Cysteinylation of a monoclonal antibody leads to its inactivation

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Abbreviations: HIC, Hydrophobic Interaction Chromatography; ESI-MS, Electrospray Ionization Mass Spectrometry; mAb, monoclonal antibody

ABSTRACT

Post-translational modifications can have a significant effect on antibody stability. A comprehensive approach is often required to best understand the underlying reasons the modification affects the antibody’s potency or aggregation state. Monoclonal antibody 001 displayed significant variation in terms of potency, as defined by surface plasmon resonance testing (Biacore), from lot to lot independent of any observable aggregation or degradation, suggesting that a post-translational modification could be driving this variability. Analysis of different antibody lots using analytical hydrophobic interaction chromatography (HIC) uncovered multiple peaks of varying size. Electrospray ionization mass spectrometry (ESI-MS) indicated that the antibody contained a cysteinylation post-translational modification in complementarity-determining region (CDR) 3 of the antibody light chain. Fractionation of the antibody by HIC followed by ESI-MS and Biacore showed that the different peaks were antibody containing zero, one, or two cysteinylation modifications, and that the modification interferes with the ability of the modified antibody arm to bind antigen. Molecular modeling of the modified region shows that this oxidation of an unpaired cysteine in the antibody CDR would block a potential antigen binding pocket, suggesting an inhibition mechanism.

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Introduction

Monoclonal antibodies, with their powerful combination of long-term stability and high ligand specificity, have proven to be effective tools for use by both the pharmaceutical and diagnostic assay industries. This is highlighted by the number of antibody therapeutics already on the market or currently in clinical studies. However, despite the obvious benefits of using antibodies, these molecules are susceptible to aggregation, degradation, and post-translational modifications that can negatively affect antibody half-life or activity. Thus, it is important to have the capability to monitor antibodies for these types of changes, as well as the ability to understand their effect on stability and function.

Changes in monoclonal antibody potency are often the result of protein aggregation or degradation, which cause physical changes to the antibody that can be readily identified by commonly used methods that assess purity, e.g., size-exclusion chromatography (SEC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In these cases, relatively large (kDa range) changes in molecule size are obvious indicators. However, there are a variety of other modifications for monoclonal antibodies that are more difficult to detect. Carboxy terminal lysine processing, deamidation, oxidation, isomerization, mutation, and changes in glycosylation are examples of modifications that can be extremely small in size (Dalton range), but can have a substantial effect on how the antibody functions. To fully characterize the nature and effects of post-translational modifications, a comprehensive analytical approach combining bioanalytical and structural studies should be considered. Analytical chromatography methods such as ion exchange and hydrophobic interaction chromatography (HIC) are effective analytical tools for uncovering changes in antibody charge variance or changes in hydrophobicity that can accompany post-translational modifications. These techniques have been used to effectively reveal antibody deamidation and methionine oxidation, among other changes. Mass spectrometry (MS) techniques can then build on this information by identifying the exact nature of the modification and its location. Information regarding antibody potency, on the other hand, is usually determined by antibody-specific methods, though plasmon resonance based assays are rapidly becoming a more general and uniform antibody potency testing method. The data obtained from each of these analytical methods can then be combined with structural approaches to develop a thorough understanding of the molecular modifications and their effect.

In the study reported here, we used a variety of techniques to describe a monoclonal antibody that had undergone an atypical antibody oxidation, cysteinylation. Unlike oxidation of methionine and tryptophan residues, cysteinylation is a rarely observed phenomenon in antibodies. This is likely due to the fact that cysteine residues are typically paired to produce...
disulfide bonds that help provide the antibody with its structural integrity. However, when antibody cysteinylation has been observed, the effect on antibody function was significant. To examine the effect of this oxidation event on the stability and activity of the antibody in question, HIC chromatography, MS and plasmon resonance-based potency testing were used to show the impact of this modification on the antigen binding capabilities of the antibody, independent of antibody aggregation or degradation. Molecular modeling was then applied in order to better understand and support the analytical data, leading to an improved understanding of this post-translational modification and its effects.

Results

Mouse monoclonal IgG 001 antibody, secreted from mouse hybridoma cells in specialized hybridoma media, was purified via Protein A affinity chromatography. When various lots of this antibody were tested for relative activity via the plasmon resonance-based Biacore system, the lots showed significant variability (Fig. 1). Compared to a high activity standard, the relative activity range observed in this group of 15 antibody lots was from 36.9% to 124.7%. Antibody potency loss can often be attributed to antibody stability issues, i.e., antibody aggregation or degradation, often resulting from formulation conditions, the different variants have been successfully fractionated to near homogeneity while maintaining activity. After application to a Dionex ProPac HIC-10 column, various mAb 001 lots were eluted with a gradient from 1 M ammonium sulfate in phosphate-buffered saline (PBS) to 0 M over one hour under ambient conditions.

