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]

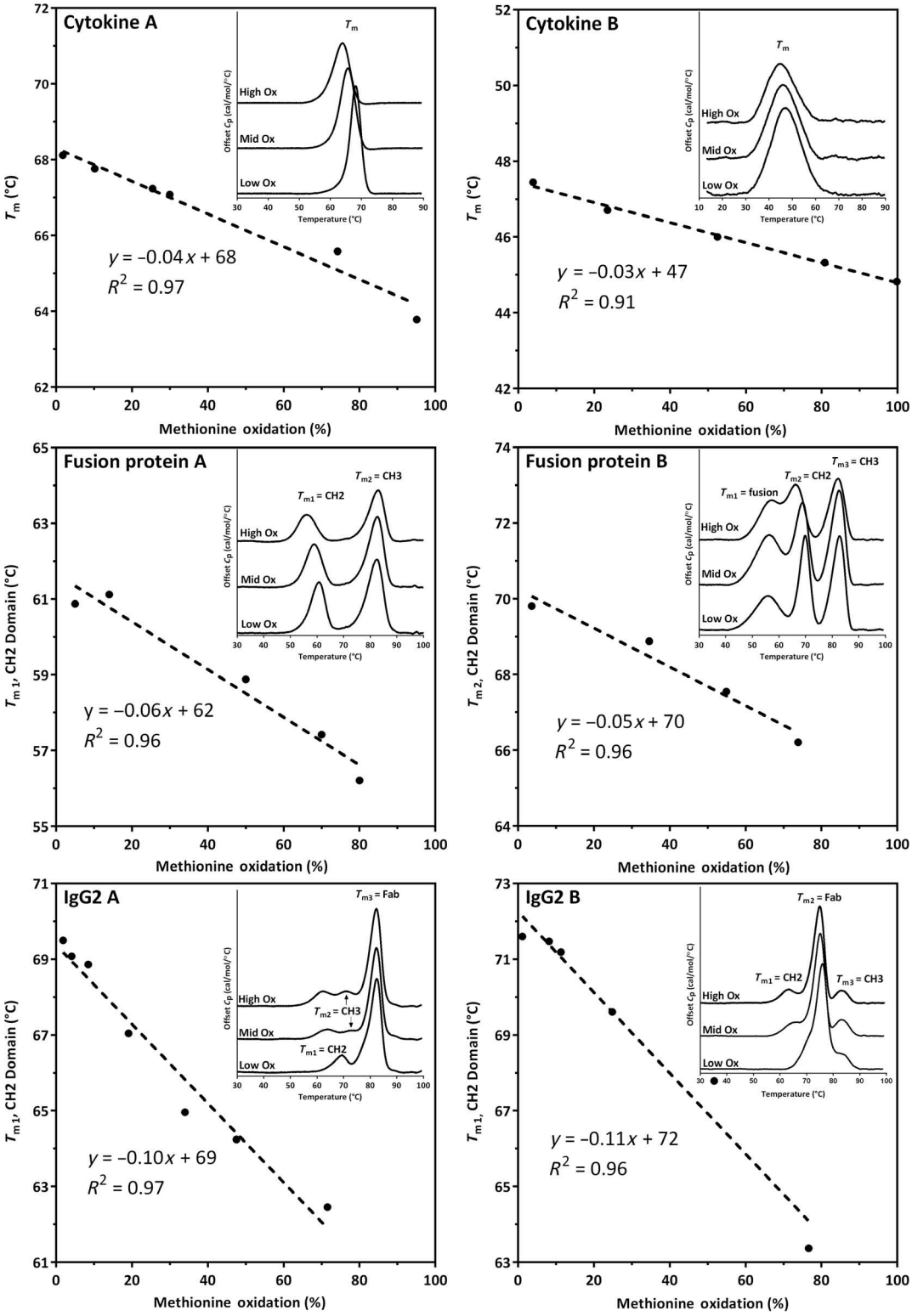

Figure 1. Differential scanning calorimetry response as a function of increased methionine oxidation. For each protein, a single solvent-exposed methionine was selected to represent the percent methionine oxidation. For the cytokines and Fc fusion proteins, the methionine most susceptible to oxidation was used. For the antibodies, M35X located in the CH2 domain, was used. Dashed lines represent the linear regression of change in T_m with respect to methionine oxidation. The equation for the best-fit line and the coefficient of determination (R^2) are provided for each protein. For each protein, three example thermograms from the larger data set, representing a low-, middle-, and high-level of oxidized methionine, are provided in the insets. For IgG2 A, the CH3 domain appears as a shoulder on the Fab domain transition under low oxidation conditions. With increasing extent of oxidation, the CH3 domain T_m decreases and the domain becomes resolved from the Fab transition. The same phenomenon occurs for the CH2 domain of IgG2 B.

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Representative DSC thermograms for the six proteins at three levels of oxidation, representing low (≤6% MetOx), middle (35%–52% MetOx), and high (72%–100% MetOx) levels of oxidation, are displayed in Figure 1. For each protein, a single solvent-exposed methionine was selected to represent the percent MetOx. Explanation of which methionine was selected, and why, is provided later in the discussion. For each of the six proteins, more than three oxidation conditions were evaluated; however, because the oxidation level increments differed across the six proteins, only three representative thermograms at similar MetOx levels for each protein were plotted for clarity. Although the three protein classes evaluated are structurally diverse, with varying numbers of methionines in their amino acid sequences, for all six proteins a change in protein stability, as evidenced by a decrease in the T_m for at least one domain, was observed with increasing levels of oxidation. For multidomain products, antibodies and Fc fusion proteins, some domains were found to be more susceptible to oxidation than others. Specifically, for all four Fc containing proteins, the CH2 domain was the most sensitive to reactive oxygen exposure, as evidenced by the greatest change in T_m between the low- and high-oxidized samples (Fig. 1). These results are in agreement with previous studies, which showed the destabilization of the CH2 domain as a result of methionine oxidation.