NOTE

Technical Decision Making with Higher Order Structure Data: Utilization of Differential Scanning Calorimetry to Elucidate Critical Protein Structural Changes Resulting from Oxidation

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ABSTRACT: Differential scanning calorimetry (DSC) is a useful tool for monitoring thermal stability of the molecular conformation of proteins. Here, we present an example of the sensitivity of DSC to changes in stability arising from a common chemical degradation pathway, oxidation. This Note is part of a series of industry case studies demonstrating the application of higher order structure data for technical decision making. For this study, six protein products from three structural classes were evaluated at multiple levels of oxidation. For each protein, the melting temperature (T_m) decreased linearly as a function of oxidation; however, differences in the rate of change in T_m, as well as differences in domain T_m stability were observed across and within structural classes. For one protein, analysis of the impact of oxidation on protein function was also performed. For this protein, DSC was shown to be a leading indicator of decreased antigen binding suggesting a subtle conformation change may be underway that can be detected using DSC prior to any observable impact on product potency. Detectable changes in oxidized methionine by mass spectrometry (MS) occurred at oxidation levels below those with a detectable conformational or functional impact. Therefore, by using MS, DSC, and relative potency methods in concert, the intricate relationship between a primary structural modification, changes in conformational stability, and functional impact can be elucidated.
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INTRODUCTION

Differential scanning calorimetry (DSC) is commonly applied in the biopharmaceutical industry to characterize protein thermal stability, overall conformation, and domain folding integrity.[1–4] The method measures heat capacity as a function of temperature, and protein unfolding transitions and/or association events are detected as changes in heat capacity. Characteristics in the DSC thermogram, such as the transition midpoint (also known as the melting temperature, T_m), can be used to assess the thermal stability of the protein under various conditions.

A common application of DSC in the biopharmaceutical industry is during formulation development, where the assay has been shown to be sensitive to changes in protein stability induced by the presence or absence of formulation excipients. In addition to formulation development, DSC has been increasingly utilized during later stages of process and product development, including for process characterization, product characterization, and product comparability evaluation.[5] In this Note, we provide one example of the sensitivity of the DSC method to a potential process-induced protein modification, oxidation, and its applicability to a comprehensive product characterization data package to support product registration. We first review the sensitivity of DSC to protein oxidation generally, across multiple protein structural classes, and then show how this chemical modification can be linked to a conformational change and subsequently to functional changes for one protein. This case study provides an example application of higher order structure (HOS) characterization, such as DSC analysis, for technical decision making in the biopharmaceutical industry and is part of a series of related articles.

MATERIALS AND METHODS

Forced Oxidation and Methionine Oxidation Determination

Six protein products representing three different structural classes, two cytokines (referred to as cytokine A and B), two Fc fusion proteins (fusion protein A and B), and two IgG2 antibodies (IgG2 A and B), were forcibly oxidized by exposure to hydrogen peroxide (Fluka 95321). All proteins were produced at Amgen Inc. (Thousand Oaks, California) and were at least 98% pure as determined by size-exclusion chromatography. Hydrogen peroxide was selected because it is a nonspecific oxidizer capable of targeting both exposed and buried regions of the protein. For each of the six proteins, the hydrogen peroxide exposure (time and concentration) was varied in order to achieve sample sets with varying levels of oxidized methionine ranging from low, less than 6%, to high, greater than 72% oxidized methionine. Samples were incubated at room temperature, protected from light, and quenched by the addition of free methionine (Sigma M5308). Because of the susceptibility of methionine to oxidation,[6–8] the extent of methionine oxidation, level of methionine sulfoxide (MetOx), was quantified for each sample and used as a marker for oxidative damage. Experimental MetOx detection parameters for each protein are provided in Table 1. Briefly, the oxidized samples and nonoxidized control were enzymatically digested, and the resulting peptides were separated by reversed-phase HPLC (RP-HPLC) with either UV or electrospray ionization–mass spectrometry

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Table 1. Experimental Conditions for Methionine Oxidation Detection Using RP-HPLC Separation

Protein	Predigestion Treatment	Digestion Enzyme	Detection
Cytokine A	Reduced (DTT)	Glu-C	UV (214 nm)
Cytokine B	–	Trypsin	UV (280 nm)
Fusion protein A	–	Lys-C	MS
Fusion protein B	Reduced (DTT) Alkylated (IAM)	Lys-C	UV (215 nm)
IgG2 A	Denatured (Gdn-HCl + EDTA) Reduced (TCEP) Alkylated (IAM)	Trypsin	MS–SIM
IgG2 B	Denatured (Gdn-HCl + EDTA) Reduced (TCEP) Alkylated (IAM)	Trypsin	MS-SIM

SIM, select ion monitoring.

(MS) detection. Note, oxidation of other amino acids, such as tryptophan, tyrosine, phenylalanine, histidine, or cysteine, can also occur under high oxidative stress[8]; however, under the conditions employed in this study (pH at or below neutral for all proteins), oxidation of amino acids other than methionine was not observed.

