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Research paper

Influence of carbohydrates on the stability and structure of a hyperglycosylated human interferon alpha mutein

Natalia Ceaglio*, Marina Etcheverrigaray, Ricardo Kratje, Marcos Oggero

Laboratorio de Cultivos Celulares, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje "El Pozo", C.C. 242, S3000ZAA Santa Fe, Argentina

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ABSTRACT

Protein physical and chemical instability is one of the major challenges in the development of biopharmaceuticals during every step of the process, ranging from production to final delivery. This is particularly applicable to human recombinant interferon alpha-2b (rhIFN-α2b), a pleiotropic cytokine currently used worldwide for the treatment of various cancer and chronic viral diseases, which presents a poor stability in solution. In previous studies, we have demonstrated that the introduction of four *N*-glycosylation sites in order to construct a heavily glycosylated IFN variant (4N-IFN) resulted in a markedly prolonged plasma half-life which was reflected in an enhanced therapeutic activity in mice in comparison with the commercial non-glycosylated rhIFN-α2b (NG-IFN). Herein, we evaluated the influence of glycosylation on the *in vitro* stability of 4N-IFN towards different environmental conditions. Interestingly, the hyperglycosylated cytokine showed enhanced stability against thermal stress, acid pH and repetitive freeze-thawing cycles in comparison with NG-IFN. Besides, microcalorimetric analysis indicated a much higher melting temperature of 4N-IFN, also demonstrating a higher solubility of this variant as denoted by the absence of precipitation at the end of the experiment, in contrast with the NG-IFN behaviour. Furthermore, far-UV circular dichroism (CD) spectrum of 4N-IFN was virtually superimposed with that of NG-IFN, indicating that the IFN structure was not altered by the addition of carbohydrate moieties. The same conclusion could be inferred from limited proteolysis studies. Our results suggest that glycoengineering could be a useful strategy for protecting rhIFN-α2b from inactivation by various external factors and for overcoming aggregation problems during the production process and storage.

1. Introduction

More than fifty years of research have contributed to the extensive characterization of human interferon alpha (hIFN-α) in terms of structure, biological activities and clinical therapeutic effects. hIFN-αs are a group of pleiotropic cytokines that were first defined by their property of inducing paracrine resistance against virus infection [1]. Later, other important functions of these cytokines were discovered, including potent antiproliferative and immunomodulatory effects [2].

Recombinant hIFN-α2 derived from genetically engineered *Escherichia coli* represents the cytokine exhibiting the longest record of use in clinical oncology, including treatment of hematological malignancies (hairy cell leukemia, chronic myeloid leukemia and some B- and T-cell lymphomas) and certain solid tumors (melanoma, renal carcinoma and Kaposi's sarcoma) [3,4]. Also, because of its antiviral properties, rhIFN-α is the treatment of choice for patients with chronic hepatitis B and C virus infections [5,6].

Effectiveness of rhIFN-α2 has been limited because of its rapid elimination from human circulation, with the consequent need of frequent high dose injections to maintain therapeutic levels. The resulting fluctuations in exogenous IFN alpha concentration during the course of treatment are believed not only to negatively impact the efficiency of the cytokine but also to be responsible of its numerous side effects, whose severity is greater at high IFN concentrations [7,8].

Abbreviations: rhIFN-α2b, human recombinant interferon alpha-2b; 4N-IFN, rhIFN-α2b with four *N*-glycosylation sites; NG-IFN, non-glycosylated rhIFN-α2b; WT-IFN, wild-type rhIFN-α2b; DG-IFN, De-*N*-glycosylated 4N-IFN; CD, circular dichroism; CHO, Chinese Hamster Ovary; mAb, monoclonal antibody; DSC, differential scanning calorimetry; PBS, phosphate-buffered saline; VSV, vesicular stomatitis virus; MDBK, Madin–Darbin bovine kidney; MEM, minimum essential medium; FCS, fetal calf serum; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; T, Tween; T_m, melting temperature; SEM, standard error of the mean.

* Corresponding author. Tel./fax: +54 342 4564397.
E-mail address: nceaglio@fbcb.unl.edu.ar (N. Ceaglio).

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We have previously reported the construction and characterization of a highly *N*-glycosylated rhIFN-α2b mutein (4N-IFN) designed to decrease the clearance of the cytokine and extend its duration of action. This IFN variant showed an improved pharmacokinetic profile in comparison with two FDA-approved rhIFN-α2b variants ($t_{1/2} = 14.57 \pm 0.78$ h compared to 0.64 ± 0.12 h for non-glycosylated *E. coli*-derived IFN and 11.39 ± 0.75 h for 12 kDa pegylated IFN). This allowed the sustainment of steady plasma concentrations for an extended period of time, resulting in a better *in vivo* antitumor efficacy in animal models (2.5-fold and 1.2-fold reduced tumor volume compared to non-glycosylated IFN and pegylated IFN, respectively) [9].

However, glycosylation engineering of pharmaceutical proteins can provide many additional advantages not only of biological relevance but also regarding the different steps of the production process.

Isolation, purification, delivery and long-term storage of proteins represent significant challenges to scientists because of the unique chemical and physical properties of these biomolecules [10,11]. Particularly, the poor structural stability of biopharmaceuticals increases the probability of aggregation and degradation in the production process, during which they are constantly exposed to environmental stresses [12]. Various physicochemical factors, including temperature, pH, ionic strength, shaking, freeze-thawing and freeze-drying, among others, can induce or speed up the protein aggregation and/or degradation pathways, thereby compromising its biological activity and leading to immune responses and even cytotoxicity [11,13,14].