[13,14] Note, for all samples, the aggregate content as a function of increased oxidation was also monitored by both sedimentation velocity analytical ultracentrifugation and size-exclusion HPLC (data not shown). Under the conditions employed in this study, no increase in aggregation as a function of increased oxidation was observed for any of the six proteins.

Interestingly, although the CH2 domain was the most susceptible to oxidation for all the Fc-containing proteins, other protein-specific differences were observed by DSC. For IgG2 A, a decrease in the CH3 domain T_m was observed as a function of oxidation, whereas the Fab domain remained unaffected. Conversely, for IgG2 B, the reverse was observed. Similarly, under highly oxidized conditions, the CH3 domain T_m of fusion protein B was decreased, whereas the CH3 domain T_m of fusion protein B was unaffected. The data presented here support that DSC thermograms, specifically changes in T_m, provide insight into changes in thermal stability of folded protein domains resulting from oxidation, uniquely for specific products as well as generally for classes of molecules. Such information can aid in evaluating the criticality of this product quality attribute.

To quantify the sensitivity of DSC to protein oxidation, the rate of change in T_m as a function of methionine oxidation was determined for each of the six proteins evaluated in Figure 1. For each protein, the T_m for the domain most sensitive to oxidation is plotted on the ordinate. For the cytokines and Fc fusion proteins, the abscissa is defined as the oxidation level measured for the methionine most susceptible to oxidation. For the antibodies, M35X ("X" is used as a placeholder as the exact amino acid position varies slightly from one antibody to another), located in the CH2 domain, was selected, as the CH2 T_m response was linear with respect to M35X oxidation for both antibodies. M25X[2], located on the CH2 domain near the hinge region, was the most susceptible methionine to oxidation; however, the response of IgG2 B with respect to M25X was not linear (data not shown, $R^2 = 0.78$).