Differential Scanning Calorimetry

Differential scanning calorimetry analysis was performed using a Capillary VP-DSC (MicroCal, Northampton, Massachusetts) with a scan rate of 1°C/min and no feedback. Cytokine A was analyzed at a concentration of approximately 1.0 mg/mL. All other samples were analyzed at a concentration of approximately 0.5 mg/mL. The DSC profiles were analyzed using MicroCal Origin v. 7.0 software (MicroCal, Northampton, Massachusetts). All thermograms were baseline corrected and normalized to the moles of protein loaded. For well-resolved transitions, the T_m was defined as the apex of the transition as determined by peak integration. The only exceptions were for IgG2 A and IgG2 B under low-oxidation conditions where one of the Fc domain transitions (CH3 for IgG2 A and CH2 for IgG2 B) was not resolved from the Fab domain unfolding and appeared as a shoulder on the front of the Fab transition (Fig. 1). For these samples, the data were fit using a Non-2-State model in Origin with three transitions. For this study, only changes in T_m were assessed; therefore, the reversibility and energetics of the unfolding events (e.g., total enthalpy of the transition) were not evaluated.

Near-Ultraviolet Circular Dichroism

Near-ultraviolet circular dichroism (UV CD) spectra from 340 to 250 nm were collected using a J-715 spectropolarimeter (Jasco Analytical Instruments, Easton, Maryland) in a 1-cm pathlength quartz cell with a scanning speed of 20 nm/min, 0.1 nm resolution, 4 s response time, and bandwidth of 1 nm. A total of eight accumulations per sample were collected and averaged. All samples were analyzed at a concentration of approximately 0.5 mg/mL, and the protein concentration was confirmed by measurement of the absorbance at 280 nm. Data analysis was carried out using Jasco Spectra Manager v. 1.53.08 software (Jasco Analytical Instruments, Easton, Maryland). A reference spectrum of formulation buffer was collected on the day of analysis and subtracted from each protein spectrum. The reference-subtracted protein spectra were normalized to mean residue ellipticity.

Intrinsic Fluorescence

Fluorescence spectra were collected using a QM4 spectrofluorometer (Photon Technologies International, Edison, New Jersey). Samples were analyzed at a concentration of approximately 0.2 mg/mL. Data analysis was conducted using FeliX32 version 1.2 build 56 software (Photon Technologies International, Edison, New Jersey). A reference spectrum of formulation buffer was collected the day of analysis and subtracted from each protein spectrum. The resulting reference-subtracted protein fluorescence spectra were smoothed using seven-point smoothing. The final spectra were intensity normalized to the emission peak maxima.

Potency by Homogeneous Time-Resolved Fluorescence

Homogeneous time-resolved fluorescence (HTRF) was used to determine the ability of IgG2 B to prevent IgG2 B antigen from binding to its receptor. In this inhibition assay, the antigen was labeled with Europium^{3+} chelate(E^{3+}) and the receptor was a soluble fusion protein with a FLAG peptide and Fc moiety secondarily labeled with an anti-FLAG antibody conjugated to an allophycocyanin (APC) fluorochrome (Prozyme PJ255). Binding of E^{3+} antigen to the receptor brings the E^{3+} into molecular proximity of the APC and allows fluorescent resonance energy transfer, which was detected by an Envision HTRF analyzer (PerkinElmer, Waltham, Massachusetts). The amount of IgG2 B present in solution is inversely related to the fluorescence intensity. Test sample activity was determined by comparing test sample fluorescence to an IgG2 B control.

RESULTS and DISCUSSION

Oxidation of amino acid side chains is a major degradation pathway for many protein therapeutics.[9–11] Exposure to reactive oxygen species can occur throughout the product life cycle, including during cell culture, purification, formulation, fill-finish operations, and storage of the final product. As a result of oxidation, product efficacy and stability can be compromised for some proteins.[11,12] The amino acid most susceptible to oxidation is methionine (M or Met), and methionine sulfoxide is the most commonly produced oxidation product.[6] Compared with methionine, methionine sulfoxide is larger, less flexible, more polar, and less hydrophobic.[11] Consequently, the conversion of methionine to methionine sulfoxide may impact the HOS of some proteins, which in turn can lead to a change in function, for example, reduced binding to another protein that confers a specific activity or impacts a clearance mechanism.[8,13,14] Oxidation of other amino acids (such as aromatic side chains or free cysteine) can occur in a lesser extent and can also affect protein conformation.[8,15–17] For the samples included in this evaluation, oxidation of amino acids other than methionine was evaluated but not detected (data not shown). Therefore, throughout this study, protein oxidation is expressed in percent oxidized methionine (MetOx), which is a sensitive marker of oxidation, and was used as a representative for the general oxidation status of the protein.[8]