In this work, we evaluated the influence of glycosylation on the stability of 4N-IFN subjected to different environmental factors. We also employed limited proteolysis experiments as a first approach to probe conformational changes in 4N-IFN with respect to the non-glycosylated rhIFN-α2b in native and denaturing conditions.

2. Materials and methods

2.1. Differentially glycosylated interferon variants

The non-glycosylated *E. coli*-derived rhIFN-α2b (NG-IFN) was kindly provided by Protech Pharma S.A. (Argentina) as a 1 mg ml^{-1} solution in phosphate buffer, pH 7–7.4. The wild-type *O*-glycosylated rhIFN-α2b (WT-IFN) and the highly *N*-glycosylated rhIFN-α2b mutant (4N-IFN, with four potential *N*-glycosylation sites due to the substitution of Pro4, Arg23, Lys70 and Asp77 by Asn) were produced in CHO cell cultures as previously described [15]. Briefly, stable CHO clones expressing both cytokines were grown in 500 cm^2 triple flasks and culture supernatants were applied onto an immunoaffinity matrix prepared by coupling the anti-rhIFN-α2b mAb CA5E6 to CNBr-activated Sepharose 4B (GE Healthcare, USA) for IFN isolation. After washing with 5 bed volumes of (A) 0.5 M NaCl, 0.2% (v/v) Triton X-100 in 25 mM Tris–HCl (pH 7.5) and (B) 0.15 M NaCl, bound rhIFN-α2b was eluted employing 0.1 M glycine (pH 2) and pH was immediately neutralized with 1 M Tris–HCl (pH 9). De-*N*-glycosylated 4N-IFN (DG-IFN) was obtained by incubation of the purified mutant with 0.5 U of PNGase F (Biolabs Inc., UK) overnight at 37 °C in non-denaturing conditions.

2.2. Stability against heat treatment

The thermal stability of NG-IFN, WT-IFN, 4N-IFN and DG-IFN was investigated by determining the residual biological activity after heating at different temperatures. Samples containing 1 μg ml^{-1} of the protein variants in phosphate-buffered saline, pH 7.4 (PBS) were incubated in a heat block (Biometra, Germany) for 10 min at 20 °C, 25 °C, 37 °C, 45 °C, 55 °C, 65 °C, 75 °C, 85 °C and 95 °C and immediately frozen at −20 °C. Samples were assessed for IFN antiviral activity and the level of heat-induced aggregation was monitored by SDS-PAGE followed by Western blot in non-denaturing conditions. T_m values (defined as the temperature at which 50% of the initial activity is lost after heat treatment) were determined from the plots of relative inactivation (%) against temperature (°C). The experiment was accomplished in duplicates.

Differential scanning calorimetry (DSC) studies of NG-IFN and 4N-IFN were carried out at a scan rate of 0.75 °C/min on a differential scanning microcalorimeter (VP-DSC, MicroCal Inc., USA). Prior to the measurement, the protein solutions were dialyzed in 50 mM phosphate/150 mM NaCl, pH 7, and concentrations were measured by UV absorbance ($\lambda = 280$ nm). DSC scans were repeated three times, normalized for concentration and baseline subtracted for comparison. The scans obtained did not show a reversibility acceptable for thermodynamic analysis, so they were studied mainly for the onset of unfolding and the overall shape of the thermogram.

2.3. Influence of pH on the stability of IFN variants

In order to evaluate the stability of IFN analogs at acid pH, NG-IFN and 4N-IFN were prepared in an initial concentration of 70 μg ml^{-1} in PBS. Then, samples were diluted to a final concentration of 50 ng ml^{-1} in 0.1 M glycine (pH 2) and incubated at room temperature. Aliquots were taken at different incubation times (0, 15, 30 min and 1, 2, 4 h) and the pH was neutralized with 1 M Tris–HCl (pH 9). Samples were stored at −20 °C until assayed for IFN antiviral activity. The experiment was carried out in duplicates.

2.4. Stability against repeated freezing and thawing cycles

The effect of repeated freezing and thawing cycles on the stability of NG-IFN and 4N-IFN was analyzed by spectrophotometry and SDS-PAGE followed by Western blot. Each sample diluted to a final concentration of 50 μg ml^{-1} in PBS was rapidly frozen in liquid nitrogen (−196 °C) and then transferred to a water bath maintained at 37 °C for thawing. These freezing and thawing cycles were repeated 15 times and aliquots were taken every five cycles. Aggregation of all samples was examined by Western blot under non-denaturing conditions. Additionally, the level of aggregation was evaluated by measuring protein concentration at 280 nm and turbidity at 405 nm of the untreated and the 15-times frozen/thawed samples.

2.5. Limited proteolysis by trypsin

Native NG-IFN and 4N-IFN were subjected to proteolysis with trypsin (Promega, USA) at a weight ratio of 1:10 (enzyme to protein). The cytokines were diluted to a final concentration of 5 μg ml^{-1} in 0.1× PBS containing 0.1 mM EDTA and incubated with an appropriate volume of trypsin 150 μg ml^{-1} dissolved in the same buffer at room temperature. Aliquots of both samples were obtained at different times (0, 15, 30 min and 1, 2, 4, 6 and 8 h) and immediately mixed with a solution consisting of 50 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.05% (w/v) bromophenol blue for SDS-PAGE/Western blot analysis. The intensities of the bands were quantified with the ImageMaster TotalLab version 1.11 software (GE Healthcare). In another experiment, NG-IFN and 4N-IFN were heated for 10 min at 75 °C and then subjected to limited proteolysis as described above.

2.6. Far-UV circular dichroism (CD)

Circular dichroism spectra were recorded in the range of 200–250 nm with a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, USA) at room temperature using a scan speed of 20 nm min^{-1} and a response time of 1 s. NG-IFN and 4N-IFN protein samples were loaded in a cuvette with 0.1 cm pathlength at a concentration of 0.33 mg ml^{-1}. The spectra shown are an average of three repeated scans that were corrected by subtraction of the buffer signal. The CD data were expressed as molar ellipticity, [θ], in degrees per square centimeter per decimole, based on a mean residual weight of 116.76 Da per amino acid residue.

