Published in final edited form as:

Anal Chem. 2008 October 1; 80(19): 7473–7481. doi:10.1021/ac801214x.

Detection and characterization of altered conformations of protein pharmaceuticals using complementary mass spectrometry-based approaches

Cedric E. Bobst¹, Rinat R. Abzalimov¹, Damian Houde², Marek Kloczewiak², Rohin Mhatre², Steven A. Berkowitz², and Igor A. Kaltashov¹

¹Department of Chemistry, University of Massachusetts-Amherst, Amherst, MA

²Analytical Development, Biogen Idec, Inc, Cambridge, MA

Abstract

Unlike small molecule drugs, the conformational properties of protein biopharmaceuticals in solution are influenced by a variety of factors that are not solely defined by their covalent chemical structure. Since the conformation (or higher order structure) of a protein is a major modulator of its biological activity, the ability to detect changes in both the higher order structure and conformational dynamics of a protein, induced by an array of extrinsic factors, is of central importance in producing, purifying, and formulating a commercial biopharmaceutical with consistent therapeutic properties. In this study we demonstrate that two complementary mass spectrometry-based approaches (analysis of ionic charge state distribution and hydrogen/deuterium exchange) can be a potent tool in monitoring conformational changes in protein biopharmaceuticals. The utility of these approaches is demonstrated by detecting and characterizing conformational changes in the biopharmaceutical product interferon β -1a (IFN- β 1a). The protein degradation process was modeled by inducing a single chemical modification of IFN- β 1a (alkylation of its only free cysteine residue with N-ethylmaleimide), which causes significant reduction in its antiviral activity. Analysis of IFN- β 1a ionic charge state distributions unequivocally reveals a significant decrease of conformational stability in the degraded protein, while hydrogen/deuterium exchange measurements provide a clear indication that the higher order structure is affected well beyond the covalent modification site. Importantly, neither technique required that the location or indeed the nature of the chemical modification be known prior to or elucidated in the process of the analysis. In contrast, application of the standard armamentarium of biophysical tools, which are commonly employed for quality control of protein pharmaceuticals, met with very limited success in detection and characterization of conformational changes in the modified IFN- β 1a. This work highlights the role mass spectrometry can and should play in the biopharmaceutical industry beyond the presently assigned task of primary structure analysis.

Introduction

Biopharmaceuticals (the vast majority of which are recombinant proteins) constitute the fastest growing segment among the new pharmaceutical products. Their share of the market is

Correspondence to: Igor A. Kaltashov.

Address correspondence to: Igor A. Kaltashov Department of Chemistry University of Massachusetts-Amherst 701 North Pleasant Street Lederle Graduate Research Tower 701 Amherst, MA 01003 Tel (413) 545-1460 Fax (413) 545-4490 Email Kaltashov@chem.umass.edu.

¹In this paper PTM will be used to imply chemical modifications that occur to protein after the biosynthesis of its polypeptide that are concerned with either signal transduction or/and degradation.

expected to continue to expand and outpace the traditional small-molecule pharmaceuticals in the foreseeable future (1,2). Since the biological activity of all proteins is intimately related to their higher order structure, the therapeutic properties of protein biopharmaceuticals are uniquely determined by their conformation. In the case of small-molecule drugs conformational issues can also be important, however they arise only in the solid state where different conformers of the same molecule can lead to different crystal states, or polymorphs (3). Polymorphs of the same drug may differ dramatically from one another in their physical properties leading in some cases to significant changes in pharmacokinetic profiles, sometimes with catastrophic consequences (4). Nevertheless, there is no evidence that these conformational differences persist in solution. This is in stark contrast to the solution behavior of protein biopharmaceuticals where partial or complete loss of a native folded structure is common and can dramatically influence their therapeutic properties (thereby adversely affecting the efficacy), their ability to elicit an immunogenic response (5), or even trigger aggregation (6). As a result, both the physical and chemical stability of a protein biopharmaceutical need to be carefully investigated and controlled during and after its production.

Protein conformations are largely determined by the primary structure (amino acid sequence), although they can also be significantly influenced by an array of post-translational modifications (PTM). While most enzymatically-controlled PTMs are central to the protein's ability to function *in vivo*, a large number of modifications are non-enzymatic and are associated with protein ageing, degradation, and ultimately, loss of function (7). Oxidation, deamidation, isomerization, and racemization of amino acids residues, as well as disulfide scrambling, are among the most common non-enzymatic PTMs inflicting damage on proteins both *in vitro* and *in vivo* (8), and have been traditionally an obvious focus of quality control efforts (9). However, in many cases detection and even localization of these PTMs is not sufficient to reveal their potential consequences to the integrity of the protein's higher order structure. Indeed, the conformational repercussions of various PTMs can vary significantly from one protein to another. In some cases non-enzymatic PTMs exert little or no effect on a protein conformation, while in other cases even subtle changes in the covalent structure of a protein can lead to profound changes in the higher order structure, many of which are far removed from the site of modification. This unpredictability in the impact of PTMs on the conformational properties of a protein biopharmaceutical (and ultimately, on its efficacy, immunogenicity, and stability) places a premium on the ability to detect and characterize changes in the higher order structure and dynamics of these proteins triggered by a host of different physical and chemical environments encountered during the manufacturing, storage, and administration of these complex drugs.

The availability of robust and reliable analytical tools capable of characterizing the higher order structure and dynamics of protein biopharmaceuticals is critical in accelerating the successful development of these novel drugs (10,11). Such tools will also play an important role in setting specification levels to which these chemical changes can be tolerated, in stability and comparability studies (when changes or optimization (7) are made to the production process), and in allowing meaningful comparison to determine if follow-on biologics (biosimilars) are in fact appropriately similar to the original innovator product (12,13).

