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Deciphering the Biophysical Effects of Oxidizing Sulfur-Containing Amino Acids in Interferon-beta-1a using MS and HDX-MS

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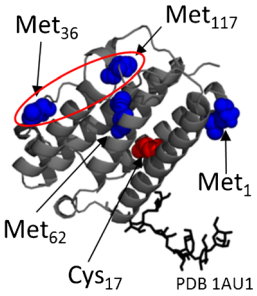

Abstract. Introduction of a chemical change to one or more amino acids in a protein's polypeptide chain can result in various effects on its higher-order structure (HOS) and biophysical behavior (or properties). These effects range from no detectable change to significant structural or conformational alteration that can greatly affect the protein's biophysical properties and its resulting biological function. The ability to reliably detect the absence or presence of such changes is essential to understanding the structure–function relationship in a protein and in the successful commercial development of protein-based drugs (biopharmaceuticals). In this paper, we focus our attention on the latter by specifically elucidating the impact of oxidation on the HOS, structural dynamics, and biophysical properties of interferon beta-1a (IFNβ-1a). Oxidation is a common biochemical modification that occurs in many biopharmaceuticals, specifically in two naturally-occurring sulfur-containing amino acids, methionine and cysteine. To carry out this work, we used combinations of hydrogen peroxide and pH to differentially oxidize IFNβ-1a (to focus on only methionine oxidation versus methionine and cysteine oxidation). We then employed several analytical and biophysical techniques to acquire information about the differential impact of these two oxidation scenarios on IFNβ-1a. In particular, the use of MS-based techniques, especially HDX-MS, play a dominant role in revealing the differential effects.

Keywords: Higher-order structure, Conformation, Conformational dynamics, Biophysical properties, Methionine and cysteine oxidation

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Introduction

The underlying structural component of protein-based drugs (referred to in this paper as "biopharmaceuticals") is its primary structure (i.e., its linear sequence of amino acids). While the primary structure is composed of 20 naturally-occurring amino acids (we will not consider non-natural amino acids in this work), post-translational modifications (PTMs) add another layer of chemical complexity [1]. Although most PTMs take place in vivo, they also occur in vitro, mostly due to non-enzymatic chemical reactions. In vitro PTMs either add no value or cause protein degradation, which is detrimental to the final drug product. Given that biopharmaceuticals are large molecules, many targets are susceptible to chemical modification, thus adding complexity to the molecule and presenting a significant challenge to its characterization. Fortunately, mass spectrometry (MS) is an effective analytical tool used in the biopharmaceutical industry because of its ability to tackle such complex issues. Different MS approaches such as intact mass analysis [2] and peptide mapping [3] are employed to characterize PTMs. Although intact mass analysis is capable of revealing changes to the protein HOS through the appearance of new charge state distributions upon PTM [4], such characterization becomes very challenging when the protein is intrinsically heterogeneous (as is the case with many biopharmaceuticals).

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Therefore, peptide mapping might be the method of choice to reduce the complexity of mass spectra. However, detecting a change in primary structure is quite different from detecting and understanding the impact of a PTM on the biopharmaceutical's HOS and its biophysical properties. Such changes may impact its efficacy, stability, and/or safety [5–8]. Consequently, significant efforts have been made to develop more informative biophysical tools that can be implemented in a practical and robust manner [9, 10]. In the last decade in particular, MS techniques such as covalent labeling [11], charge state distribution [12], ion mobility [13], and hydrogen deuterium exchange monitored by mass spectrometry (HDX-MS) [14] have been developed to better characterize changes in biophysical properties of biopharmaceuticals upon chemical changes due to PTMs [15–17]. In several of our previously published works, we have used IFNβ-1a as a model biopharmaceutical to demonstrate the capability of some of these techniques. For instance, we have previously demonstrated that alkylation and oxidation of IFNβ-1a causes significant alterations in the protein's conformation and dynamics [4, 18].

In this paper, we investigate in more detail the specific effects of methionine and cysteine oxidation on IFNβ-1a using primarily HDX-MS and liquid chromatography (LC)-MS peptide mapping as key characterization tools. More specifically, we focus on the differentiating effects of methionine versus cysteine oxidation with regards to the effects on the HOS, structural dynamics, and biophysical properties of IFNβ-1a. Differentiation between methionine versus cysteine oxidation was possible because of the unique effect that pH has on the reaction between H_2O_2 and each of these two amino acids [19]. For instance, under mildly acidic conditions (e.g., pH 4.8), cysteine exists predominantly in the sulfide form (-SH), thus reducing its susceptibility to oxidation. At this pH, only solvent-accessible methionine residues are susceptible to oxidation by H_2O_2. On the other hand, at pH near or above neutrality (e.g., pH 7.2), the sulfhydryl group of the cysteine residues exists mostly in the thiolate form ($-S^-$). In this case, both methionine and cysteine residues are subject to oxidation by H_2O_2. Therefore, we have been able to selectively oxidize either methionine residues alone or both methionine and cysteine residues, by controlling the pH at which we carried out the oxidation reaction. By adopting this strategy, we were able to elucidate the differentiating effects of methionine versus cysteine oxidation on the HOS, conformational dynamics, and biophysical properties of IFNβ-1a. Our results further support the growing interest in (and capability of) MS-based techniques to characterize complex drugs in the biopharmaceutical industry [20–24].