2.7. Protein analysis

2.7.1. In vitro antiviral activity

The antiviral activity of rhIFN-α2b was determined by its ability to inhibit the cytopathic effect caused by vesicular stomatitis virus (VSV) on MDBK cells [16,17]. For this, 2.5×10^4 cells per well were seeded into culture microtiter plates in minimum essential medium (MEM; Gibco, USA) supplemented with 10% (v/v) FCS and incubated at 37 °C overnight. After removing culture supernatants, 1:2 serial dilutions of rhIFN-α2b WHO international standard (NIBSC 95/566) from 20 U/ml to 0.16 U/ml or 1:2 serial dilutions of rhIFN-α2b test samples in 2% (v/v) FCS-supplemented medium were added. Plates were incubated for 6 h at 37 °C and, after removal of supernatants, an appropriate dilution of VSV virus was added. Virus replication was allowed to proceed until the cytopathic effect was clearly observable in control wells (no IFN). The medium was discarded and cells were fixed and stained simultaneously with a solution of 0.75% (w/v) crystal violet in 40% (v/v) methanol. After 10 min, plates were washed with water and the remaining dye was solubilized in 20% (v/v) acetic acid. The plates were read at 540 nm with a microtiter plate reader and the signal intensity of each dilution was reported as the mean of the absorbance measured in five wells.

2.7.2. Electrophoresis and Western blotting

Polyacrylamide gel electrophoresis was performed according to the standard method [18] using 15% (w/v) resolving gels and 5% (w/v) stacking gels. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). Blots were blocked for 1 h with 5% (w/v) non-fat milk in Tris-buffered saline (TBS) and then probed with rabbit anti-rhIFN-α2b polyclonal antibodies. After 1 h, blots were incubated with peroxidase-labelled goat anti-rabbit immunoglobulins (DAKO, Denmark). Immunoreactive bands were visualized using an ECL™ Chemiluminescent Western Blotting Analysis System (GE Healthcare). Washes between steps were performed with TBS containing 0.05% (v/v) Tween 20 (TBS-T). Dilutions were prepared in TBS-T containing 0.5% (w/v) non-fat milk.

3. Results and discussion

3.1. Thermal stability of NG-IFN, WT-IFN, 4N-IFN and DG-IFN variants

3.1.1. Determination of residual biological activity after heat treatment

Temperature is the most important factor affecting protein stability in the biopharmaceutical industry and although there is no general mechanism to describe its effect, normally the higher the temperature, the lower the stability of the polypeptide [11]. Following heat treatment, the secondary and tertiary structure of a protein may change or form aggregates, usually resulting in the irreversible loss of biological activity [19,20]. To investigate the thermal behaviour of IFN derivatives with different type and content of glycosylation, we incubated each protein at different temperatures (20–95 °C) for 10 min and evaluated the residual biological activity (Fig. 1A).

All proteins preserved nearly intact antiviral activity after 10 min incubation at 55 °C and lower temperatures. However, incubation at 65 °C caused the partial loss of biological activity of non-glycosylated rhIFN-α2b, which exhibited a T_m of 62.5 ± 4.4 °C. *O*-glycosylation did not affect the cytokine's heat resistance, as the CHO cells-derived protein showed a similar T_m (62.9 ± 1.2 °C). On the contrary, 4N-IFN showed a substantial increased thermal resistance, preserving more than 65% of residual activity after incubation at 95 °C. De-*N*-glycosylation of this IFN variant produced a protein with similar thermal behaviour compared to NG-IFN and WT-IFN (T_m = 68.2 ± 0.4 °C, no statistical differences among the three variants). These results indicate that *N*-glycans protect 4N-IFN from high temperature inactivation and that the amino acid substitutions which generate the four *N*-glycosylation sites do not affect the protein's thermal stability. Besides, it can be assumed that potential structural differences either due to the expression system (*E. coli* and CHO cells) or caused by different folding processes (post-folding addition of *O*-glycans and co-translational attachment of *N*-glycans) do not seem to be related to the observed phenomenon.