Until very recently, however, there was a lack of simple, effective and reliable experimental tools to meet this analytical objective. X-ray crystallography is obviously biased towards the most stable crystal conformations (which may allow the non-native conformations in solution to escape detection), while high-resolution NMR spectroscopy still suffers from rather unforgiving molecular weight limitations and high material requirements. Although the higher order structure of protein drugs can also be probed using separation techniques, such as size exclusion chromatography (SEC), native gel electrophoresis (14), and analytical centrifugation

(15), a variety of optical spectroscopic methods, such as circular dichroism and fluorescence (16,17), as well as light scattering techniques (18) and calorimetry (19), the structural information deduced from these measurements is usually very limited and lacking in both sensitivity and specificity.

This technological gap is being filled by mass spectrometry (MS), which in recent years has matured into a technique capable of providing structural information on biopolymers at a variety of levels (20,21). Although most current applications of MS in the analysis of biopharmaceuticals are limited to characterization of covalent (primary) structure, including both amino acid sequence and PTMs (22), rapid progress in developing MS-based approaches to probe protein conformation and dynamics suggests that its potential spans well beyond the routine sequencing tasks. Among several MS techniques used to assess protein higher order structure, hydrogen/deuterium exchange (HDX) (23) and analysis of ionic charge state distributions in electrospray ionization (ESI) mass spectra (24) appear to be particularly promising. A unique advantage of these techniques is the ability to detect very small amounts of non-native conformers within the background of natively folded protein species. This is in contrast to MS structural techniques based on chemical cross-linking (25) or chemical modification (26), which produce low yields of the target protein and thus would preclude the detection of conformers present in minute quantities. Furthermore, combination of HDX with protein fragmentation prior to MS analysis (proteolysis in solution under slow exchange conditions (27) or ion dissociation in the gas phase (28)) provides an opportunity to obtain polypeptide backbone flexibility maps and offers the capability to specifically identify segments of a protein that have unfolded. As an analytical technique, HDX MS continues to enjoy spectacular growth, as suggested by the rapid expansion of both the number of applications and the scope of biophysical information that is extracted from these measurements. It is therefore surprising that HDX MS has seen very few applications in the field of biopharmaceutical analysis, where a capability to detect and characterize conformational changes is urgently needed (29). In addition, the use of ESI MS to assess the integrity of higher order structure by analyzing a protein's ion charge state distributions (30) has never been utilized as a means to detect conformational changes in protein biopharmaceuticals resulting from chemical modifications and/or exposure to the array of physical and chemical environments during all phases of its manufacturing and storage.

In the present work we investigate structural changes that result from the chemical modification of interferon $\beta 1a$ (IFN- $\beta 1a$), a cytokine defined by its anti-viral activities. Essential to its biological activity is the recruitment of a heterodimeric cell surface receptor composed of two transmembrane subunits, IFN receptor 1 (IFNAR1) and IFN receptor 2 (IFNAR2), to form the ternary IFNAR-1/IFN- $\beta 1a$ /IFNAR-2 complex (31). Specific covalent modification of the sole free cysteine of IFN- $\beta 1a$ leads to a protein with impaired biological activity as well as a suspected structural change. Utilizing this altered IFN- $\beta 1a$ we demonstrate that ion charge state distribution analysis in ESI MS in combination with HDX MS is a sensitive and reliable toolset for monitoring changes in the higher order structure of this commercial biopharmaceutical product. The high sensitivity, accuracy, and ability to provide detailed structural information make these MS-based methods very attractive additions or potential alternatives to the "classical" biophysical techniques commonly employed by the industry to assess and monitor comparability and stability of the higher order structure of protein biopharmaceuticals.

Materials and Methods

IFN- $\beta 1a$ sample preparation and treatment

The IFN- $\beta 1a$ used in this study was obtained from Biogen Idec, Inc. (Cambridge, MA). N-ethylmaleimide (NEM) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical grade or higher. IFN- $\beta 1a$ alkylation with NEM

was carried out in buffer A (100 mM sodium phosphate, 200 mM sodium chloride, pH 7.2) at an approximate protein concentration of 0.25 mg/mL with an NEM final concentration of 0.5 mM (using a stock NEM solution of 100 mM). The reaction was then allowed to proceed for 1 hour at ambient temperature in the dark. After incubation, the NEM-IFN- β 1a sample was buffer exchanged into buffer A using a 4 mL 10K MWCO centrifugal membrane filters (Amicon, Millipore). The buffer exchange was performed at 5 °C and 4,000 rpm and was repeated 3 times to ensure removal of excess NEM. The concentration of NEM-IFN- β 1a was determined by UV absorption measurements at 280 nm using the extinction coefficient of 1.50 OD₂₈₀ nm/mg/mL (samples were typically concentrated to a final concentration of about 0.25 mg/mL). NEM-IFN- β 1a was then characterized by mass spectrometry to determine the extent of alkylation (in terms of both NEM : IFN- β 1a stoichiometry and the fraction of modified IFN- β 1a molecules). Intact mass analysis and peptide mapping confirmed that a single NEM molecule was covalently attached to IFN- β 1a cysteine 17 and that the labeling efficiency at this site was > 95 %. SEC analysis was then conducted on NEM- IFN- β 1a (*vide infra*) to ensure quantitative retention time shift of the IFN- β 1a material and its homogeneity.

Size exclusion chromatography

SEC analyses were carried out using a G2000SWxl column (Tosoh Bioscience, San Francisco, CA) at ambient temperature in a mobile phase of buffer A (flow rate of 1.0 mL/min) with sample injection volumes ranging from 25 to 100 μ L. Protein elution profiles were recorded by monitoring absorption at a wavelength of 280 nm.