Experimental

Samples and Buffers

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. H_2O_2 (30% solution) was purchased from Acros Chemicals (a Thermo Fischer Scientific company, Pittsburg, PA USA). IFNβ-1a samples were overexpressed in CHO cells at Biogen. The two different formulation buffers were: pH 4.8 buffer (consisting of 20 mM Na acetate, 150 mM L-arginine-HCl, pH 4.8), and pH 7.2 buffer (consisting of 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 200 mM NaCl pH 7.2). Samples were dialyzed into each of the two buffers as described below.

Sample Preparation and Strategy

IFNβ-1a at 0.27 mg/mL pH 4.8 was concentrated to ~1 mg/mL with an Amicon centrifugal device (molecular weight cutoff, MWCO, of 3 kDa) from Millipore (Billerica, MA, USA). The sample volume was split into two aliquots, one of which was buffer-exchanged into pH 7.2 buffer. Buffer exchange was carried out in the centrifugal devices described above by employing three consecutive washes with the pH 7.2 buffer. Then, each aliquot was split in half: the first was subjected to oxidation whereas the other aliquot served as control material. Oxidation was carried out for 4 h at 20 °C by incubating each of the IFNβ-1a aliquots with H_2O_2 at a final concentration of 0.1% (note: an equivalent volume of H_2O was added to each control sample). The oxidation reaction was quenched by addition of approximately 1.0 mg of L-methionine to the mixture (methionine was also added to the control). After ~10 min of gentle mixing, the control and oxidized samples in each aliquot were buffer-exchanged at the corresponding pH to remove additives, using an Amicon centrifugal device (MWCO of 3 kDa) with three consecutive buffer washes.

Instrumentation and Data Processing

LC-MS Peptide Mapping

Peptide mapping by LC-MS was performed on reduced and lysyl endopeptidase (ELC)-digested IFNβ-1a using an Agilent 1100 HPLC coupled to a mass spectrometer (LCQ Deca XP MAX ion-trap, Thermo Fisher Scientific, Pittsburg, PA, USA). Briefly, 100 µg of IFNβ-1a was concentrated to approximately 45 µL, denatured with 90 µL of 8 M guanidine HCl, and reduced with 5 µL of 100 mM DTT at 25 °C for 60 min. Fifty mM Na phosphate, pH 7.2 was added to bring the volume to 140 µL. The sample was then diluted with the addition of 235 µL of 50 mM Na phosphate, pH 7.2, and digested with 12 µL of 0.8 mg/mL ELC at 25 °C for 22 ±2 h. Ninety µL of digested IFNβ-1a was injected onto a 2 × 250 mm Vydac C18 5 µm 300 Å column and peptides were separated under reversed-phase conditions using a linear gradient of water (HPLC water, 0.1% TFA) and acetonitrile (90% acetonitrile, 0.1% TFA).

Size Exclusion Chromatography (SEC) Analysis

IFNβ-1a samples were analyzed by SEC on a Waters 1525 HPLC system. 10 µL of 10 µM (1.0 mg/mL) IFNβ-1a

sample were injected onto a TSK Gel G2000SW column from TOSOH Bioscience. An isocratic mode separation in 100 mM sodium phosphate, 200 mM NaCl, pH 6.8 buffer proceeded for 15 min and the eluent was monitored online with both UV and light scattering detection (LS, Wyatt mini-dawn). UV was monitored at 280 nm for protein quantitation and 90° scattering was used for assessing information about MW of species in the SEC chromatograms.

Analytical Ultracentrifugation (AUC)

AUC experiments were performed on a Beckman-Coulter XL-I centrifuge operated at 40,000 rpm and 20 °C. Twelve-mm double sector charcoal-filled Epon centerpieces were used with sapphire windows. UV data was collected at 280 nm with a radial data spacing of 0.003 cm. Sedimentation coefficients were determined by processing the data with SEDFIT (v14.1) [25]. Note, AUC experiments were performed only once for each sample.

HDX-MS Analysis

IFNβ-1a samples were equilibrated at ambient temperature (20 ±1 °C) for 1 h before labeling with deuterated formulation buffer (pH 4.8 buffer consisting of 20 mM Na acetate, 150 mM L-arginine-HCl, or pH 7.2 buffer consisting of 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 200 mM NaCl, both prepared in D_2O instead of H_2O). The samples were then diluted 1:12.5 with deuterated formulation buffer, allowing hydrogen exchange to occur, and incubated for various amounts of time (0.167, 1, 10, 60, and 240 min). The reaction was then quenched with a 1:1 dilution of 200 mM Na phosphate, 8 M guanidinium HCl, and 0.5 M TCEP, pH 2.4. Quenched samples were held for 0.5 min and further diluted 1:1 with 0.1% formic acid before injecting them onto the LC-MS system for analysis. Approximately 20 pmol of exchanged/quenched IFNβ-1a were injected onto an immobilized pepsin column, where the digestion and peptide trapping were performed for 3 min at a flow rate of 0.1 mL/min in 0.1% formic acid at 10 °C. The peptic peptides were trapped on an ACQUITY BEH C18 1.7 µm peptide pre-column trap (Waters Corp., Milford, MA, USA) maintained at 0 °C [26]. Flow was diverted by a switching valve and the trapped peptides flushed from the trap onto an ACQUITY BEH C18 1.7 µm, 1 mm × 100 mm column (Waters Corp.) to separate the peptides at 0 °C using a 9 min linear acetonitrile gradient (2%–55%) with 0.1% formic acid at a flow rate of 40 µL/min. The eluate from the C18 column was directed into a Waters Synapt G2S mass spectrometer with electrospray ionization and lock-mass correction (using Glu-fibrinogen peptide). Mass spectra were acquired from *m/z* 255 to 1800. Every time point was analyzed in triplicate. Peptic peptides were identified using a combination of exact mass and MS^E [27]. HDX data were processed using DynamX software from Waters Corp. HDX-MS performed as outlined here is referred to as "local HDX-MS" using a bottom-up approach [14].