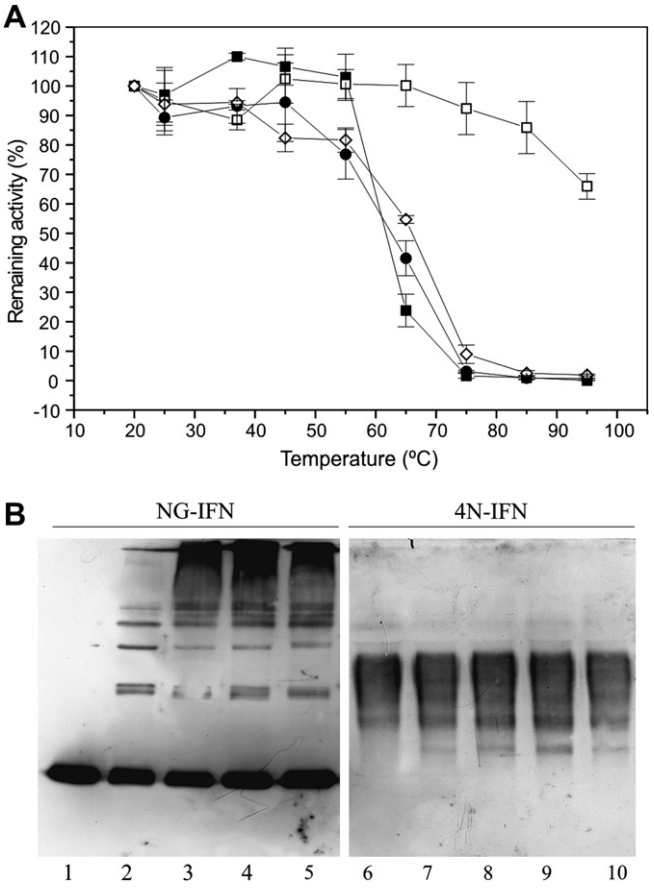

Fig. 1. Thermal stability of NG-IFN (■), WT-IFN (●), 4N-IFN (□) and DG-IFN (◇) at various temperatures. Samples containing 1 μg ml^{-1} of the protein variants in PBS were heated for 10 min at the temperatures indicated. (A) Residual antiviral activities against temperature (calculated as the percentage of the initial value). Results are expressed as mean ± SEM for two independent experiments. (B) SDS-PAGE/Western blot analysis after heat treatment: lanes 1, 6: 20 °C; lanes 2, 7: 37 °C; lanes 3, 8: 65 °C; lanes 4, 9: 75 °C; lanes 5, 10: 85 °C.

It has been previously shown that *in vitro* deglycosylation of naturally glycosylated proteins (including yeast external invertase, bovine serum fetuin, glucoamylase, chicken egg white ovotransferrin and avidin) decreases protein thermal stability, as judged by the decrease in denaturation temperature and denaturation enthalpy [21]. This destabilizing effect has proved to be dependent on the carbohydrate content, with maximum effects for heavily glycosylated proteins and no significant differences for proteins with modest glycan content (10% and less). However, these studies have been carried out with glycoproteins already existing in nature. Here we demonstrate that addition of potential *N*-glycosylation sites to a poorly *O*-glycosylated protein, rhIFN-α2b, results in an increased heat resistance, indicating a general effect of stabilization of the attached carbohydrate chains towards denaturing agents. Indeed, there are a number of examples involving chemical glycosylation of α-chymotrypsin and β-lactoglobulin [22,23] which also demonstrate an artificially enhanced thermostability in a manner that depends on both the amount of glycans and their molecular size. Mechanistically, it has been postulated that the increment of the glycosylation degree stabilizes the protein native state by increasing the internal non-covalent forces and rigidifying the protein structure. Specifically, carbohydrates seem to act as a molecular spacer between the protein electrostatics and the solvent electrostatics, so that dielectric shielding increases the strength of internal interactions and thus decreases structural dynamics, leading to improved conformational stability. Besides, increasing the glycan molecular size appears to destabilize the protein unfolded state, being this phenomenon also dependent on the location of the oligosaccharide chains. However, studies carried out by Shental-Bechor and Levy [23] using chemically glycosylated variants of the SH3 domain protein have demonstrated an average increment of T_m of 0.6–0.9 °C per added glycan. In our case, we achieved an increase in T_m of more than 8 °C per *N*-glycosylation site added. Whether these differences are related to the glycosylation process (chemical or biological, respectively) or are protein-dependent remains to be determined.

3.1.2. Assessment of heat-induced aggregation

Protein aggregation results from intermolecular association of partially denatured protein chains through hydrophobic interaction [24]. It has been reported that rhIFN-α has a tendency to acquire multiple partially unfolded states, the extent of which is greater at higher temperatures [25]. Besides, it has been demonstrated that high temperature is responsible for almost complete aggregation of recombinant consensus IFN-α after ultrasonic nebulization [26]. Since protein aggregates may have no or reduced bioactivity [11] we evaluated the level of heat-induced aggregation of non-glycosylated and highly glycosylated IFN by SDS-PAGE under non-reducing conditions. As shown in Fig. 1B, after incubation at 20 °C NG-IFN migrated as a monomer but when it was heated at 37 °C some aggregates were formed, particularly dimers and trimers. Heat treatment at temperatures above NG-IFN T_m (65 °C, 75 °C and 85 °C) resulted in the appearance of high amounts of high molecular mass multimers. As it was also reported by Ruiz et al. [14], SDS and mercaptoethanol failed to dissolve those aggregates (data not shown), suggesting that these substances do not have access to the interfaces of protein molecules because of strong hydrophobic interactions. In contrast, 4N-IFN did not produce any aggregates following heat treatment at any of the temperatures tested, correlating with preservation of its biological activity. WT-IFN and DG-IFN behaved similarly to the non-glycosylated variant, indicating that the presence of *N*-glycans determine the 4N-IFN enhanced molecular stability (data not shown).

Although the mechanism by which glycosylation affect 4N-IFN thermal stability cannot be inferred from this methodology, it is likely that carbohydrates moieties, in addition to an apparent stabilizing effect, may prevent the unfolded or partially folded molecules from aggregation [27]. This effect has been largely described for recombinant human erythropoietin (rhEPO), which retains complete activity after incubation at 70 °C for 15 min, while asialo rhEPO and deglycosylated rhEPO maintain only 35% and 11% of the initial activity, respectively [28]. Many authors have proposed that the high hydrophilicity of sialic acids present in complex-type oligosaccharides structures may contribute to block aggregation by shielding hydrophobic residues [29]. This hypothesis could be applied to 4N-IFN, whose major carbohydrate structures are tri- and tetraantennary *N*-glycans with a high terminal sialylation ratio (10.6 mol sialic acid per mol of protein) [9].