For all six proteins, the T_m of the domain most susceptible to oxidation decreased linearly with increasing oxidation, suggesting the DSC obtained T_m can be used to easily and effectively monitor higher order structural changes as a consequence of oxidation (Fig. 1). Interestingly, even though the same domain, CH2, was used for both the antibodies and Fc fusion proteins, the sensitivity to oxidation was more similar within a structural class than across structural classes. Of the six proteins evaluated, the antibodies showed the greatest changes in thermal stability upon oxidation, with a response of approximately −0.1°C/% Ox. The precision of the DSC method for T_m measurement of the CH2 domain is approximately ±0.3°C (data not shown). Assuming differences greater than two standard deviations represent detectable changes in stability by DSC, the method is capable of detecting oxidation-induced structural changes for antibody samples with a 6% increase in M35X oxidation. Similarly, for the Fc fusion proteins, DSC can detect changes as a consequence of oxidation with 10%–12% increase in oxidation (calculation assumes the precision of the measurement for fusion protein Fc domains is ±0.3°C, data not shown), and a 20%–27% increase in oxidation for the cytokines evaluated (calculation assumes precision of the measurement for cytokines is ±0.4°C, data not shown).

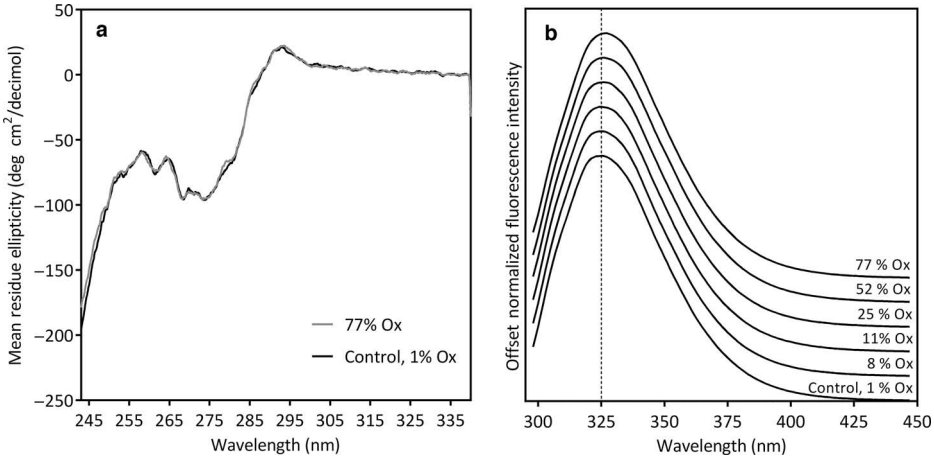

Figure 2. (a) Overlaid near-UV CD spectra for IgG2 B nonoxidized control (black line, ~1% MetOx) and the most highly oxidized sample (gray line, ~77% MetOx). (b) Offset overlaid intrinsic fluorescence spectra for IgG2 B. From bottom to top, nonoxidized control, 8%, 11%, 25%, 52%, and 77% MetOx. Dotted vertical line represents wavelength of maximum intensity for the nonoxidized control.