The appearance of multiple peaks with varying peak areas suggests the presence of one or more post-translational modifications. In an attempt to reveal more detail regarding the nature of this modification, multiple lots of mAb 001 were subjected to electrospray ionization MS (ESI-MS). An interesting observation from this analysis was the presence of a 119 Da modification seen on the light chain of the antibody at various levels (Fig. 3A). This molecular weight suggested cysteinylation, a form of oxidation not often observed with antibodies. IgG1 isotype antibodies like mAb 001 contain a total of 12 cysteine residues, all of which are involved in either inter- or intramolecular disulfide bonds. While there is evidence suggesting antibodies possess a low level of unpaired cysteines, the presence of the significant level of oxidation seen here suggests the presence of an additional cysteine residue in the molecule that is not typically present. Indeed, sequencing confirmed the presence of an additional cysteine residue (C96) in complementarity-determining region (CDR) 3 of the antibody light chain (Fig. 3B). It was unexpected that this type of oxidation event would be observed using ESI-MS, where the antibody is reduced during sample preparation under conditions that should have normally reduced this form of oxidation. Nevertheless, tryptic peptide mapping followed by MS analysis suggested that this additional cysteine was being cysteinylated (data not shown). MS/MS analysis confirmed that in addition to cysteinylation, this cysteine residue is also oxidized to either cysteine sulfenic (+32 Da) or sulfonic acid (+48 Da) (data not shown).

To more thoroughly study the HIC chromatogram peaks, the three major peaks observed, Peaks 1, 3, and 5 were fractionated and analyzed (Fig. 4). After fractionation, the peaks were greater than 90% homogeneous when reinjected onto the HIC column (Fig. 4, data not shown). When tested for relative activity by Biacore, the activity ranged from negligible (10.7%) for Peak 1, to near 50% (45.4%) for Peak 3, to high activity (76.5%)
for peak 5. When these fractions were then tested for the presence of cysteinylation by ESI-MS, Peak 1 was almost completely modified via cysteinylation (Fig. 4 insert), whereas Peak 3 appeared to have near equal amounts of modified versus unmodified light chain. This data suggests that Peak 1 represents cysteinylation of both light chains, Peak 3 the cysteinylation of one of the two light chains, and Peak 5 is the uncysteinylated form of the antibody. Though Peak 5 had little cysteinylation, the antibody exhibited evidence of extensive oxidation, judging by the size of the +32 dalton and +49 dalton ESI-MS peaks. The presence of these peaks is potentially indicative of the cysteine 96 residue not being cysteinylated, but instead oxidized to either cysteine sulfenic or sulfonic acid, respectively. It cannot be confirmed whether this oxidation was introduced during fractionation or was already present within the mAb 001 Peak 5 fraction. Interestingly, while this oxidation of Peak 5 is substantial (~67% of the total light chain), the impact on activity, unlike with cysteinylation, is relatively minor (~25% loss of activity). Peaks 2 and 4 were also fractionated. ESI-MS testing of these two peaks indicates that Peak 2 represents antibody with little to no cysteinylation, but significant light chain oxidation, with a majority of the light chain shifted +32 Daltons (data not shown). The antibody from Peak 4 also appears to possess negligible cysteinylation, but has significant light chain oxidation, but not to the extent observed with the Peak 2 fraction (data not shown).

The occurrence of oxidation in the reduced ESI-MS samples suggests that the modification may be partially or totally

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**Figure 2.** mAb 001 lots show varying profiles when tested by analytical HIC. Three lots of mAb 001 are shown: 159194, 159218, and 159231. These lots show distinct differences when run on an analytical HIC column. However, when the chromatograms are overlaid (Overlay), a consistent pattern of five peaks are observed, with peak height varying from antibody lot to lot.

**Figure 3.** A. mAb 001 lots show varying amounts of a 119 Da modification that suggests cysteinylation. Three lots of mAb 001 were tested on ESI-MS. The light chains of the three lots show different amounts of a 118-119 Da modification. This is consistent with antibody cysteinylation. B. mAb 001 contains a cysteine residue at position 96 of light chain CDR3. The 108 amino acid light chain variable domain contains CDR1 at positions 24 to 34, CDR2 at positions 50 to 56, and CDR3 containing Cysteine 96, at positions 89 to 97.