3.1.3. Differential scanning microcalorimetry

DSC is a commonly used technique to probe conformational changes in proteins with temperature and to obtain information about their thermodynamic stability. DSC studies of both NG-IFN and 4N-IFN rendered similar results than the ones obtained through residual activity measurements and SDS-PAGE evaluation of aggregation after heat treatment (data not shown). By using a scan rate of 0.75 °C/min, the non-glycosylated protein exhibited a T_m (calculated at the maximum of the heat capacity) of about 66 °C, similar to the one found in activity measurements (Fig. 1A) and to the ones reported in the literature for rhIFN-α2b at pH 7.5 ($T_m = 65.4$ [30]) and for rhIFN-α2b at pH 5 ($T_m = 66.1$ [31]). Besides, denaturation caused the aggregation and precipitation of NG-IFN in the cell after its T_m, so that the scans obtained were not acceptable for thermodynamic analysis. On the other side, 4N-IFN exhibited a much higher T_m (around 90 °C) and no precipitation was observed above its T_m, although there was some aggregation at the end of the experiment.

3.2. Influence of pH on the stability of IFN variants

Type I human interferons are characterized by their property of exhibiting high stability at low pH values [32]. We therefore investigated the remaining antiviral activity of NG-IFN and 4N-IFN after incubation in a solution of 0.1 M glycine, pH 2, at room temperature during different time periods (Fig. 2). These conditions

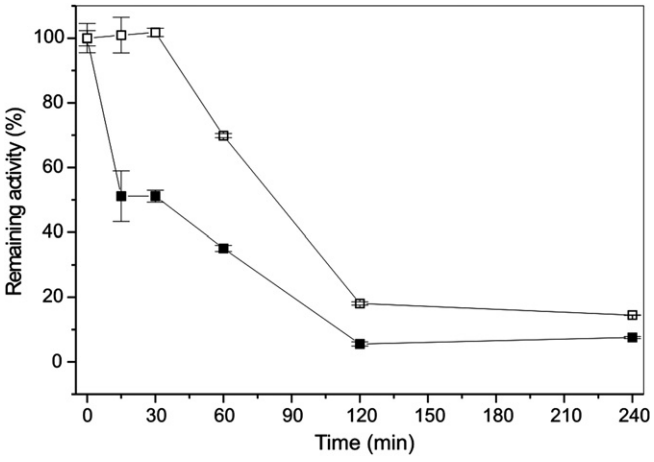

Fig. 2. Stability of NG-IFN (■) and 4N-IFN (□) at acid pH. Samples were diluted to a final concentration of 50 ng ml^{-1} in 0.1 M glycine, pH 2. Aliquots were taken at the indicated times, immediately neutralized with 1 M Tris–HCl, pH 9, and assessed for IFN antiviral activity. Results are expressed as residual activity (calculated as the percentage of the initial value) versus time (mean ± SEM for two independent experiments).

simulate the ones employed for the elution of the cytokine from the immunoaffinity matrix used for purification.

The non-glycosylated protein lost about 50% of its initial activity after 15 min of incubation at acidic pH, exhibiting 30% of remaining antiviral activity 1 h after the beginning of acid treatment. This did not occur for the *N*-glycosylated mutein, which preserved intact bioactivity after 30 min of incubation, but showed some degree of inactivation after 1 h, retaining about 70% of residual activity at this time. However, both NG-IFN and 4N-IFN were almost completely inactivated (5% and 20% of residual activity) after 2 h of low pH treatment, contrarily to what it has been reported in literature for NG-IFN. Nonetheless, it has been described that pH 2 induces some conformational changes of the hIFN-α2 molecule (but not hIFN-α1) that result in different antigenic forms, though not affecting its bioactivity on human A549 cells [33,34]. However, activity assays using bovine MDBK cells have not been carried out by these authors. Again, WT-IFN and NG-IFN showed a similar behaviours compared to NG-IFN (data not shown).

The reduced short-term lability of 4N-IFN under acidic conditions could be of great relevance during the purification process of the cytokine, which in industrial production may include many steps comprising exposure to low pH values. In particular, elution of 4N-IFN during the immunoaffinity chromatography using mAb CA5E6 is accomplished by lowering the pH, so that time to neutralize fractions is a critical factor for preserving its activity.

3.3. Stability against repeated freeze-thawing cycles

There are several points during preparation, sterilization and storage of proteins in the biopharmaceutical industry in which freeze-thawing stresses may arise, either by design or accidentally. Proteins in aqueous solutions are often frozen for long-term storage or for holding batches prior to preparation of a large lot. Also, there are some risks of accidental freeze-thawing during shipping. Therefore, pharmaceutical proteins are routinely exposed to repetitive freezing and thawing cycles that can eventually induce protein instability and aggregation [20,21,35]. To compare the resistances of NG-IFN and 4N-IFN against this form of stress, both proteins were subjected to 15 cycles consisting of freezing in liquid nitrogen and thawing in a water bath at 37 °C.

The turbidity of NG-IFN sample at 405 nm was greatly increased after 15 freeze-thawing cycles, showing an optical density almost 50-times higher than at the beginning of the experiment (Fig. 3A). This indicated the formation of insoluble aggregates [36] which were not detected by SEC-HPLC due to the previous filtration of the sample carried out to remove any particulates before the analysis (data not shown). On the other hand, 4N-IFN showed a much lower increase in optical density after multiple freeze-thawing cycles (less than 7-times), suggesting that *N*-glycans prevent aggregation of rhIFN-α2b.

Non-reducing SDS-PAGE analysis showed the appearance of a high molecular mass band corresponding to a dimeric form of NG-IFN after 5 freeze-thawing cycles according to the molecular mass standards, being the amount of dimers higher as the number of freeze-thawing cycles increased (Fig. 3B). Again, reducing agents such as β-mercaptoethanol could not dissolve those aggregates (data not shown), suggesting the presence of non-covalent dimers. In contrast, 4N-IFN demonstrated much higher stability under this form of stress, with no visible aggregates after 15 freeze-thawing cycles.