Circular dichroism spectrophotometry

All CD measurements were carried out using a Jasco J-715 spectropolarimeter (Jasco, Inc., Easton, MD). Sample concentrations for the near-UV CD measurements were 20 μ M in buffer A; the spectra were recorded in the 250-360 nm range in 0.1 nm steps using a 1 cm quartz cuvette. Sample concentrations for far-UV measurements were 4 μ M in 100 mM ammonium acetate (pH 6.8). The data were collected in the 190-250 nm range in 0.5 nm steps (1 nm bandwidth, 20 nm min⁻¹ scan rate) using a 0.1 cm quartz cuvette. Three scans were averaged for each spectrum to ensure adequate signal-to-noise ratio. All samples were kept at 25 °C during the data acquisition.

Fluorescence

The fluorescence emission spectra were recorded with Jasco FP-750 spectrofluorometer (Jasco, Inc., Eaton, MD) using 1 cm optical path length quartz cuvettes. The instrument settings were as follows: excitation bandwidth, 10 nm; emission band, 5 nm; response speed, slow; excitation wavelength, 280 nm; spectroscopic data spacing, 1 nm; scanning speed, 60 nm/min; and sensitivity settings, medium. Three consecutive scans (300-480 nm range) were recorded and averaged for each spectrum. No correction for the observed fluorescence photo bleaching effect was introduced. Protein concentration used for measurements was 0.023 mg/ml in 50 mM sodium phosphate, 100 mM NaCl buffer (pH 6.8).

Analytical ultracentrifugation

All AUC experiments were conducted in a Beckman/Coulter (Fullerton, CA) XL-I analytical ultracentrifuge at 20 °C, at a speed of 50K rpm. UV sedimentation profiles were recorded using UV absorption at a wavelength of 238 nm. Sample concentrations were in the range of 0.2 to 0.3 mg/mL in buffer A. Centrifugation cell consisted of a 12 mm charcoal filled epon centerpiece and sapphire windows. Data analysis was conducted with the computer program SEDFIT developed by Peter Schuck and co-workers (32).

HDX MS

HDX for intact proteins was initiated by diluting the protein stock solutions (50 μ M in 20 mM ammonium acetate) 10-fold into exchange buffer (20 mM ammonium acetate in D₂O) equilibrated to 25°C. At a designated time the sample was placed in an ice bath for 15 seconds and then an equal volume of pre-chilled quench solution (acetonitrile containing 0.2% formic acid) was added and rapidly mixed, yielding a pH of 2.4. Using a denaturing quench dramatically increased the resolution of the mass measurements, which were recorded immediately following the quench. Forward exchange was measured for each protein by preparing a sample in which the protein was added last to a prechilled, quenched solution. Plots of increase in mass over time were prepared by subtracting the average mass of the forward exchange control sample from the average mass calculated at various HDX time points.

To probe protein conformational stability locally, HDX was initiated by diluting the protein stock solutions (50 μ M in 100 mM sodium phosphate pH 7.2, 100 mM NaCl) 10-fold into exchange buffer (50 mM sodium phosphate D₂O, pD 7.2, uncorrected for the isotope effect) equilibrated to 25 °C. At a designated time an aliquot of the solution was placed in an ice bath for 15 sec and then an equal volume of pre-chilled quench solution (3.6 M guanidine HCl, 0.5 % trifluoroacetic acid, 50 mM tris(2-carboxyethyl) phosphine HCl) was added and rapidly mixed, yielding pH 2.4. All subsequent steps were performed in an ice bath. The quenched sample was digested online using two immobilized protease columns in tandem, first pepsin and then type XIII fungal protease (33), at a flow rate of 0.16 ml min⁻¹ using 0.1 % formic acid as the mobile phase. The resulting peptides were collected and desalted with an inline nanotrap cartridge then resolved by a C18 column (Armor Advance, 5 μ m, 50 $\times$ 2.1 mm) using a rapid gradient from 5% to 65% acetonitrile containing 0.1 % formic acid and a flow rate of 0.3 ml min⁻¹. The total time for the digest and desalting was 3 min, all peptides had eluted from the C18 column by 9 min. MS measurements were carried out with a hybrid quadrupole/time-of-flight mass spectrometer (QStar-XL, MDS Sciex/Applied Biosystems, Toronto, Canada) equipped with a standard Turbospray™ source.

Two data sets were used in our comparison of IFN- β 1a with NEM-IFN- β 1a by local HDX MS. One data set consisted of 3 exchange time points (10 s, 30 s, and 120 s) for each sample. The other consisted of a single 5 min exchange time point performed in triplicate for each sample. Proteolytic peptides were identified using a combination of exact mass and MS/MS data for unlabeled protein utilizing a shallower elution gradient (such proteolytic fragments cover over 75 % of the protein sequence). The extent of deuterium incorporation was calculated by monitoring the average mass increase for each identified peptide. The theoretical maximum deuterium incorporation value was calculated for each peptide based on the dilution factor and number of exchangeable amides, with the assumption that exchange of N-terminal amide is lost by back exchange during sample workup(34).

Direct ESI MS measurements for the analysis of IFN- β 1a ion charge state distributions and IFN- β 1a/IFNAR-2 binding studies

The extracellular portion of IFNAR-2, which was expressed and purified at Biogen Idec (35), and NEM-IFN- β 1a were buffer exchanged into 100 mM ammonium acetate pH 6.8 through repeated concentration and dilution using Centricon (Millipore) centrifugal filters with a 10 kDa cut-off (fixed angle rotor operated at 4,000 g and 4 °C). A mixture of 4 μ M IFNAR-2 and 1.5 μ M NEM-IFN- β 1a prepared in 100 mM ammonium acetate was analyzed on the QStar-XL (MDS Sciex/Applied Biosystems, Toronto, Canada) equipped with a nanospray source.