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**Table 1.** The chromatogram peaks shown in Fig. 2 were analyzed and quantified. The highest percentage peak is in bold.

| Peak No. | Peak 159218 | Peak 159194 | Peak 159231 |
|---------|-------------|-------------|-------------|
| 1       | 42.25       | 11.95       | 0.74        |
| 2       | 27.62       | 15.50       | 1.92        |
| 3       | 21.76       | 33.92       | 13.40       |
| 4       | 5.86        | 17.14       | 18.03       |
| 5       | 2.51        | 21.48       | 65.90       |

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The occurrence of oxidation in the reduced ESI-MS samples suggests that the modification may be partially or totally.
solvent inaccessible. As shown previously, antibody cysteinyla-
tion can be partially reversed under mild reduction conditions,
at least in some situations. To test whether the cysteinylation
of mAb 001 could be reversed, the highly-modi-
fi
ed, low-activity mAb 001 antibody lot 159218 was exposed to mild reducing
conditions (50 mM cysteine in PBS, pH 8.0 for 90 minutes at
ambient temperature). SEC and Biacore testing indicated that
mock treatment of the mAb 001 lot (no cysteine addition) did
not affect its aggregation levels or activity (data not shown). As
seen in Fig. 5, the reducing conditions did partially reverse the
total oxidation compared to the mock-treated sample. The
amount of Peak 1, representing the antibody population in
which both light chains are cysteinylated, is decreased in the
reduced sample by $\sim$20%, while Peak 2 is decreased by greater
than 40% (Table 2). When mild denaturing conditions (1 M
guanidine) are included with the sample reduction, the extent
of both cysteinylation and non-cysteinylation oxidation rever-
sal was significantly increased compared to reduction alone.
Both Peaks 1 and 2 were reduced by greater than 60%, while
the amount of non-cysteinylated sample (Peak 5) was increased
greater than 13-fold over the control sample, compared to a
5.6-fold increase for the reduced sample alone (Table 2). These
data suggest that the cysteine 96 residue is only partially solvent
accessible and potentially explains why the oxidation event was
not reversed during ESI-MS sample preparation.

To further explore the possibility that the cysteine 96 residue
is partially buried, structural analysis was employed. While X-
ray crystallography may be considered the optimal approach in

Figure 4, mAb 001 fractionation by oxidation state leads to isolation of non-cysteinylated, singly- and doubly- cysteinylated species that show significant differences in activity. Various lots of mAb 001 were fractionated by HIC chromatography. The peaks labeled Peak 1 (P1), Peak 3 (P3), and Peak 5 (P5) were isolated from these various antibody lots, depending on the lot and the amount of the particular peak present. Lot 159251B is shown for peak comparison purposes. Representative chromatograms of the three fractionated peaks are shown. These fractionated lots were tested for potency by Biacore testing and for the presence of the 119 Da modification by ESI-MS.
examining the effect of post-translational modifications on antibody structure, these studies can be extremely work intensive and time consuming. An alternative approach is molecular modeling. This method has been shown to be effective in describing antibody CDR structures. Thus, modeling of the antibody 001 CDR domains was employed. As seen in Fig. 6, the molecular model indicates that the cysteine 96 residue is found at the bottom of a structural pocket in the antibody light chain CDR 3 domain. This pocket appears essential for antigen binding, for cysteinylation of the cysteine 96 residue, which appears to block this pocket, impedes ligand binding as indicated from the Biacore and ESI-MS results of the fully cysteinylated antibody fraction.

**Discussion**

The pharmaceutical industry is focused on the development of monoclonal antibodies, as well as antibody fragments and single variable domains, as injectable drugs. It is thus absolutely critical to thoroughly characterize the molecules under evaluation and understand underlying stability issues that may compromise antibody function. This evaluation should begin with the amino acid sequence of the molecule, where the presence of reactive amino acids, i.e., cysteines, asparagines, and lysines are noted. These residues can be particularly concerning when located in the CDRs of the antibody, where their solvent accessibility make them prime candidates for post-translational modification. However, neither the presence of these amino acids, nor the observation of post-translational modifications to the molecule necessarily means that the antibody’s stability or potency will be negatively affected. Many post-translational modifications have negligible effect on antibody performance. However, when identified, it is imperative to determine the nature, location, and impact of the post-translational modification.