Results and Discussion

LC-MS Peptide Mapping

To assess our ability to selectively control the oxidation of IFNβ-1a's only cysteine (Cys_{17}) sulfhydryl group, oxidation was carried out at two different pH values. Samples of unmodified or non-oxidized IFNβ-1a (referred to hereinafter as "control") and H_2O_2-treated (referred to hereinafter as "oxidized") IFNβ-1a at pH 4.8 and pH 7.2 were digested by ELC and analyzed by LC-MS peptide mapping. Figure 1 represents the MS total ion chromatograms (TIC) for the two pH conditions analyzed (4.8 and 7.2). The chromatogram of IFNβ-1a control (blue) is compared with the oxidized IFNβ-1a (red, bottom) at each pH condition. At pH 4.8 the traces of the two IFNβ-1a samples (control and oxidized) are generally found to be comparable in terms of the number of peaks detected. However, differences in peak intensities observed between the control and oxidized samples at pH 4.8 are due to increased levels of oxidation. On the other hand, at pH 7.2 the MS trace of the oxidized sample reveals a few additional peaks that were not detected at pH 4.8. Accurate MS and MS/MS analysis indicated that these peaks represent different versions of oxidized peptide 8 (i.e., peptide 8 with oxidized Cys_{17} and Met_1).

Peptide mapping by LC-MS enabled us to monitor the extent of oxidation on each of the four methionine residues and the one cysteine residue in IFNβ-1a (note, the other two Cys residues are in the cystine form). This was achieved by monitoring peptides that contain methionine and cysteine amino acids highlighted in different colors in Figure 1: purple for peptide 4 (residues 116–123 containing Met_{117}, at ~21 min), yellow for peptide 6 (residues 34–45 containing Met_{36}, at ~25 min), gray for peptide 8 (residues 1–19 containing Met_1, at ~35 min), and green for peptide 10 (residues 53–99 containing Met_{62}, at ~52 min). Note that unlike the oxidation at pH 4.8, when IFNβ-1a is oxidized at pH 7.2, we observe a new unique peak that matches the mass of peptide 8 + 4 oxygen atoms (1 for Met_1:sulfoxide and 3 for Cys_{17}:sulfonic acid). Table 1 lists the relative percent oxidation for each of these amino acids, calculated from the area under the peak in the extracted ion chromatograms of each of the oxidized and control peptides. This data represents, in the case of pH 7.2, our most recent understanding of IFNβ-1a oxidation and is an update to the oxidation data that we previously reported [18]. Of particular importance is the oxidation level of the only free sulfhydryl group in IFNβ-1a, which showed that Cys_{17} is highly susceptible to oxidation at pH 7.2, but not at pH 4.8 (see peptide 8 Cys_{17} in Table 1). Such results are consistent with the known chemical reactivity of sulfhydryl groups [28, 29]. In addition, it should be noted that similar results for IFNβ-1a under more acidic conditions (pH 3.8) have also been reported, even in the presence of significantly higher levels of the oxidizing agent (H_2O_2) [30].