Biological activity assay

Antiviral activities of IFN- β 1a and NEM-IFN- β 1a were assessed using the method of Alam *et al.* (36). In summary, the assay measures the ability of IFN- β 1a to protect human lung

carcinoma (A549) cells from the cytopathic effect (CPE) of the encephalomyocarditis (EMC) virus. IFN- β 1a samples are diluted in cell growth medium to the working range of the assay (10000 U/mL). On day 1 of the assay, samples are serially diluted in cell growth medium on assay plates along with an assay standard and control. A549 cells (37) are added to the plates and incubated 15-20 hours at which point the EMC virus is added to the plates and incubated an additional 30 hours. Each plate then is stained with crystal violet, washed and dried, and then read visually to determine the lowest concentration of IFN- β 1a protecting the cells from the CPE of EMC virus.

Results and Discussion

IFN- β 1a is a 23 kDa glycoprotein (38), several forms of which are used for treatment of relapsing forms of multiple sclerosis (39); it also exhibits anti-viral and anti-cancer activity (31). While recent attempts to introduce IFN- β 1a directly into targeted tissue using gene transfer techniques showed much promise (40), administration of recombinant forms of interferons remains the only approved form of treatment, with several follow-on versions currently in development. As is the case with most proteins, interferons have a problematic propensity to misfold, which leads to activity loss, aggregation and increased immunogenic response (5). IFN- β 1a misfolding can be accelerated by a variety of factors, including chemical modifications, interaction of proteins with surfaces, exposure to elevated temperatures, lyophilization, *etc.*

Earlier studies of interferon degradation identified several amino acid residues that are common targets of chemical modifications, including three (out of four) methionine residues and a single free cysteine residue (41), a conclusion that was confirmed in our more recent study. While it is impossible to achieve a selective oxidation of any single methionine side chain (or their combination) in IFN- β 1a, protein alkylation with N-ethylmaleimide (NEM) can produce an almost homogenous product, where >95% of protein molecules are modified at cysteine at position 17, without affecting the rest of the protein. Despite the limited range of alkylation targets in IFN- β 1a (compared to oxidation), it was found to result in *ca.* 50% loss in activity as detected by standard anti-viral assays. Therefore, this alkylated form of IFN- β 1a (NEM-IFN- β 1a) renders itself as an attractive model of protein degradation, whereby any change in protein activity and conformation must necessarily originate from the single covalent modification.

Biological activity loss resulting from IFN- β 1a alkylation is likely mediated by changes in the protein higher order structure. However, classical biophysical tools provide little information on conformational consequences of IFN- β 1a alkylation. Although alkylation of IFN- β 1a with NEM does result in a reduction of SEC elution time (Figure 1A), a similar shift was also observed in H₂O₂-treated samples, which do not suffer any detectable activity loss (data not shown). Analytical ultracentrifugation analysis indicated only a very slight reduction (of about 0.6%) in the sedimentation coefficient of the NEM-IFN- β 1a monomer in comparison to the native IFN- β 1a monomer. Although this difference is very small, it has been reproducibly repeated and is consistent with the SEC data suggesting a possible increase in hydrodynamic volume on alkylation. Such an increase in hydrodynamic volume without a change in molecular weight (as suggested by light scattering measurements) would yield a reduced sedimentation coefficient; however, given the magnitude of the SEC shift a much larger difference in the sedimentation coefficients between IFN- β 1a and NEM-IFN- β 1a was anticipated. Fluorescence measurements (Figure 1B) yield a *ca.* 15% reduction in the NEM-IFN- β 1a emission intensity and a very small blue shift (*ca.* 3 nm). Although these results are consistent with the notion of less stable tertiary structure of NEM-IFN- β 1a (*e.g.*, by indicating transfer of aromatic residues from a hydrophobic to aqueous environment), the paucity of the spectral changes makes them unsuited for gauging the integrity of the higher order structure.

In the case of the far-UV CD spectra for NEM- IFN- β 1a a small reduction in the total helical content is indicated (Figure 1C), with the difference between the far-UV spectra of intact and alkylated forms of IFN- β 1a bearing signature features of a small increase in random coil on alkylation (see inset in Figure 1C). This result suggests a helix-to-coil conversion in some (yet unidentified) region(s) of the protein triggered by alkylation. However, the amplitude decrease of the negative bands at 208 nm and 222 nm, which are signatures of α -helical structure, is not very significant and is not well suited to serve as a marker of the structure loss, especially in a situation when stress-affected IFN- β 1a molecules constitute only a fraction of the entire protein population. Ellipticity changes in the near-UV region indicate a partial loss of the protein's tertiary structure (or, more precisely, a decrease in the order in the spatial arrangement of its aromatic residues) upon alkylation, and the relative magnitude of this change is much more pronounced compared to the differences observed in the far-UV CD spectra (Figure 1D). However, the intrinsic weakness of the near-UV CD signal makes it again hardly suitable for reliable detection of unfolding, especially if structurally compromised species represent only a fraction the entire ensemble of protein molecules.

Overall, the classical biophysical measurements provided little insight as to where these changes were occurring on IFN- β 1a and in some cases were questionable as to their significance. However, in the case of ESI-MS analysis evidence of partial unfolding of IFN- β 1a triggered by its alkylation is readily provided. Protein ion charge state distributions in the mass spectra of NEM-IFN- β 1a acquired under near-native conditions are bimodal (Figure 2), suggesting that at least two protein conformations are populated at equilibrium (42,43). The compactness of one of these states is indistinguishable from that of intact IFN- β 1a, whose ionic charge state distribution is characteristic of a natively folded protein. Higher charge density ionic population of NEM-IFN- β 1a (charge states +11 through +14) represents a less compact state with a diminished tertiary structure (30). Although this population of high charge-density ions is also present in the spectrum of intact IFN- β 1a, the relative abundance is significantly lower, making these ions barely detectable. Interestingly, the presence of this non-native state could not be detected following protein oxidation with H_2O_2 , which in comparison to NEM treatment does not appear to compromise IFN- β 1a activity as reported by Orr et. al. (29) and confirmed by our own work (data not shown).

