glycosylated IFN α-2b incubated at 37°C for 48 h. Lane 5, O-linked glycosylated IFN α-2b incubated with deglycosylation enzymes at 37°C for 48 h. (2.2 μg protein/lane for IFN α-2b, 1.6 μg protein/lane for glycosylated IFN α-2b).

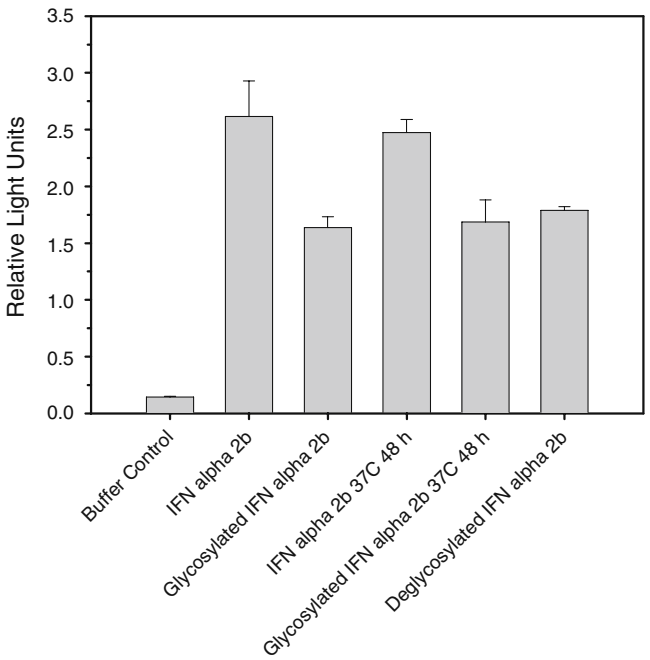

Fig. 5 Mean potency of IFN α-2b, O-linked glycosylated IFN α-2b and deglycosylated IFN α-2b at 50 IU/ml as measured by an interferon stimulating response element (ISRE) promoter coupled to a firefly luciferase gene. Error bars represent standard deviation of at least six replicates.

glycan reduced the stability of the protein, measured through chemical denaturation, by approximately 1 kcal/mol. They attributed this loss of stability to changes in the packing of the protein resulting in increased exposure of hydrophobic residues to solvent and an increase in the radius of gyration leading to a decrease in contact between hydrophobic residues (25).

The reporter gene assay used in this study indicated decreased potency for O-linked glycosylated IFN and an inability of this product to attain the maximal signal observed for its non-glycosylated counterpart even at increased concentrations (data not shown). Maximal potency was not restored with removal of the O-linked glycan. These observations were unexpected, particularly in view of the fact that naturally occurring IFN α-2b is similarly glycosylated at the same site and the published evidence that the IFN β homologue is more potent when glycosylated (23). However, others have found that IFN α-2 mutants with engineered N-linked glycosylation sites have decreased *in vitro* activity (26). However, these hyper-glycosylation variants showed increased *in vivo* antitumor activity in a mouse model (27), suggesting that many other factors besides thermal stability and *in vitro* potency may be at play in the *in vivo* situation. The decrease in potency of O-linked glycosylated IFN in the luciferase-based reporter gene assay used here are also in contrast to the results of Loignon *et al.*, who found that the O-linked glycosylated IFN had the same or slightly higher potency than an *E. coli*

produced form in both a reporter gene assay and in an antiviral assay (8). There are several differences between the assay used by Loignon and coworkers and ours that may account for this apparent discrepancy: the differences in reporter genes (one resulting in a secreted protein, the other not) the presence or absence of serum in the medium, etc. More importantly, Loignon and coworkers used a different non-glycosylated version of IFN α-2b as a comparator than we did. Their comparator was a commercially produced product not intended for human use that had not been verified in terms of potency. We used the EDQM reference standard, a reference standard produced by a licensed manufacturer of therapeutic IFN α-2b under contract to the EDQM. It was examined by an international study and calibrated against the WHO potency standard for IFN α-2b (28) and, thus, is able to give a better indication of the relative biological activity of the O-linked glycosylated IFN before and after deglycosylation.

In both naturally occurring IFN α-2b and the O-linked glycosylated IFN α-2b used in this study a relatively small carbohydrate moiety is covalently attached to the hydroxyl oxygen of threonine 106 in the human IFN protein. This residue is in a loop region joining helix 3 to helix 4 in the folded helical bundle structure determined for IFN α-2b MMDB ID: 56471(29). This loop contains a number of hydrophobic residues that appear to be involved in interactions of the loop with the rest of the folded protein. Glycosylation of this threonine would significantly increase the hydrophilic character of the side chain and may interfere with the hydrophobic interactions that stabilize this loop within the overall folded structure. Introduction of the carbohydrate moiety might also decrease the conformational flexibility of the loop region. Previous studies have shown that the flexibility of loops connecting helices in helical bundle proteins can be critical in allowing proper packing of one helix against another (30) and can result in decreased protein stability like that observed for the O-linked glycosylated IFN α-2b used in this study. Removal of the carbohydrate would be expected to remove these steric and hydrophilic constraints and result in the increased stability observed when the O-linked carbohydrate was enzymatically removed from the glycosyalted IFN α−2b sample.

The decrease in *in vitro* potency observed for O-linked IFN α-2b is less easily explained using structural considerations. The potency assay used here depends upon the ability of the interferon variant to bind to its natural cellular receptor and thereby trigger the normal activation of the interferon sensitive response element that has been artificially placed in front of a luciferase reporter gene. There are three major regions of the interferon molecule (residues 29–35, 78–95, and 123–140, referred to as IRRP1, IRRP2, and IRRP3, respectively) that have been identified as critical for its interaction with the receptor (31), all of which appear to be structurally remote from the site of glycosylation. While glycosylation at threonine 106 may affect the formation of the loop in which it is situated and hence the positioning of the helix containing IRRP3, it is not immediately clear why removal of the sugar would not allow restoration of the normal positioning of this element and concomitant restoration of full potency.

CONCLUSION

We investigated the effect of O-linked glycosylation on the thermal stability of interferon alpha 2b and concluded that the presence of the carbohydrate decreased the thermal stability of the glycosylated IFN variant as compared to the non-glycosylated variant; thermal stability was largely restored after enzymatic deglycosylation. However, the relative lower potency for the glycosylated variant did not rebound after deglycosylation, indicating the effects on *in vivo* function may not be reversible. We believe the effects on thermal stability may be due to steric constraints that are introduced with glycosylation and affect the folding of one helix against another. The effects of glycosylation on potency in the *in vitro* assay are not as easily attributable to structural considerations and remain the subject of further investigation.

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