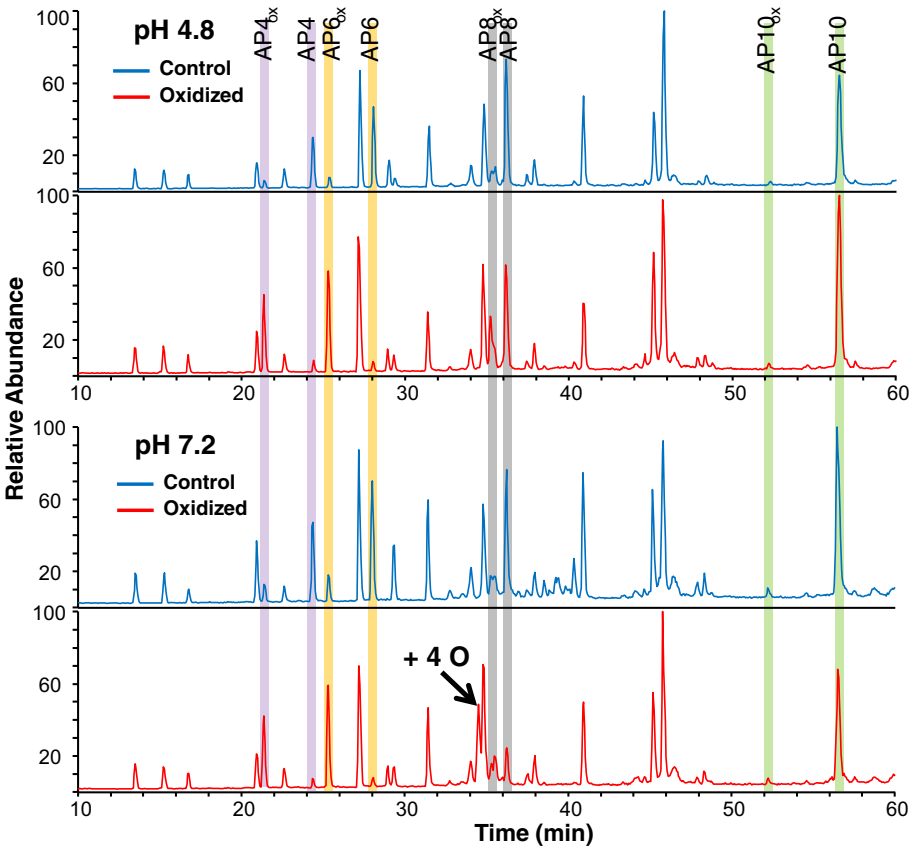

Figure 1. TIC traces of IFNβ-1a control (in blue) and oxidized IFNβ-1a (in red) in both formulation buffers: pH 4.8 (top panel) and pH 7.2 (bottom panel). Highlighted in purple, yellow, grey, and green are the peaks corresponding to the control and oxidized sulfur-containing peptides AP4, AP6, AP8, and AP10, respectively. Levels of oxidation listed in Table 1 are calculated by measuring the area under the peak of the oxidized material relative to the control. Note that for the oxidized material at pH 7.2, accurate MS/MS analysis determined that the newly observed peak (black arrow, +4O) corresponds to the oxidized AP8 peptide that includes the sulfonic acid form (+3O) of the free sulfhydryl group on Cys_{17} and Met_1 oxidation (+1O). In addition, the extra peaks that appear between 37 and 40 min for the pH 7.2 control sample are impurities that do not belong to the protein

Table 1. Relative Percent Oxidation of Susceptible Amino Acid Residues Present in Each of the 4 sulfur-containing peptides (highlighted In Figure 1)

Condition	Peptide ID	AA first	AA last	Susceptible oxidation site	% Oxidation
Control	AP4	116	123	Met 117	4.5 ± 0.4
	AP6	34	45	Met 36	6.3 ± 0.3
	AP8	1	19	Met 1	2.5 ± 0.8
	AP8	1	19	Cys 17	NA*
	AP10	53	99	Met 62	3.1 ± 0.9
Oxidation at pH 4.8	AP4	116	123	Met 117	89.8
	AP6	34	45	Met 36	91.6
	AP8	1	19	Met 1	28.9
	AP8	1	19	Cys 17	0.9
	AP10	53	99	Met 62	2.0
Oxidation at pH 7.2	AP4	116	123	Met 117	90.7
	AP6	34	45	Met 36	92.9
	AP8	1	19	Met 1	65.3
	AP8	1	19	Cys 17	51.3
	AP10	53	99	Met 62	0.1

* NA = not available.

Note: Peptide mapping was performed in triplicate on the control sample only, to establish the baseline oxidation levels of methionine residues (due to sample handling, for example) and to assess reproducibility of the experiment (expressed in terms of one standard deviation). Oxidation levels for the forced oxidation at both pH 4.8 and pH 7.2 are also listed. Note that AP8 contains both Met_1 and Cys_{17}. Because Met_{62} is buried in the core of the protein, it is not susceptible to oxidation under either pH condition. Oxidation levels are fairly similar for Met_{117}, Met_{36}, and Met_{62} between both pH conditions. However, the level of oxidation increases at pH 7.2 for both Met_1 and Cys_{17} relative to pH 4.8