While the appearance of the high charge-density protein ions in ESI mass spectra of NEM-IFN- β 1a indicates partial unfolding, consistent with the partial loss of higher order structure observed in CD measurements, ESI MS measurements provide significantly more information on the alkylation-induced conformational change. The bimodal charge state distribution of NEM-IFN- β 1a ions clearly indicate that the conformation of the chemically modified protein is not permanently altered. In other words, instead of locking the protein in a non-native state, alkylation makes the native conformation less stable and increases the frequency of sampling less ordered conformations, thereby elevating their Boltzmann weight and making them visible in ESI MS. At the same time, the ionic signal representing the compact, native-like state of IFN- β 1a remains more abundant than the signal corresponding to the non-native species following protein alkylation. Fractional concentration of the partially unfolded NEM-IFN- β 1a molecules in solution is difficult to estimate, but it is unlikely to be higher than the relative abundance of the corresponding ions in ESI MS (44).

The direct ESI MS measurements and the subsequent analyses of NEM-IFN- β 1a ionic charge state distributions are very informative, as they clearly reveal the presence of two protein conformations at equilibrium and unequivocally link this behavior to the chemical modification event. However, the direct measurements can only be carried out in volatile buffer systems, thereby disallowing analysis of protein Pharmaceuticals in the context of their specific matrices. Structural integrity of therapeutic proteins may be compromised by a variety of factors, including composition of their formulations (45). Therefore, it may be argued that the

transfer of a protein drug from its formulation-specific environment to the ESI-compatible solution may lead in some cases to a false-negative or false-positive detection of unfolding. Furthermore, no structural characteristics of the non-native protein state can be deduced from such measurements, information that can be very valuable for the optimization of the production and storage processes.

To enable detection and characterization of conformational changes triggered by alkylation of IFN- β 1a in the relevant environment, the backbone dynamics of IFN- β 1a and NEM-IFN- β 1a were probed with HDX MS. Global HDX MS measurements provide clear evidence of increased flexibility of the modified protein, as suggested by the accelerated kinetics of deuterium incorporation (Figure 3). In order to understand how the local dynamics of different protein segments are affected by chemical modification at a single site, the spatial distribution of deuterium incorporation was evaluated for both unmodified and alkylated forms of IFN- β 1a. Deuterium content measurements of various proteolytic fragments yield valuable stability information throughout the range of structural elements within native IFN- β 1a, as well as the effect exerted by the alkylation on local dynamics in various parts of the protein. While most of the proteolytic fragments exhibited a low-to moderate increase in deuterium uptake kinetics (< 20%) as a result of protein alkylation, the effect is much more significant in several peptides. An example is presented in Figure 4, where the kinetics of deuterium uptake within the peptic fragment spanning fifteen amino acid residues (88-102) is very slow in the native unmodified IFN- β 1a, but is accelerated dramatically in the alkylated form of the protein. A closer examination of the time evolution of the isotopic distribution of this peptide fragment derived from NEM-IFN- β 1a reveals the presence of two distinct peptide ion populations. Deuterium content of one of them is indistinguishable from that of the same peptic fragment derived from the unmodified form of IFN- β 1a; another population exhibits significantly lower protection levels. Such bimodal isotopic distributions usually signal the occurrence of the so-called correlated exchange, a signature of the EX1 exchange regime (46). In our case, *ca.* 5% of the ion population may also represent a contribution of unmodified IFN- β 1a present in the NEM-IFN- β 1a sample.

Bimodal isotopic distributions were also observed for several other peptides whose exchange kinetics are influenced by protein alkylation (marked with asterisks in Figure 5). Although the differences between the two forms of the protein can be seen as early as 10 sec of the exchange reaction (Figure 4), a clear distinction between the two populations of (88-102) peptide ions can be made following 5 min of exchange. Therefore the HDX MS data presented in Figure 5 shows the levels of deuterium incorporation in IFN- β 1a and NEM-IFN- β 1a at 5 min. The results of HDX MS measurements for IFN- β 1a and NEM-IFN- β 1a presented in Figure 5 were mapped onto the crystal structure of IFN- β 1a (38) (Figure 6A and B) and provide a clear indication that the backbone flexibility is not distributed evenly throughout the polypeptide chain.

Comparison of the flexibility maps of the unmodified and alkylated IFN- β 1a is a very useful tool for visualizing the structural elements whose stability is affected by the chemical modification (Figure 6C). It is not surprising that the flexibility of the protein segments containing the Cys¹⁷ residue increase following IFN- β 1a alkylation. However, the affected regions are not confined by primary structure to the polypeptide region containing the chemically modified residue (Cys¹⁷). One of the most affected segments (88-102) is very distant in sequence from the alkylated residue, but is proximally located in the structure on helix C facing the Cys¹⁷ side chain. Alkylation of Cys¹⁷ with NEM apparently introduces a significant steric constraint in the protein interior, which distorts helix packing, thereby adversely affecting the stability of the higher order structure. The flexibility maps also allow one to identify several other protein segments (e.g., helix D), whose stability is adversely affected by the chemical modification of Cys¹⁷ through some yet unknown allosteric

mechanisms, despite being distant from the modification site both in the sequence and in the 3D structure.