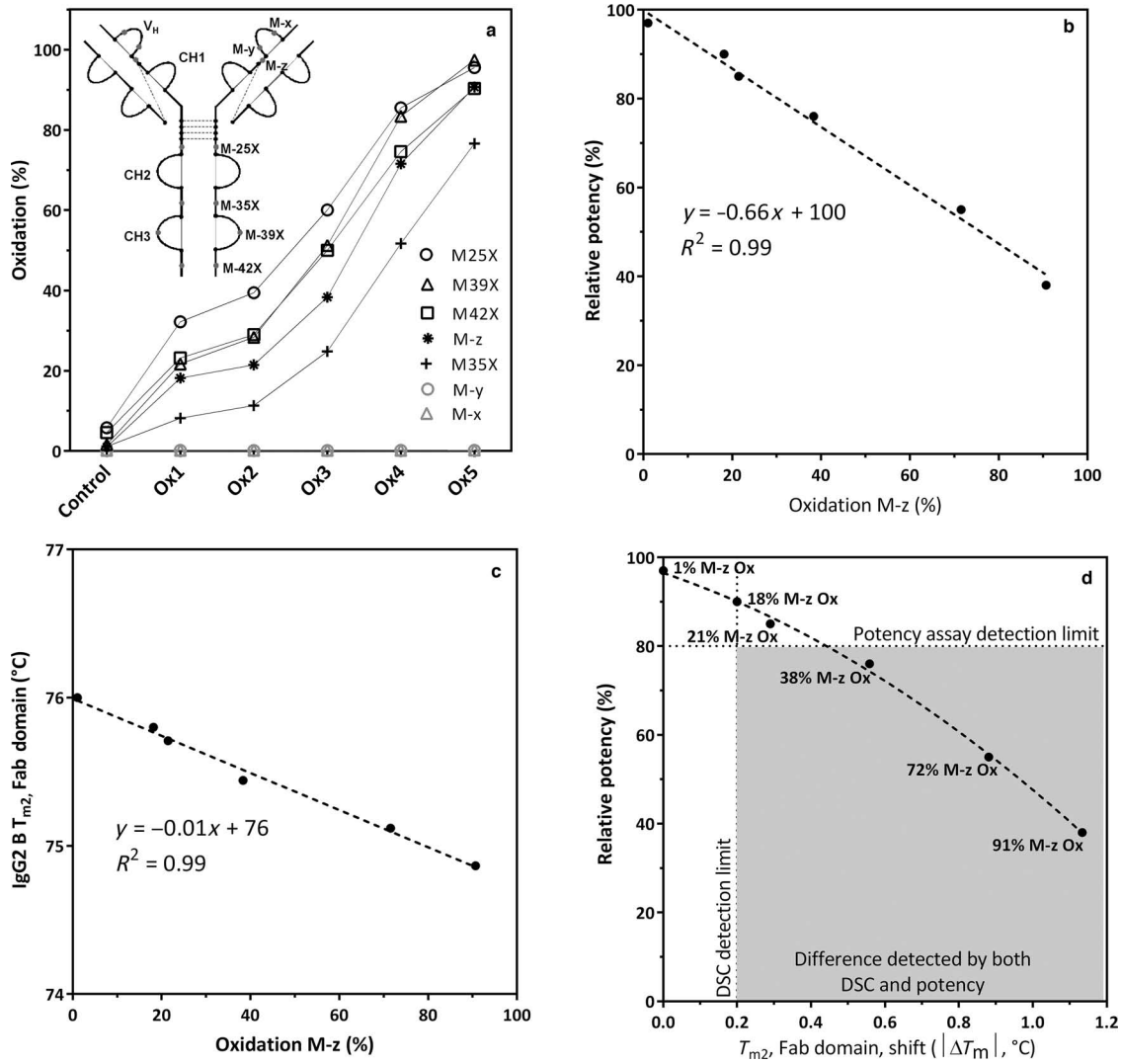

Figure 3. (a) Measured percent oxidized methionine by LC/MS–select ion monitoring for the seven IgG2 B heavy chain methionines across five different samples and one nonoxidized control. (b) IgG2 B HTRF relative potency as a function of M-z oxidation. Dashed line represents the linear regression of change in potency with respect to methionine oxidation with the expression for the best-fit line and R^2 provided. (c) IgG2 B Fab domain T_m measured by DSC as a function of M-z oxidation. Dashed line represents the linear regression of change in Fab T_m with respect to methionine oxidation with the expression for the best-fit line and R^2 provided. (d) IgG2 B relative potency as a function of oxidation-induced shift in Fab T_m. Dotted lines represent assay detection thresholds for differences. Dashed line represents the best-fit nonlinear regression (second-order polynomial). Shaded gray region represents conditions under which an oxidation-induced change is observed by both DSC and HTRF.

Additional studies were carried out using IgG2 B to further understand the sensitivity of DSC to protein oxidation, as well as determine the relationship between changes in the primary sequence (MetOx), observed conformational changes, and functional impact. To assess the sensitivity of DSC to oxidation compared with orthogonal methods sensitive to changes in tertiary structure, the IgG2 B oxidized samples were analyzed by both near-UV CD spectroscopy and intrinsic fluorescence spectroscopy. Secondary structure of the oxidized samples was also evaluated by Fourier transform infrared spectroscopy. No detectable difference in secondary structure as a result of oxidation was observed. At the highest level of IgG2 B oxidation included in this study (77% MetOx), near-UV CD did not detect a difference between the oxidized sample and the control (Fig. 2a). Spectral similarity was quantitatively evaluated using a weighted spectral difference calculation.[18] By intrinsic fluorescence spectroscopy, the spectral profiles were highly similar at each level of oxidation, with a slight but detectable red shift in the maximum wavelength intensity (2 nm) for the sample with the highest level of oxidation (Fig. 2b). Although fluorescence spectroscopy confirmed that a conformational change as a result of oxidation had occurred, these differences in method response to oxidation highlight the differences in method capabilities between fluorescence spectroscopy and DSC. Furthermore, although orthogonal, the two methods monitor different properties of the molecule. In this case, DSC was shown to be more sensitive and appropriate for monitoring oxidation-induced conformational changes compared with near-UV CD and fluorescence spectroscopy. This observation underscores the importance of employing a well-designed and