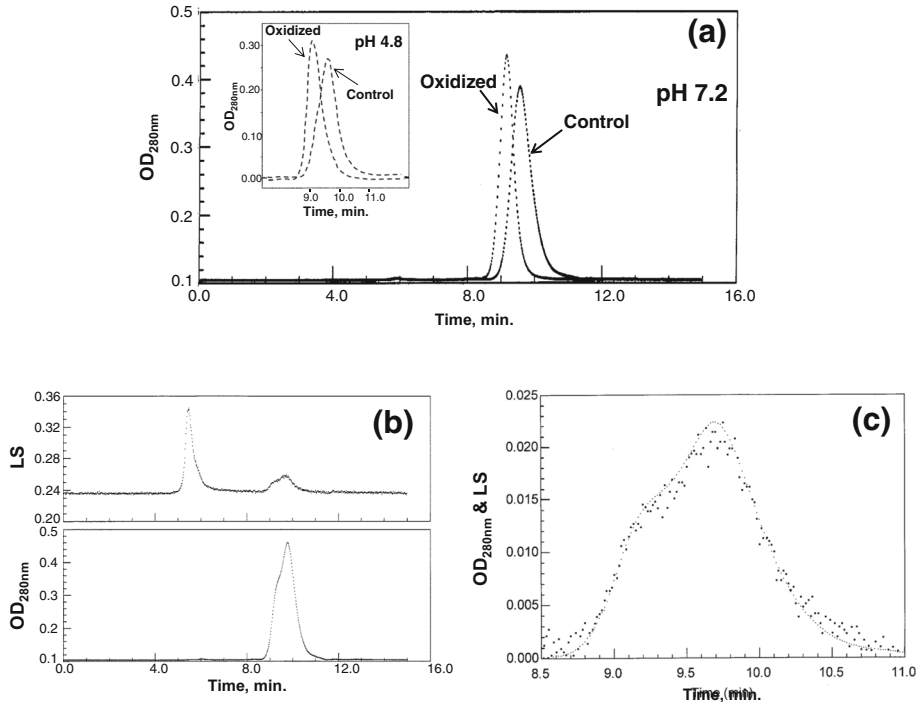

Figure 2. SEC chromatograms of control and oxidized IFNβ-1a under both pH conditions (4.8 and 7.2). **(a)** An overlay of UV chromatograms ($OD_{280\ nm}$) of the fully oxidized and control IFNβ-1a showing a shift in the peak to shorter RT upon oxidation. The full scale chromatogram corresponds to the pH 7.2 oxidation condition, whereas the insert corresponds only to the zoomed region of importance for the pH 4.8 oxidation condition. **(b)** A mixture of the control and fully oxidized IFNβ-1a at pH 7.2 with LS (top) and UV (bottom) detection. **(c)** After correction for the interdetector volume and appropriate normalization (so the peak max value near RT 9.7 min, in both cases had the same value), the results show that both the fully oxidized and control material have effectively the same MW as indicated by the good overlay of LS and UV chromatograms giving a fairly constant LS to UV ratio of 1.0 across the elution profile [34]

Size Exclusion Chromatography

Previously, we reported that IFNβ-1a oxidized at pH 7.2 displays a change in biophysical behavior characterized by a shift to shorter SEC retention time (RT) relative to the control IFNβ-1a elution profile (Figure 2a) [18]. In addition, it has also been noted that the shifted peak is sharper and more symmetrical than the control peak. A shorter RT in SEC is typically indicative of an increase in the hydrodynamic volume (physical size) of the sample molecules, possibly because of an increase in their MW (i.e., aggregation) or because of a conformational change (e.g., unfolding). However, the other possible explanation, which we eluded to in our earlier work [4], is that the control IFNβ-1a is chemically interacting (weakly) with the SEC chromatographic material, causing it to be slightly retained, and thus resulting in a longer RT. A weak interaction between IFNβ-1a and the chromatographic material would also explain the resulting broader and more asymmetric IFNβ-1a monomer peak shape that we and others [31] have observed under similar mobile-phase conditions using the same type of SEC column. To better understand which of these possible scenarios is responsible for the observed SEC RT shift, we performed several experiments on IFNβ-1a samples oxidized at both pH 4.8 and 7.2.

First, characterization of the SEC RT shift was carried out using an IFNβ-1a sample containing a mixture of control and oxidized IFNβ-1a (at pH 7.2) with on-line LS and UV detection, Figure 2b. The resulting overlay of LS and UV chromatograms [after the detector volume delay was properly accounted for and the corresponding chromatograms were appropriately normalized, so that the ratio of the IFNβ-1a peak maximum, near RT 9.7 min, in the LS and UV chromatogram (LS/UV) equaled 1.0] shows that the LS/UV ratio across the resulting SEC chromatogram is maintained at a fairly constant value close to 1.0, Figure 2c. Because the LS/UV ratio is directly related to the weight-averaged MW of the eluting material, our results indicate that the SEC RT shift observed upon oxidizing IFNβ-1a is not caused by an increase in MW (i.e., the RT shift is not due to aggregation).

Because our LC-MS experiments have shown that at pH 7.2, both methionine and cysteine residues are oxidized, it is essential to determine whether the reduction in SEC RT can be attributed to the oxidation of methionine alone, cysteine alone, or both. We have previously shown that highly specific and quantitative alkylation of the sulfhydryl group in Cys_{17} of IFNβ-1a also induces the same SEC RT shift [4]. Therefore, initially we speculated that the sulfhydryl oxidation of Cys_{17} might likely account for the observed SEC RT shift [18]. To better evaluate this possibility, we conducted SEC analysis in the present work on INFβ-1a material that was oxidized at pH 4.8, under which conditions we demonstrated (by peptide mapping) that Cys_{17} is not oxidized. Results from this SEC

experiment revealed that the IFNβ-1a material oxidized at pH 4.8 also exhibits a highly similar SEC RT shift (see insert in Figure 2a). From these results, we have concluded that the observed SEC RT shift in INFβ-1a is attributed to the oxidation of one or more methionine residues and does not depend on the oxidation of Cys_{17}.

Based on the above results, we considered the possibility that the oxidation-induced IFNβ-1a SEC RT shift could be a result of an increase in hydrodynamic volume caused by a large conformational change (e.g., partial unfolding of IFNβ-1a's compact structure, upon methionine oxidation). To determine whether methionine oxidation at either pH causes a change in the hydrodynamic volume of IFNβ-1a, and is thus the underlying cause for the SEC RT shift, we performed AUC experiments via boundary velocity sedimentation. The sedimentation coefficient, expressed in Svedberg units, S, for all matching sample sets (control versus oxidized IFNβ-1a at both pH conditions) were found to differ from each other by less than ±1% (data not shown). At this level of difference, a change in the hydrodynamic volume of IFNβ-1a upon oxidation at either pH is unlikely to be large enough to explain the observed much larger and striking SEC RT shift, which has weaker dependence on size than the sedimentation coefficient.

Finally, because we found no significant change in MW (at pH 7.2) or hydrodynamic volume (at both pH values) between the control and oxidized IFNβ-1a, we are left with the explanation that the SEC RT shift observed for INFβ-1a is caused by a weak secondary chemical interaction between INFβ-1a and the surface of the chromatographic material. Under this scenario, methionine oxidation likely reduces and/or eliminates these interactions. To better understand the structural differences observed between methionine (pH 4.8) and methionine plus cysteine (pH 7.2) oxidation of INFβ-1a, we performed HDX-MS experiments on all INFβ-1a samples at both pH conditions.