The backbone flexibility maps also provide some clues regarding the molecular mechanism of IFN- β 1a inactivation following its alkylation. We note that the two helices forming the interface with high-affinity IFN- β 1a receptor IFNAR-2 (A and E (38)) are among the most dynamic (least protected) elements of IFN- β 1a secondary structure. Elevated polypeptide chain flexibility has been suggested in the past to play an important role in protein interactions (47), and transient structural disorder was shown in the past to be an important facilitator of protein binding to small ligands (48) and other proteins (49). Should the IFN- β 1a chain flexibility be a factor catalyzing the IFN- β 1a/IFNAR-2 binding as well, a modest mobility increase within a short segment of helix A induced by IFN- β 1a alkylation would be unlikely to disrupt the binding. Indeed, preliminary studies utilizing direct ESI MS as well as SEC indicate the presence of a NEM-IFN- β 1a/IFNAR-2 complex, which is readily formed in a manner comparable to that of unmodified IFN- β 1a (data not shown). Should this be the case, the increased backbone mobility observed for NEM-IFN- β 1a may have a deleterious effect towards IFNAR-1 recognition, which in contrast to IFNAR2 could proceed through a classical docking mechanism (50). Among the structural elements of IFN- β 1a proposed to form the IFNAR-1 binding interface, helix C exhibits the most significant gain in backbone mobility upon protein alkylation (Figure 6).

The involvement of helix C in IFN- β 1a/receptor interaction and its prominent role in partial unfolding of the protein following its alkylation make it a convenient reporter of compromised integrity of native conformation in response to IFN- β 1a degradation. Evolution of the isotopic distribution of peptic fragments located in this structural element of NEM-IFN- β 1a (Figure 4) provides very readily conclusive evidence of diminished structural stability. Importantly, the bimodal character of the isotopic distributions observed at up to 5 min of HDX provides an easy and reliable way to make a distinction between the two forms of the protein, one whose structural integrity has not been compromised (low deuterium uptake level) and the conformer characterized by high backbone mobility in this region (high deuterium uptake level). This feature would allow the signature of partial unfolding to be seen even in the background of protein molecules whose structural integrity has not been compromised. In fact, the abundance ratio of the two ionic populations may be used to calculate the relative amount of the degraded protein in the sample.

Conclusions

In the present study we have demonstrated that HDX MS along with ion charge state distribution analysis can be confidently used to monitor the stability of protein biopharmaceutical products. In particular, HDX MS provided reliable and detailed characterization information on the native IFN- β 1a conformation and dynamics. In addition, the direct comparison of native IFN- β 1a to a form chemically modified at its free cysteine is particularly enlightening in assessing the impact of this simple modification. Although some evidence of the diminished conformational stability of NEM-IFN- β 1a could be obtained from measurements employing classical biophysical tools, specifically by CD, HDX MS is unrivaled in its ability to detect and characterize the large scale dynamic events within the protein that affect its function. Such specific detailed information on the higher order structure of protein biopharmaceuticals cannot be obtained by the standard biophysical characterization commonly used in the biopharmaceutical industry. Since this experimental technique probes conformational stability directly without relying on covalent markers of protein degradation, it may be used in a variety of settings, including situations where protein structural integrity is compromised while its covalent structure remains intact (structural changes that arise solely from noncovalent changes). This capability opens up a host of new and exciting opportunities

in the field of characterization of the higher order structure and stability of protein Pharmaceuticals that could have significant benefit to protein design, stability, and comparability studies.

Acknowledgements

This work was supported in part by a grant from the National Institutes of Health R01 GM061666. The authors would like to also thank Dr. Darren Baker (Biogen Idec, Molecular Discovery) for samples of IFNAR2 and Peter Reavey (Biogen Idec, Bioanalytical Quality Control) for conducting the antiviral assay on IFN- β 1a and NEM-IFN- β 1a samples.

References

- Walsh G. Biopharmaceutical benchmarks 2006. *Nat. Biotech* 2006;24:769–776.
- Lawrence S. Pipelines turn to biotech. *Nat. Biotech* 2007;25:1342–1342.
- Singhal D, Curatolo W. Drug polymorphism and dosage form design: a practical perspective. *Adv. Drug Del. Rev* 2004;56:335–347.
- Bauer J, Spanton S, Henry R, Quick J, Dziki W, Porter W, Morris J. Ritonavir: an extraordinary example of conformational polymorphism. *Pharm. Res* 2001;18:859–866. [PubMed: 11474792]
- Maas C, Hermeling S, Bouma B, Jiskoot W, Gebbink MFBG. A role for protein misfolding in immunogenicity of biopharmaceuticals. *J. Biol. Chem* 2007;282:2229–2236. [PubMed: 17135263]
- Morozova-Roche L, Malisauskas M. A false paradise - mixed blessings in the protein universe: the amyloid as a new challenge in drug development. *Curr. Med. Chem* 2007;14:1221–1230. [PubMed: 17504142]
- Soskic V, Groebe K, Schratzenholz A. Nonenzymatic posttranslational protein modifications in ageing. *Exp. Gerontol* 2008;43:247–257. [PubMed: 18215483]
- Hipkiss AR. Accumulation of altered proteins and ageing: Causes and effects. *Exp. Gerontol* 2006;41:464–473. [PubMed: 16621390]
- Federici MM. The quality control of biotechnology products. *Biologicals* 1994;22:151–159. [PubMed: 7917231]
- Walsh G. Second-generation biopharmaceuticals. *Eur. J. Pharm. Biopharm* 2004;58:185–196. [PubMed: 15296948]
- Szymkowski DE. Creating the next generation of protein therapeutics through rational drug design. *Curr. Opin. Drug Discov. Devel* 2005;8:590–600.
- Roger SD, Mikhail A. Biosimilars: opportunity or cause for concern? *J. Pharm. Pharm. Sci* 2007;10:405–410. [PubMed: 17727803]
- Kuhlmann M, Covic A. The protein science of biosimilars. *Nephrol. Dial. Transplant* 2006;21:v4–8. [PubMed: 16959791]
- Oliva A, Farina JB, Llabres M. New trends in analysis of biopharmaceutical products. *Curr. Pharm. Anal* 2007;3:230–248.
- Berkowitz SA. Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals. *AAPS J* 2006;8:E590–605. [PubMed: 17025277]
- Luykx DMAM, Goerdal SS, Dingemans PJ, Jiskoot W, Jongen PMJM. HPLC and tandem detection to monitor conformational properties of biopharmaceuticals. *J. Chromatogr. B* 2005;821:45–52.
- Raso SW, Abel J, Barnes JM, Maloney KM, Pipes G, Treuheit MJ, King J, Brems DN. Aggregation of granulocyte-colony stimulating factor in vitro involves a conformationally altered monomeric state. *Protein Sci* 2005;14:2246–2257. [PubMed: 16131655]
- Ye H. Simultaneous determination of protein aggregation, degradation, and absolute molecular weight by size exclusion chromatography-multiangle laser light scattering. *Anal. Biochem* 2006;356:76–85. [PubMed: 16839514]
- Brewer JM. The use of differential scanning calorimetry (DSC) to determine the correctness of folding of cloned proteins. *Biotechnol. Appl. Biochem* 1999;30:173–175. [PubMed: 10512798]