Here, we described the post-translational modification of the monoclonal antibody 001. The lack of aggregation or degradation associated with mAb 001 suggested that perhaps a post-translation modification was responsible for the extreme variability in antibody potency seen from lot to lot. This theory was supported by the results obtained from analytical HIC. HIC has been shown to be an effective tool for analyzing various post-translational modifications, including oxidation. Unexpectedly, ESI-MS showed the presence of a 119 Dalton modification, implicating the unlikely candidate of cysteinylation as the post-translational modification involved. The presence of oxidation in a reduced antibody sample notwithstanding, sequencing

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Table 2. The HIC chromatographic profiles from Fig. 5 were analyzed and quantified. % Diff. refers to the percent difference between the sample peak areas of the variously treated antibodies and the untreated control.

| Sample           | Peak 1  | % Diff. | Peak 2  | % Diff. | Peak 3  | % Diff. | Peak 4  | % Diff. | Peak 5  | % Diff. |
|------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Untreated        | 47.50   | 0.00    | 21.03   | 0.00    | 22.49   | 0.00    | 7.10    | 0.00    | 1.88    | 0.00    |
| Mock             | 46.86   | −1.34   | 20.75   | −1.32   | 22.19   | −1.32   | 7.21    | 1.51    | 2.98    | 58.78   |
| Reduced          | 38.04   | −19.92  | 12.14   | −42.27  | 30.26   | 34.54   | 8.89    | 25.12   | 10.66   | 467.55  |
| Reduced & Denatured | 18.41   | −61.23  | 7.18    | −65.87  | 37.94   | 68.70   | 10.89   | 53.29   | 25.58   | 1261.51 |

Figure 5. mAb 001 oxidation is partially reversible by mild reduction and denaturation. A low-potency, highly-cysteinylated lot of antibody 001 (lot 159218) was exposed to either no reducing agent (Mock), mild reduction with 50 mM cysteine (Reduction), or mild reducing and denaturing conditions (Reduction, Denaturing) before the antibody was desalted back into its original buffer formulation for analysis. The overlay shows the change in peaks.
confirmed the presence of an additional and thus unpaired cysteine in the CDR3 of the light chain of the antibody. This amino acid is an obvious target for cysteinylation, as well as a likely candidate for the other forms of oxidation recorded by ESI-MS.

Though cysteinylation of antibodies is rarely observed, it has been documented with a variety of other proteins, linked with the presence of reactive oxygen species or with changes in extracellular redox conditions.27,28 In antibodies, however, multiple cysteines are employed in the form of disulfide bridges to help support antibody structure and stability. Unpaired cysteines are to be avoided, especially in the antibody’s CDR domains, where the high degree of reactivity associated with this amino acid can have a negative effect on antibody stability and activity, as has been described here.

A similar antibody cysteinylation event was previously described by Gadgil et al.,11 and the biophysical impact of the modification was described by Banks et al.12 Though the authors’ antibody, MAB007, and our mAb 001 are both clearly negatively affected by cysteinylation, there are several key differences between the two events. MAB007 was found to be cysteinylated on an additional, unpaired cysteine residue in the heavy chain CDR3, compared to the light chain CDR 3 of our mAb 001. Furthermore, the apparent high solvent accessibility of the MAB007 cysteinylation site contrasts with the partially inaccessible nature of mAb 001’s unpaired cysteine site. The impact of the cysteinylation appears to be more significant with mAb 001, where the modification inhibits antigen binding nearly completely, as determined by the Biacore relative activity results, suggesting a different mechanism for antibody inactivation.

The analysis of the fractionated peaks of the mAb 001 HIC chromatography was extremely informative in describing the effect of cysteinylation on antibody activity. Singly modified antibodies, which correspond to ~50% modified light chain as seen by ESI-MS, had 50% relative activity compared to unmodified antibody lots. Antibody with both light chains modified, corresponding to nearly 100% modification, had negligible activity. These results suggest the model described in Fig. 7. Antibody cysteinylation, potentially due to metal-catalyzed oxidation or as the result of UV exposure during expression or storage in mammalian tissue culture, leads to inactivation of the modified antibody arm, leaving the antibody capable of binding only one antigen. When both arms of the antibody are modified, the antibody becomes completely inactive. Whether cysteinylation is truly occurring while the antibody is in the cell culture media or whether it is occurring intracellularly is a question we are currently exploring.