Local HDX-MS

HDX-MS is an outstanding tool to detect and assess differences in the conformation and conformational dynamics of proteins [14]. Peptide maps generated in this work (and in previous work [18]) provided sequence coverage for all samples of IFNβ-1a that was equal or greater than 95%. The amount of deuterium uptake by each peptide was determined by subtracting the average MW of the undeuterated peptide from that of the experimentally determined one at each of the various HDX incubation periods. Deuterium uptake data was then plotted as a function of the sample's exposure time to D_2O. Five representative peptide fragments are shown in Figure 3. We immediately conclude that for these five peptide fragments the exchange profiles of control and oxidized IFNβ-1a look identical when the protein is oxidized at pH 4.8 (Figure 3, top). However, when the oxidation is carried out at pH 7.2, major differences in deuterium uptake for the same peptides between control and oxidized IFNβ-1a are observed (Figure 3, bottom).

An important point in assessing the observed HDX differences seen in Figure 3 between the control and oxidized IFNβ-1a sample at each pH is the strong dependence of the intrinsic rate of HDX on pH. In fact, the HDX reaction is much slower (~2.4 orders of magnitude) at pH 4.8 than at pH 7.2 [32]. Consequently, differences in HDX at pH 4.8, if any, typically require longer incubation periods in D_2O to be observed. In addition, possible differences in the extent and rate of HDX for the same peptide might be also due to conformational differences and/or differences in conformational dynamics of IFNβ-1a at each pH. Although strategies to elucidate pH-dependent conformational changes by HDX-MS and deconvolve them from the pH effect on the intrinsic rate of HDX have been published [33], they reflect indirect approaches for dealing with this problem. To further confirm the absence of pH-induced conformational changes when IFNβ-1a is oxidized at pH 4.8, we undertook a more direct approach involving the simple buffer-exchange of the oxidized and control IFNβ-1a at pH 4.8 into pH 7.2 buffer. These samples were then characterized by HDX-MS at pH 7.2. Results generated from this experiment also reveal no significant HDX differences between control and oxidized IFNβ-1a material (see Discussion and Figure 4, middle).

To allow for the complete comparison of all common peptic peptides detected between control and oxidized IFNβ-1a at each pH, we generated a relative deuterium exchange difference plot as explained previously [18]. In Figure 4, the x-axis

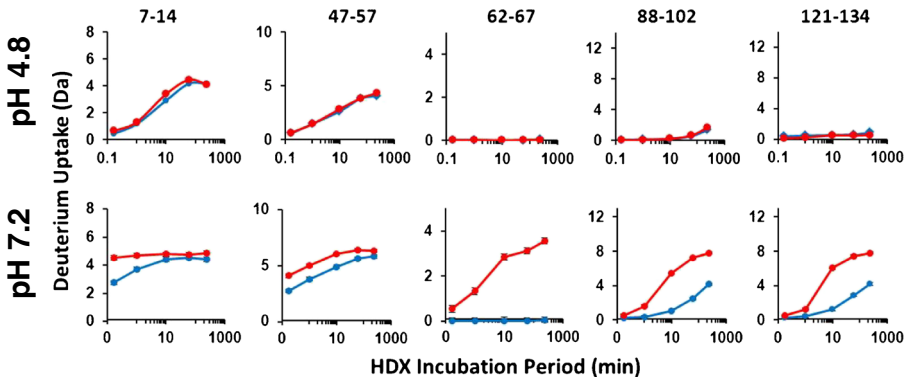

Figure 3. Deuterium uptake profiles as a function of incubation period for the same five representative peptic peptides of IFNβ-1a at pH 4.8 (top) and pH 7.2 (bottom): control is in blue and oxidized is in red. Major differences in deuterium uptake are observed at pH 7.2 but not at pH 4.8