20. Kaltashov IA, Eyles SJ. Studies of biomolecular conformations and conformational dynamics by mass spectrometry. *Mass Spectrom. Rev* 2002;21:37–71. [PubMed: 12210613]
21. Kaltashov, IA.; Eyles, SJ. *Mass spectrometry in molecular biophysics: conformation and dynamics of biomolecules*. John Wiley; Hoboken, N.J.: 2005.
22. Srebalus-Barnes CA, Lim A. Applications of mass spectrometry for the structural characterization of recombinant protein Pharmaceuticals. *Mass Spectrom. Rev* 2007;26:370–388. [PubMed: 17410555]
23. Wales TE, Engen JR. Hydrogen exchange mass spectrometry for the analysis of protein dynamics. *Mass Spectrom. Rev* 2006;25:158–170. [PubMed: 16208684]
24. Kaltashov IA, Abzalimov RR. Do ionic charges in ESI MS provide useful information on macromolecular structure? *J. Am. Soc. Mass Spectrom* 2008;19in press
25. Andrea S. Chemical cross-linking and mass spectrometry to map three-dimensional protein structures and protein-protein interactions. *Mass Spectrom. Rev* 2006;25:663–682. [PubMed: 16477643]
26. Konermann L, Tong X, Pan Y. Protein structure and dynamics studied by mass spectrometry: H/D exchange, hydroxyl radical labeling, and related approaches. *J. Mass Spectrom.* 2008in press
27. Engen JR, Smith DL. Investigating protein structure and dynamics by hydrogen exchange MS. *Anal. Chem* 2001;73:256A–265A.
28. Kaltashov IA, Eyles SJ. Crossing the phase boundary to study protein dynamics and function: combination of amide hydrogen exchange in solution and ion fragmentation in the gas phase. *J. Mass Spectrom* 2002;37:557–565. [PubMed: 12112737]
29. Tobler SA, Holmes BW, Cromwell MEM, Fernandez EJ. Benzyl alcohol-induced destabilization of interferon- γ : a study by hydrogen-deuterium isotope exchange. *J. Pharm. Sci* 2004;93:1605–1617. [PubMed: 15124217]
30. Konermann L, Douglas DJ. Acid-Induced unfolding of cytochrome *c* at different methanol concentrations: Electrospray ionization mass spectrometry specifically monitors changes in the tertiary structure. *Biochemistry* 1997;36:12296–12302. [PubMed: 9315869]
31. Borden EC, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR, Stark GR. Interferons at age 50: past, current and future impact on biomedicine. *Nat. Rev. Drug Discov* 2007;6:975–990. [PubMed: 18049472]
32. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys. J* 2000;78:1606–1619. [PubMed: 10692345]
33. Cravetto L, Lascoux D, Forest E. Use of different proteases working in acidic conditions to improve sequence coverage and resolution in hydrogen/deuterium exchange of large proteins. *Rapid Commun. Mass Spectrom* 2003;17:2387–2393. [PubMed: 14587084]
34. Bai Y, Milne JS, Mayne L, Englander SW. Primary structure effects on peptide group hydrogen exchange. *Proteins* 1993;17:75–86. [PubMed: 8234246]
35. Arduini RM, Strauch KL, Runkel LA, Carlson MM, Hronowski X, Foley SF, Young CN, Cheng W, Hochman PS, Baker DP. Characterization of a soluble ternary complex formed between human interferon-beta-1a and its receptor chains. *Protein Sci* 1999;8:1867–1877. [PubMed: 10493588]
36. Alam J, Goelz S, Rioux P, Scaramucci J, Jones W, McAllister A, Campion M, Rogge M. Comparative pharmacokinetics and pharmacodynamics of two recombinant human interferon beta-1 a (IFN beta-1 a) products administered intramuscularly in healthy male and female volunteers. *Pharm. Res* 1997;14:546–549. [PubMed: 9144748]
37. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer* 1976;17:62–70. [PubMed: 175022]
38. Whitty, A.; Karpusas, M. The structure of human interferon-b-1a (AvonexTM) and its relation to activity: a case study of the use of structural data in the arena of protein Pharmaceuticals. In: Chasman, DL., editor. *Protein Structure: Determination, Analysis and Applications for Drug Discovery*. Marcel Dekker; New York-Basel: 2003. p. 483-519.
39. Bermel RA, Rudick RA. Interferon-b treatment for multiple sclerosis. *Neurotherapeutics* 2007;4:633–646. [PubMed: 17920544]
40. Serman DH, Recio A, Carroll RG, Gillespie CT, Haas A, Vachani A, Kapoor V, Sun J, Hodinka R, Brown JL, Corbley MJ, Parr M, Ho M, Pastan I, Machuzak M, Benedict W, Zhang X.-q. Lord EM,