**Materials and methods**

**Materials**

Mouse monoclonal antibody mAb 001, is an IgG1 isotype antibody expressed in mouse hybridoma cells grown in custom hybridoma serum-free medium (Life Technologies, Grand Island, NY). This antibody was produced by the Abbott Diagnostics Division Bioprocess Design Cell Culture group. It was purified by Protein A chromatography and was processed into 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4. Antibody concentrations ranged from 3 to 8 mg/mL. All chemicals used were reagent grade or higher.
Active antigen binding concentration assay using surface plasmon resonance

A Biacore 2000 instrument (GE Healthcare, Piscataway, NJ) was used to determine active antibody activity, or relative binding of antibody to its immobilized antigen. The antigen was adhered on a CM5 chip to a level of 17,124 RU, using amine immobilization. A mouse IgG-coated surface was used for automatic blank subtraction. mAb 001 samples were diluted to 40 nM in HBS-EP buffer (GE Healthcare, Piscataway, NJ) and injected at 5 µL/min over the sensor chip surfaces for 3 minutes. Active antibody concentration was determined by comparing the slope of the initial response units over time of a sample of unknown activity to those of a standard curve generated from a high activity lot of mAb 001 loaded at concentrations ranging from 0 nM to 60 nM and expressed as a percentage of standard activity.

HPLC assays

A Dionex Ultimate 3000 HPLC instrument (Thermo Scientific Inc., Waltham, MA) equipped with a photodiode array detector was used for these experiments. Data analysis was performed using Thermo Chromeleon software version 6.80.

Size-exclusion chromatography

SEC was performed using a TSKgel G3000SWxl column, 7.8 mm × 30 cm, 5 µm (TOSOH Bioscience LLC, King of Prussia, PA). Sample elution was carried out isocratically with 100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 at a flow rate of 1.0 mL/min. Absorbance was measured at 214 nm. Total run time was 20 minutes and chromatographic peak areas were used to determine levels of high molecular weight aggregation.

HIC chromatography

HIC samples were directly injected onto a Dionex ProPac HIC-10 column, 7.5 mm × 75 mm. Sample elution was carried out at 0.75 mL/min with a mobile phase consisting of buffer A (100 mM sodium phosphate, pH 7.0) and buffer B (buffer A plus 1 M ammonium sulfate). A linear gradient from 0% to 100% buffer A was performed over 60 minutes at ambient temperature. Peak detection was performed at 214 nm. Integration was performed manually due to incomplete peak separation. Baseline-to-valley integration with an extended baseline was used. Fractionation, when performed, was based on time.

De-cysteinylation reaction

mAb 001 was diluted to a concentration of 1 mg/mL in 25 mM sodium phosphate, 150 mM sodium chloride, pH 8.0 (Mock), with 50 mM N-acetyl cysteine in PBS, pH 8.0 (Reduction), or with 50 mM N-acetyl cysteine, 1 M guanidine in PBS, pH 8.0 (Reduction, Denaturing). The samples were incubated at room temperature for 90 minutes. Desalting of the samples into 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 and subsequent concentration was performed using an Amicon ultra 30 kDa membrane.

ESI-MS

The samples were desalted using Amicon Ultra centrifugal filter devices (MWCO 10 KDa, Merck KGaA, Darmstadt, Germany). For the reduction of samples for the analysis of light and heavy chains, the reducing agent TCEP (tris(2-carboxyethyl)phosphine, Thermo Scientific Inc., Waltham, MA) solution was added to the samples at a final concentration of 50 mM and incubated for 10 minutes at room temperature prior to the desalting of samples. The ESI-MS experiments were performed using an AB SCIEX (Framingham, MA) QSTAR® Pulsar hybrid quadrupole-TOF LC-MS/MS mass spectrometer using positive mode. A C18 trap column was used to further desalt and concentrate the samples. The mass spectrometer key parameters were set as follows: IS 5500 V, GSI 15, DP 80 V, FP 300 V, and DP2 15 V. The ESI-MS analysis results were processed using Analyst QS 2.0 software.

Molecular modeling

A molecular model was constructed for the VhVI domain using the BioLuminate software package (Schrödinger, Inc., New York, NY). The template was derived from coordinates in the public PDB with accession codes 1OAK and 1C1E. The
resulting model retained reasonable geometry as assessed by visual inspection along with Ramachandran plot and steric clash analysis of atom positions. Cysteinylation was modeled for visualization by simple graphical fit of the rigid body without spatial clash to the surface of the VhVl model.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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