During the freezing process, as ice crystals are formed, the concentration of all solutes increases dramatically and the pH is altered, all of this destabilizing the protein and leading to its denaturation [20,35]. Thus, it may be postulated that the presence of *N*-linked carbohydrates protects from cold denaturation-induced aggregation.

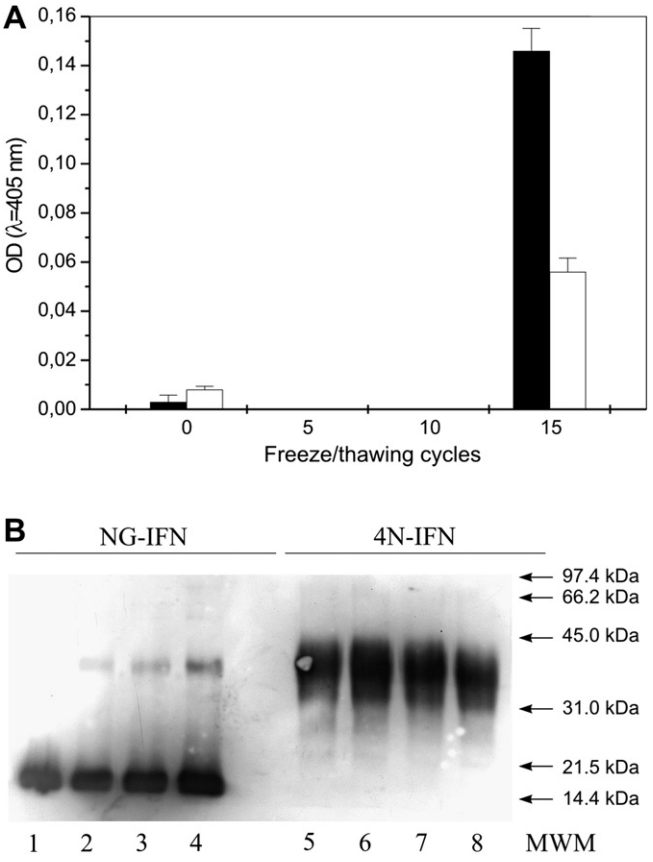

Fig. 3. Effect of repetitive freeze-thawing cycles on the aggregation of NG-IFN (■) and 4N-IFN (□). Each protein was diluted to a final concentration of 50 μg ml^{-1} in PBS, and exposed to repetitive cycles of rapid immersion in liquid nitrogen and in a water bath at 37 °C. (A) Analysis of protein aggregation by turbidity measurement at 405 nm at the beginning and end of treatment. Results are means ± SEM for two independent experiments. (B) SDS-PAGE followed by Western blot after every 5 cycles of freeze-thawing. Lanes 1, 5: control (0 cycles); lanes 2, 6: 5 cycles; lanes 3, 7: 10 cycles; lanes 4, 8: 15 cycles; MWM: molecular weight marker (Bio-Rad).

3.4. Limited proteolysis for assessment of structural changes

Limited proteolysis is still a widely used powerful tool for probing the higher order structure of proteins [37], based on the paradigm that proteolysis of native globular proteins occurs at "hinges and fringes", that is to say, segments of amino acid residues in an accessible, flexible conformation such as exposed surface loops and domain linking segments [38,39]. When a protein is destabilized, either by heating, by chemical denaturation or because of the introduction of mutations critical for the preservation of its structure, the resulting unfolding increases its inherent susceptibility to proteolytic attack, generally accelerating its degradation [40,41].

With the aim of comparing limited proteolysis susceptibility of NG-IFN and 4N-IFN as a first approach to evaluate the impact of amino acid substitutions and carbohydrate addition on the conformational status of the hyperglycosylated cytokine, both proteins were subjected to trypsin digestion under controlled conditions (low enzyme:substrate ratio and room temperature). We chose a 1:10 enzyme:substrate ratio because lower values (1:100 and 1:50) were not able to initiate degradation after 4 h of treatment. To follow the time course of protein degradation,

samples were withdrawn from the tryptic digestion mixture at intervals and subjected to SDS-PAGE/Western blot (Fig. 4A). The intensities of the bands were semiquantified by densitometric analysis and plotted as the percentage of intact protein versus time (Fig. 4B).

As the digestion proceeded, the amount of both intact NG-IFN and 4N-IFN decreased progressively over time, preserving about 10% of intact protein after 8 h of treatment. No visible digestion products were detected for NG-IFN, suggesting its degradation into small fragments which could not be resolved by SDS-PAGE. Contrarily, 4N-IFN showed a main proteolytic product with an apparent molecular mass of about 30 kDa. The appearance of this fragment could be the consequence of replacing Arg23 and Lys70 by Asn, thus eliminating two potential trypsin cleavage sites while simultaneously adding two complex-type carbohydrate structures. Nonetheless, no apparent differences were observed between the rate of breakdown of both full-length cytokines, indicating that the tridimensional structure of the hyperglycosylated variant was not significantly altered compared to the unmodified IFN.