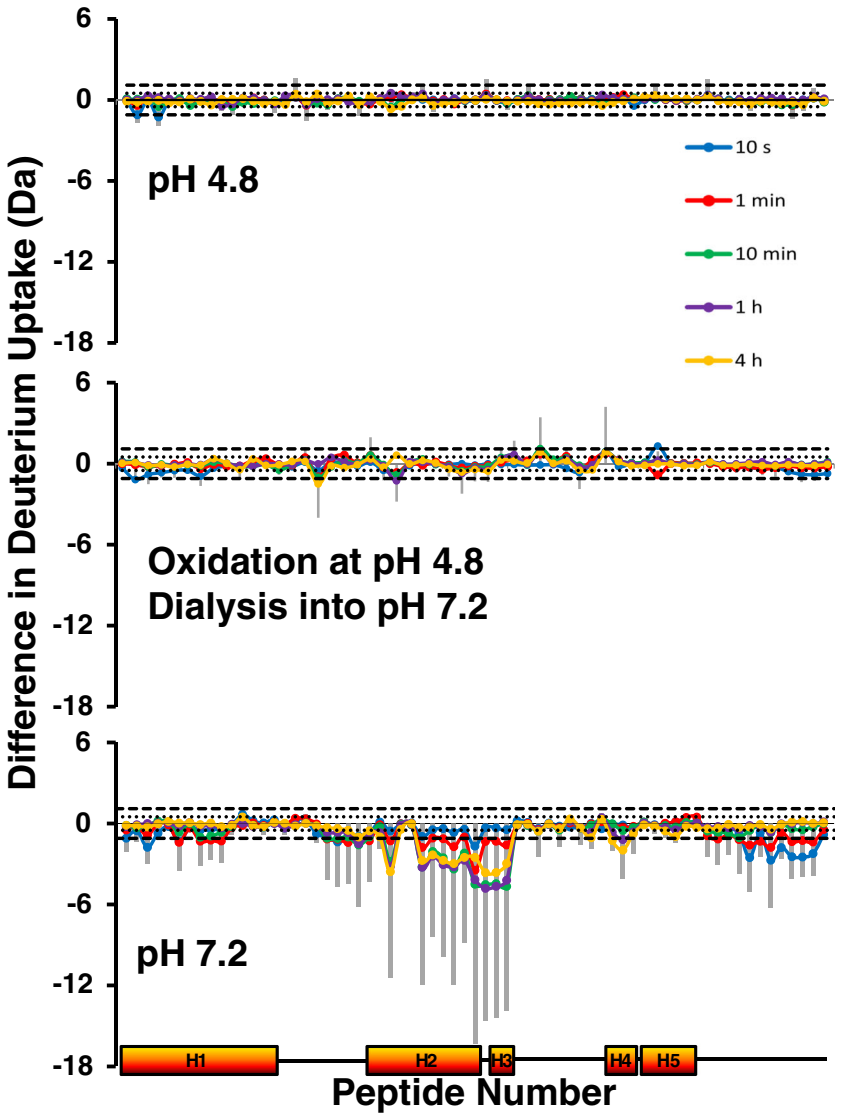

Figure 4. Deuterium exchange difference plot between control and oxidized IFNβ-1a at pH 4.8 (top), control and oxidized IFNβ-1a at pH 4.8 that was then dialyzed into pH 7.2 buffer (middle), and control and oxidized IFNβ-1a at pH 7.2 (bottom). Differences in deuterium uptake values for each of the 67 peptides (>95% sequence coverage) at each of the five incubation periods (10 s, 1 min, 10 min, 1 h, and 4 h) are represented in blue, red, green, purple, and yellow, respectively. The sums of differences in deuterium uptake are represented by grey bars. Labels H1–H5 located at the bottom of the graph along the x-axis represent the five helices of IFNβ-1a that are based on the peptides that belong to each helix. At pH 7.2, individual differences in deuterium uptake that exceed the statistically determined ±0.5 Da threshold (dotted lines) and sum of differences that exceed the ±1.1 Da threshold (dashed lines) indicate major conformational changes in the oxidized material relative to the control. At pH 4.8, differences for the same peptides are all insignificant

corresponds to the individual peptides analyzed and the y-axis corresponds to the difference in deuterium uptake between the two protein states (control and oxidized) that are being compared at each pH condition. The grey bars are the sum of the exchange differences across all HDX time points for each peptide and can be either positive or negative. Negative values indicate that the oxidized IFNβ-1a sample exchanges more rapidly and extensively than the control, thus suggesting that the oxidized protein has a more open or flexible structure, and vice versa. The blue, red, green, purple, and yellow lines correspond to the individual difference data at a given time point (0.167, 1, 10, 60, and 240 min, respectively). For a protein sample to exhibit a statistically significant difference between the two states being compared, the following criteria must be met. At least one time point (along the blue, red, green, purple, and yellow lines) must fall outside of the ±0.5 Da threshold (grey dotted lines) for a specific peptide. In addition, the corresponding sum of differences (i.e., the black vertical bars) for that same peptide must also exceed the ±1.1 Da threshold (black dashed lines) [18].

The top HDX-MS difference plot shown in Figure 4 indicates that the changes in deuterium uptake induced by

oxidation of IFNβ-1a at pH 4.8 are negligible. This conclusion is further supported by the same result obtained when the sample oxidized at pH 4.8 was dialyzed into the pH 7.2 buffer before HDX analysis (Figure 4, middle). This data negates the need to run HDX for longer D_2O incubation periods at the lower pH to alleviate concerns about the pH effect on the intrinsic rate of HDX, and proves that oxidation at pH 4.8 does not alter the HDX dynamics of IFNβ-1a. In comparison, however, oxidized IFNβ-1a at pH 7.2 undergoes a significant change in conformational dynamics compared with the control as clearly indicated in the bottom HDX-MS difference plot shown in Figure 4. In both oxidized samples (one at pH 4.8 and one at pH 7.2) the same methionine residues are oxidized effectively to the same extent (see Table 1). The only significant difference in methionine oxidation is the increase in the oxidation level of Met_1 from 29% at pH 4.8 to 65% at pH 7.2 (see Table 1). Therefore, the structural differences revealed by the changes in HDX at pH 7.2 must be solely attributed to the oxidation of the free sulfhydryl of Cys_{17} that exhibited an increase in oxidation level from 0% at pH 4.8 to 51% at pH 7.2. We reached this conclusion irrespective of the noted change in the oxidation level of Met_1. In fact, the absence of any significant HDX differences observed when IFNβ-1a is oxidized at pH 4.8 and kept in the same buffer or buffer-exchanged into pH 7.2 suggests that even when Met_1 was already 29% oxidized, no significant changes in HDX were detected. Consequently, without observing an effect on HDX caused by an oxidation level of 29% for Met_1, we would be hard-pressed to invoke that any changes would be caused by an increase in the level of Met_1 oxidation to 65%.