thorough characterization plan to elucidate the critical quality attributes of a product and demonstrate how those attributes may be monitored during production and storage.

Finally, to evaluate the impact of oxidation on relative *in vitro* potency, the IgG2 B oxidized samples were analyzed by a noncell-based HTRF competitive binding assay. For the antibodies (IgG2 A and IgG2B), in addition to oxidation of methionines in the Fc, the primary structure analysis found one methionine located in the Fab domain, M-z (the three methionines located in the variable region of the antibody are referred to as M-x, M-y, and M-z), was oxidized, whereas the other two were protected. Oxidation levels for all methionines in IgG2 B are provided in Figure 3a. The HTRF competitive binding assay showed a linear decrease in relative potency with respect to increased M-z oxidation (Fig. 3b). Similarly, by DSC, the Fab domain T_m declined linearly with respect to M-z oxidation (Fig. 3c). As the HTRF assay monitors binding of the antigen to the Fab domain, for each oxidized sample, the HTRF relative binding was plotted as a function of the shift in Fab domain T_m compared with the nonoxidized control (Fig. 3d). The curvature of the line in Figure 3d (flatter slope at low oxidation levels that becomes steeper with increased oxidation), implies that a conformational change, as evidenced by a shift in the T_m, precedes the loss in binding (decrease in relative potency). In contrast, a linear relationship would indicate that the conformational changes and loss in binding occur concurrently. Statistical analysis confirmed that the relationship between the Fab domain T_m shift and relative potency decrease was nonlinear ($p = 0.02$). These results support the conclusion that DSC can be a leading indicator of decreased antigen binding, capable of detecting oxidation-induced changes just prior to seeing an impact on product potency.

CONCLUSIONS

Oxidation of amino acid side chains is a major degradation pathway for protein therapeutics. As a result of oxidation, product efficacy and stability can be compromised; therefore, careful method selection should be applied to aid in evaluating this quality attribute. Here, we showed the sensitivity of DSC to oxidation-induced conformational stability changes across multiple protein classes. For all six proteins evaluated, the T_m determined by DSC declined linearly with increasing MetOx levels. In comparison, orthogonal spectroscopic structural characterization methods, near-UV CD and fluorescence spectroscopy, were far less sensitive to oxidation-induced conformational changes, suggesting the methods have different capabilities and monitor different properties of the molecule. Therefore, for monitoring protein oxidation, DSC is a more appropriate structural characterization method compared with these spectroscopic methods. Furthermore, for IgG2 B, detected changes in T_m by DSC, were found to precede a loss in relative potency demonstrating that DSC is a leading indicator of decreased antigen binding. These results suggest that the T_m measured by DSC can be a useful and predictive tool for elucidating the impact of chemical changes detected by mass spectrometry (or other primary structure analysis) on protein HOS and relating conformational changes to potential functional impact. Finally, it is possible that further information regarding protein conformational stability can be obtained by studying additional characteristics of the DSC thermogram, such as the enthalpy of unfolding. However, the results presented here support the conclusion that monitoring changes in T_m by DSC provides a simple, measurable, linear, and meaningful response.

In conclusion, this case study provides an example of the utility of DSC analysis during process and product development and characterization. We show how an understanding of protein HOS can be used to elucidate the critical structural attributes that change following a chemical modification. Furthermore, the same HOS methods can be used for non-routine characterization testing to monitor product quality attributes during production and storage.

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