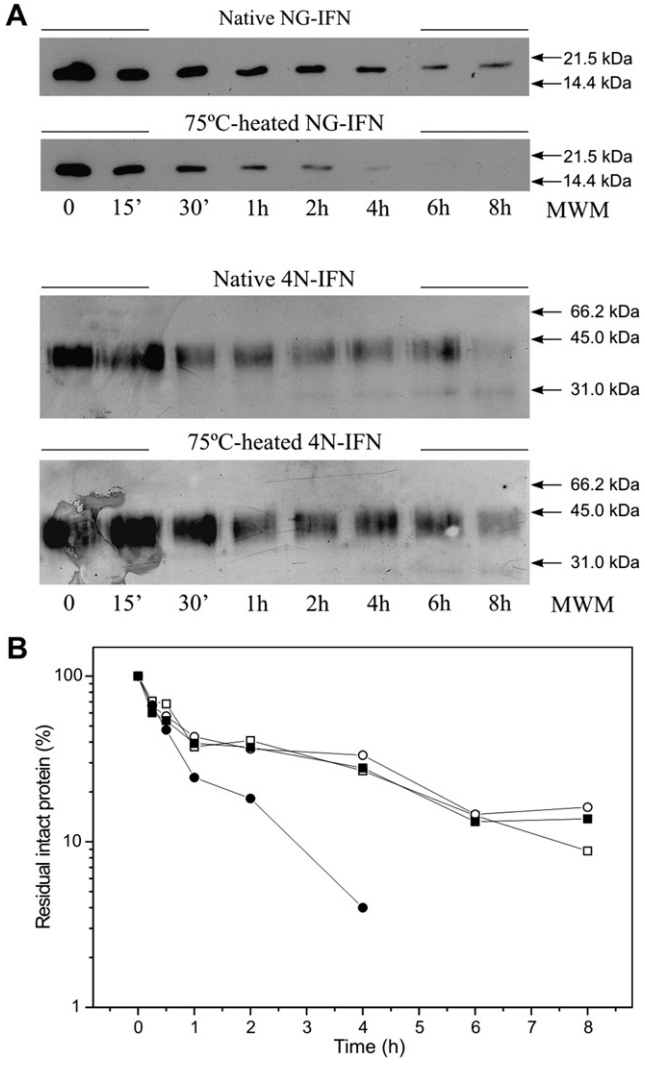

Fig. 4. Time course of tryptic digestion of native and 75 °C-heated NG-IFN and 4N-IFN. (A) SDS-PAGE/Western blot analysis of samples taken at different time points (0, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 8 h) during treatment with trypsin at a ratio of 1:10 enzyme:cytokine at room temperature. MWM: molecular weight marker. (B) Percentage of intact protein versus time, as determined by densitometric analysis: (■) native NG-IFN, (●) NG-IFN heated 10 min at 75 °C, (□) native 4N-IFN and (○) 4N-IFN heated 10 min at 75 °C.

After heating for 10 min at 75 °C, NG-IFN was more rapidly degraded than in native conditions, with only 4% of intact protein remaining after 4 h of digestion. This was in accordance with the complete loss of activity of the unmodified cytokine incubated at temperatures above 65 °C, indicating that heat inactivation probably renders unfolded states which are more susceptible to protease degradation and have a tendency to form aggregates, as we previously demonstrated (Fig. 1B). On the contrary, the trypsin inactivation profile of 4N-IFN previously heated for 10 min at 75 °C was identical to the corresponding one for the native cytokine, corroborating the increased heat resistance of this IFN mutein. These results are in agreement with the general notion that protease nicksites are located at highly flexible areas and at protein regions devoid of structure, the amount of which could raise after physical or chemical denaturation, ligand interaction and introduction of single, double or multiple-point mutations, among other factors [38,39,42,43].

3.5. Characterization of 4N-IFN with far-UV CD spectroscopy

In order to further corroborate the absence of secondary structural changes in the hyperglycosylated IFN mutein in comparison with the non-glycosylated variant, both proteins were studied by CD spectroscopy. As it can be seen in Fig. 5, the average CD spectrum of 4N-IFN is practically overlapped with that of NG-IFN, with similar molar ellipticities in the whole range studied. This indicates that glycosylation does not disrupt the secondary structure of the cytokine, which maintains a conformation close to the native protein. Indeed, in agreement with the 3D structure of IFN-α2b published in protein databases, both NG-IFN and 4N-IFN exhibited the typical spectrum shape expected for a mostly alpha-helical protein [44–46], with pronounced negative peaks around 210 and 218 nm. These results are in accordance with the similar limited proteolysis profiles of NG-IFN and 4N-IFN, both methodologies suggesting the absence of unfolded regions within the alpha helices of the hyperglycosylated protein which could have been induced by point mutations and/or carbohydrate attachment. This is particularly important for biopharmaceutical proteins such as rhIFN-α2b, for which structural changes could lead to undesired immunological responses in patients, with consequences ranging from acute toxicities to complete therapy failure due to the development of neutralizing antibodies [27,47].

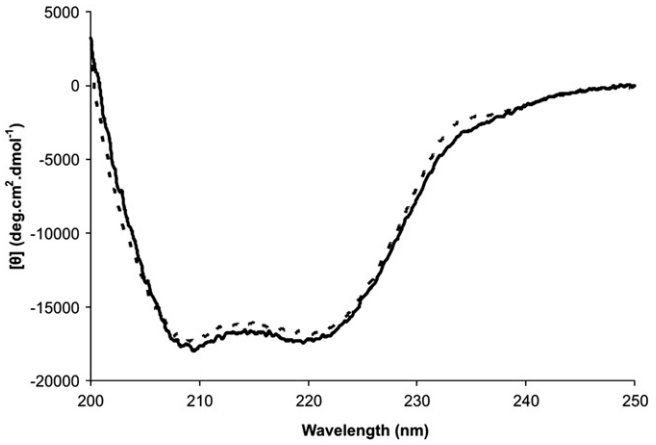

Fig. 5. Circular dichroism profile of NG-IFN (- - -) and 4N-IFN (—). Far-UV CD spectra were obtained using a Jasco J-810 spectropolarimeter at room temperature in the range of 200–250 nm. Buffer spectra were also collected and subtracted from each sample spectrum. Spectra data were analyzed using Microsoft® Excel version 5.1 software (Microsoft Corp., USA).