Litzky LA, Heitjan DF, June CH, Kaiser LR, Vonderheide RH, Albelda SM. A phase I clinical trial of single-dose intrapleural IFN-*b* gene transfer for malignant pleural mesothelioma and metastatic pleural effusions: high rate of antitumor immune responses. *Clin. Cancer Res* 2007;13:4456–4466. [PubMed: 17671130]

41. Orru S, Amoresano A, Siciliano R, Napoleoni R, Finocchiaro O, Datola A, De Luca E, Sirna A, Pucci P. Structural analysis of modified forms of recombinant IFN-*b* produced under stress-simulating conditions. *Biol. Chem* 2000;381:7–17. [PubMed: 10722045]
42. Dobo A, Kaltashov IA. Detection of multiple protein conformational ensembles in solution via deconvolution of charge state distributions in ESI MS. *Anal. Chem* 2001;73:4763–4773. [PubMed: 11681449]
43. Mohimen A, Dobo A, Hoerner JK, Kaltashov IA. A chemometric approach to detection and characterization of multiple protein conformers in solution using electrospray ionization mass spectrometry. *Anal. Chem* 2003;75:4139–4147. [PubMed: 14632127]
44. Kuprowski MC, Konermann L. Signal response of coexisting protein conformers in electrospray mass spectrometry. *Anal. Chem* 2007;79:2499–2506. [PubMed: 17288464]
45. Frokjaer S, Otzen DE. Protein drug stability: A formulation challenge. *Nat. Rev. Drug Discov* 2005;4:298–306. [PubMed: 15803194]
46. Xiao H, Hoerner JK, Eyles SJ, Dobo A, Voigtman E, Mel'cuk AI, Kaltashov IA. Mapping protein energy landscapes with amide hydrogen exchange and mass spectrometry: I. A generalized model for a two-state protein and comparison with experiment. *Protein Sci* 2005;14:543–557. [PubMed: 15659380]
47. Shoemaker BA, Portman JJ, Wolynes PG. Speeding molecular recognition by using the folding funnel: the fly-casting mechanism. *Proc. Natl. Acad. Sci. U.S.A* 2000;97:8868–8873. [PubMed: 10908673]
48. Xiao H, Kaltashov IA. Transient structural disorder as a facilitator of protein-ligand binding: native H/D exchange-mass spectrometry study of cellular retinoic acid binding protein I. *J. Am. Soc. Mass Spectrom* 2005;16:869–879. [PubMed: 15907702]
49. Griffith WP, Kaltashov IA. Protein conformational heterogeneity as a binding catalyst: ESI-MS study of hemoglobin H formation. *Biochemistry* 2007;46:2020–2026. [PubMed: 17253776]
50. Camacho CJ, Vajda S. Protein-protein association kinetics and protein docking. *Curr. Opin. Struct. Biol* 2002;12:36–40. [PubMed: 11839487]

Figure 1.

Comparison of IFN-β1a (blue traces) and NEM-IFN-β1a (red traces) using classical biophysical techniques: SEC profiles (A), fluorescence emission spectra (B), far-UV CD spectra (C) and near-UV CD spectra (D). Gray trace in panel A corresponds to incompletely alkylated protein. Inset in panel C shows the difference between the two spectra (normalized to protein concentration).

Figure 2. Charge state distributions of NEM-IFN-β1a (top) and intact IFN-β1a (bottom) ions in ESI MS acquired under near-native conditions following buffer exchange of protein solutions to 100 mM ammonium acetate.

Figure 3.

Global HDX kinetics of intact IFN- β 1a (blue) and NEM-IFN- β 1a (red). The top panel shows raw HDX MS data (charge state +9), where the m/z scale for IFN-NEM ions was offset such that the observed difference in the ion peak position is due to the different rates of deuterium incorporation. Using the same color coding, the bottom panel shows the incorporation of deuterium by each protein versus time, calculated as described in the materials and methods.

Figure 4.

Evolution of isotopic distributions of a peptide fragment (88-102) derived from unmodified IFN- β 1a (blue) and NEM-IFN- β 1a (red) at different time points of the exchange reaction. The gray trace shows the isotopic distribution of the fully exchanged peptide (HDX end-point).

Figure 5.

Levels of deuterium incorporation following 5 min of exchange within segments representing all observed proteolytic fragments derived from unmodified IFN- β 1a (blue) and NEM-IFN- β 1a (red). Gray bars plot the difference between IFN- β 1a and NEM-IFN- β 1a. Peptides that exhibited bimodal exchange in the NEM-IFN- β 1a sample are indicated with an asterisk.

Figure 6.

Segments representing all observed proteolytic fragments of unmodified NEM-IFN-β1a (A) and IFN-β1a (B) colored according to their deuteration level following 5 min of exchange mapped on the 1° and 3° structures of the protein. Color coding reflects flexibility of various segments ranging from high protection levels (blue) to very low protection (red). The bottom diagram (C) shows protein segments where flexibility is changed as a result of alkylation. These are colored by difference in % exchange as indicated by the scale, except for the residues in pink, which exhibited a marked difference in exchange though the fast exchange kinetics in this region prevented an accurate difference calculation. In all 3° structures residues not observed were colored in black. Helical segments are indicated by wavy lines above the amino

acid sequence and labeled alphabetically by convention. Cysteine residues involved in a disulfide bond are colored in orange circles, the free (alkylated) cysteine residue is marked with a red pentagon, and the first residue of the CHO chain is marked with a blue hexagon.