Significance of the Present IFNβ-1a Oxidation Results Relative to Earlier Studies

In our previous work, the oxidation of IFNβ-1a, performed only at pH 7.2, induced an observable SEC RT shift and significant conformational changes in the protein structure as indicated by the large changes in HDX. These changes were attributed to the oxidation of the protein's methionine and/or its only free cysteine residues [18]. In addition, we demonstrated that alkylation of IFNβ-1a with *N*-ethylmaleimide (NEM) at pH 7.2, which only targets its sole free sulfhydryl group (Cys_{17}), also induces the same SEC RT shift and fairly similar changes in HDX compared with the oxidation work [4]. At the time, these two observations provided a preliminary indication that at pH 7.2, the oxidation of the free sulfhydryl (on Cys_{17}) is also the likely cause for the observed change in HDX and SEC RT shift, not the oxidation of the methionine residues.

In the present work, LC-MS peptide mapping results revealed that the free sulfhydryl group on Cys_{17} of IFNβ-1a, which is oxidized in the presence of H_2O_2 at neutral pH (7.2) [18], does not get oxidized under mildly acidic conditions (pH 4.8). Nevertheless, at both pH conditions, the same three out of the total four methionine residues in IFNβ-1a (Met_1, Met_{36}, and Met_{117}) are oxidized, with two of the methionine residues (Met_{36} and Met_{117}) being nearly completely oxidized at both pH conditions, while Met_1 oxidation is reduced from 65% at pH 7.2 to 29% at pH 4.8. SEC characterization of IFNβ-1a oxidized at pH 4.8 showed that this material still displays the same SEC RT shift (change in biophysical properties), which was previously reported when IFNβ-1a was oxidized at pH 7.2 [18]. Consequently, we conclude that the SEC RT shift can be solely induced by the oxidation of methionine residues in IFNβ-1a. In addition, our current HDX-MS results, which compare the control and oxidized IFNβ-1a at pH 4.8, show no significant changes in deuterium uptake. Therefore, the observed SEC RT shift appears to be caused by a surface chemistry alteration on IFNβ-1a, rather than to a conformational change that increases the protein's hydrodynamic volume. The surface chemistry change imparted to IFNβ-1a upon oxidation of Met_1, Met_{36}, and/or Met_{117} residues must somehow interfere with weak secondary chemical interactions that existed between the surface of control IFNβ-1a and that of the SEC chromatography material. Consequently, the minor additional retention observed for the control IFNβ-1a material beyond that achieved through classic steric SEC chromatography can be attributed to the weak secondary surface chemistry interaction described above. Furthermore, because the SEC RT shift observed in IFNβ-1a at both pH values appears to be nearly quantitative and highly similar, we postulate that the methionine residues responsible for the shift must also undergo the same complete oxidation. The only methionine residues that approach quantitative oxidation levels at both pH conditions are Met_{36} and Met_{117}. Consequently, it would appear that one or both of these residues are likely responsible for the SEC RT shift. Nevertheless, we cannot overrule the possibility that at pH 7.2, the oxidation of Cys_{17} could also on its own facilitate the observed SEC RT shift, especially given the results that we have previously reported [4] concerning the specific alkylation effect of this one amino acid.

Conclusion

From a conformation and/or conformational dynamics point of view, we conclude from our HDX-MS work that oxidation of the sulfhydryl group of Cys_{17}, which occurs when IFNβ-1a is incubated in H_2O_2 at pH 7.2, is the key factor for the changes observed in this protein's HOS. This perturbation in HOS, which appears to be widespread over a large portion of IFNβ-1a's structure, is relatively small in amplitude (in terms of actual change in physical size), given the inability to detect significant changes in IFNβ-1a's hydrodynamic volume via classic biophysical size/shape techniques such as standard boundary sedimentation velocity AUC. Similarly, it should be noted that an earlier AUC experiment was also unable to reliably detect a change in IFNβ-1a's hydrodynamic volume (only a 0.6% difference in sedimentation coefficient between control and alkylated IFNβ-1a) when a similar HOS perturbation in IFNβ-1a was observed (by HDX-MS) upon specific NEM alkylation of the same sulfhydryl group of Cys_{17} [4].

Results presented in this paper also allow us to conclude the following: (1) multiple orthogonal techniques are needed to assess changes in biopharmaceuticals, especially biophysical changes, (2) high-resolution MS-based techniques play an important role in the elucidation of minor changes in protein conformation that are otherwise invisible to other biophysical tools, and (3) changes in the biophysical properties of biopharmaceuticals can manifest themselves not only by conformational changes resulting from primary structural changes but also by simple surface chemistry changes that lead to no significant conformational changes, as demonstrated here in the case IFNβ-1a methionine oxidation at pH 4.8.

Furthermore, even when the same key structural element in a biopharmaceutical is perturbed (in this case Cys_{17}), and the resulting structural changes are somewhat similar, subtle variations can account for striking differences in the protein function. This can be realized here by comparing the HDX-MS results of oxidized (at pH 7.2) and alkylated Cys_{17} of IFNβ1-a. In this comparison, an initial coarse review of the observed HDX-MS data reveals somewhat similar alterations in the protein's HOS (see Supplemental Figure S1). However, while INFβ-1a oxidized at pH 7.2 had fairly comparable biological activity to the control, NEM alkylation of the same free Cys_{17} of IFNβ-1a showed significant reduction in biological activity compared with the control [4]. The importance of such subtle changes in the biophysical properties of biopharmaceuticals makes the task of determining what is (and is not) a critical quality attribute (CQA) for this class of drugs a very demanding activity to insure their efficacy, stability, and safety.

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