Our findings are also consistent with those obtained by other authors for natural glycoproteins, whether with predominant β-sheet or alpha-helical secondary structure, for which no significant changes were observed in the far-UV CD spectra before and after deglycosylation [21]. Yet, other glycoprotein mutants in which one or all *N*-glycosylation sites have been genetically removed (including human lecithin-cholesterol acyltransferase [48] and human choriogonadotropin [49]) have shown dramatic changes in their conformation assessed by the same methodology, indicating that carbohydrates contribute to the correct folding of the nascent polypeptide chain. In the present study, non-natural oligosaccharides do not seem to influence the 4N-IFN folding, which, in this case, constitutes an advantage in order to avoid the arrangement of potential immunogenic epitopes.

4. Conclusions

Protein instability is one of the major concerns during every step in commercial manufacturing, from purification to the packaging and delivery of pharmaceutical proteins. Thus, the design of proteins that exhibit increased stability against environmental stresses represents an important strategy for the development of next-generation biotherapeutic products.

Here we demonstrated that glycoengineering can be used not only to enhance rhIFN-α2b therapeutical properties such as elimination half-life and *in vivo* bioactivity, as we have previously reported [9,15], but it can also constitute a successful technology to improve the cytokine's stability and resistance towards many physicochemical factors encountered during its production process.

For some therapeutic proteins, including rhIFN-α2b, partial unfolding and aggregation are the prevalent physical instability reactions in liquid protein formulation, usually resulting in the irreversible loss of the protein's biological activity with major economic and technical consequences for the biotechnology industry [11,50]. More importantly, the aggregation of parenterally administered proteins can cause adverse patient reactions, such as unexpected immune responses, hypersensibilization or even anaphylactic shock. During long-term treatment of viral or malignant diseases with rhIFN-α2b, nearly 20% of patients generate anti-IFN antibodies [51] and this immunogenicity has been reported to be elicited by the cytokine misfolding and aggregation [52,53]. Glycosylation engineering has previously been used to create a long-acting IFN analog, 4N-IFN, with enhanced *in vivo* antitumor activity [9]. However, it is largely known that one of the functions of glycosylation is to aid in the folding and secretion of the emerging polypeptide and to stabilize the conformation of the mature protein, protecting it from thermal and hydrolytic inactivation [54,55]. Besides, the stabilizing effect of glycosylation has demonstrated to be highly protein-dependent [11]. Therefore, the stability of 4N-IFN was evaluated in response to several stresses that are known to induce protein unfolding and aggregation, such as high temperature, pH and repetitive freeze-thawing cycles. In this way, in this study we demonstrated that 4N-IFN exhibits an increased resistance to high temperature inactivation ($T_m \approx 90$ °C) in comparison with the NG-IFN ($T_m \approx 66$ °C), mostly by preventing heat-induced aggregation of the hyperglycosylated cytokine. Also, glycosylation protects 4N-IFN from low pH-inactivation, an important advantage for the purification of the cytokine which comprises elution from the chromatography matrix at acidic pH. Repeated freeze-thawing cycles caused the rapid formation of great quantities of insoluble aggregates of NG-IFN, while the turbidity of the 4N-IFN solution was only slightly increased at the end of the experiment. Unfortunately, the methodologies used in our work mainly account for increased physical stability rather than for chemical stability such as oxidation and crosslinking. In general, the ability of glycosylation to prevent this type of instability has been much less investigated and more studies are needed to demonstrate and understand the mechanisms involved.

Several protein stabilization mechanisms of the attached carbohydrates have been proposed. These include the formation of hydrogen bonds with the polypeptide backbone or surface hydrophilic amino acids and steric interaction with the adjacent peptide residues [56]. Also, the negative charge of sialic acids probably increases the solubility of the protein, which is known to be proportional to the square of the net charge on the protein [57], by increasing its hydrophilicity. This may cause intermolecular interaction unfavorable, abolishing the formation of stress-induced aggregates.

Other strategies have been employed in order to stabilize proteins and make them more resistant to environmental factors. The use of a tagging system comprising the fusion of a stabilizing peptide such as the C-terminal acidic tail of α-synuclein, human serum albumin and the constant region of human immunoglobulins, has been recently reported for many proteins of clinical value, including human growth hormone (hGH), human granulocyte-colony stimulating factor (G-CSF) [36], hIFN-α1 [57], hIFN-α2b [58], insulin [59] and leptin [60]. Protein engineering through the mutation of critical amino acid residues has also been used for enhancing stability of cytokines [61] and therapeutic antibodies [62]. Likewise, proteins are protected against potential stresses by the addition of proper excipients or additives [61].

Although changing the production process of rhIFN-α2b from *E. coli* to CHO cells might seem a drawback for 4N-IFN industrial application representing a significant cost increase, there are numerous examples of glycoproteins for which a mammalian cell production platform is widely justified. Such is the case of human IFN-β, a naturally highly glycosylated protein which is 10 times more active when produced in CHO cells than when it is obtained as a non-glycosylated variant in bacteria [63]. Furthermore, deglycosylation causes a decrease in total activity primarily due to insoluble disulfide-linked IFN precipitates, reduces the protein's half-life and results in an increased sensitivity to thermal denaturation. Given these facts, glycosylated rhIFN-β is a well-marketed product despite its high production costs and might be also a potential candidate for application of glycoengineering strategies.

One important aspect of our work was to demonstrate that the introduction of the carbohydrate moieties did not affect the structure of the protein, but rather appeared to somewhat stabilize its conformation, potentially diminishing the risk of raising harmful immune responses.

To sum up, the results presented here show that 4N-IFN not only presents enhanced properties in terms of therapeutic efficacy compared to the non-glycosylated cytokine, but it also exhibits an improved *in vitro* stability. This represents an important advantage during all the steps of the productive process, including isolation, purification and long-term storage.

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