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Comparison of methionine oxidation in thermal stability and chemically stressed samples of a fully human monoclonal antibody

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Abstract

Methionine (Met) oxidation is a major degradation pathway of protein therapeutics. Met oxidation of a fully human recombinant monoclonal antibody was investigated under both chemically stressed conditions using *tert*-butylhydroperoxide (*t*BHP) and thermal stability conditions where the sample was incubated in formulation buffer at 25 °C for 12 months. This antibody has one Met residue on each of the light chains and four Met residues on each of the heavy chains. In the thermal stability sample, only Met residues 256 and 432 in the Fc region were oxidized to form methionine sulfoxide, while Met residues in the Fab region were relatively stable. The susceptibility of Met residues 256 and 432 was further confirmed by incubating samples with *t*BHP, which has been shown to induce Met oxidation. Further analysis revealed that the susceptible Met residues of each heavy chain were randomly oxidized in samples incubated with *t*BHP, while in the thermal stability sample, the susceptible Met residues of one heavy chain were preferentially oxidized.
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Keywords: Recombinant monoclonal antibody; Methionine oxidation; Mass spectrometry

1. Introduction

Methionine (Met) is the most susceptible amino acid for oxidation in proteins including recombinant proteins used in therapeutics [1–3]. The major product of Met oxidation is methionine sulfoxide. Compared to Met, methionine sulfoxide has a larger, more polar and less flexible side chain with decreased hydrophobicity and increased hydrogen bonding capability [4]. Susceptibility of Met residues is highly dependent on their solvent exposure and location in the protein's three-dimensional structure. For example, in the study of two closely related hormones, human growth hormone (hGH) and human chorionic somatomammotropin (hCS), Teh et al. [5] reported that Met170, which was present in both molecules, was completely resistant to oxidation by incubation with hydrogen peroxide (H_2O_2). While the other two Met residues in hGH as well as the other five Met residues in hCS had dramatically different oxidation rates depending on their microenvironments. Different oxidation rates of Met residues in the same protein were also obtained for human parathyroid hormone [6], recombinant human granulocyte colony stimulating factor [7], recombinant interferon γ and recombinant tissue-type plasminogen activator [8], human cystatin C expressed in *E. Coli* [9], recombinant human leptin [10], recombinant coagulation factor exposed to H_2O_2 [11] and wheat germ calmodulin [12]. Met oxidation is a concern for protein therapeutics due to the possible adverse effects such as in the case of recombinant human leptin [10], where decreased thermostability and a significant loss of *in vitro* bioactivity was observed when Met1 and Met69 were both oxidized. In other proteins, oxidation of Met has been shown to cause conformational changes [9,12–16], decreased stability [4,7,10,12,17], and decrease or loss of biological functions [5,7,10,11,18–22]. These changes are highly site specific. Oxidation of some Met residues has little to no conformational and/or biological consequences [5,7,8,10,20,23].

There are reports on Met oxidation of recombinant monoclonal antibodies. Kroon et al. [24] reported that one Met (Met164) in the light chain and four (Met34, Met316, Met360

Abbreviations: Met, methionine; *t*BHP, *tert*-butylhydroperoxide; DTT, dithiothreitol; TFA, trifluoroacetic acid; NGA2F, fucosylated-bianntennary-oligosaccharide with 0 galatose; CPB, carboxypeptidase B

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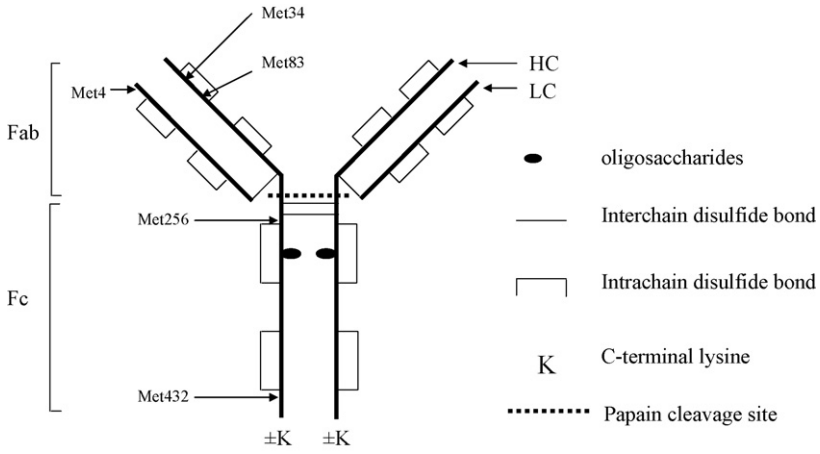


Fig. 1. Diagram of an IgG_1 molecule. The major features of the fully human recombinant monoclonal IgG_1 antibody are shown. Papain as shown in this diagram cleaves this antibody above the two hinge region interheavy chain disulfide bonds. Locations of Met residues on one side of the molecule are shown in the diagram.

and Met408) in the heavy chain of a mouse monoclonal antibody orthoclone OKT3 were oxidized when the samples were stored at 2–8 °C for 14 months to 3 years. Met34 of the heavy chain is located in the complementarity determining region (CDR), therefore oxidation of this Met residue may affect the biological activity of OKT3. Roberts et al. [25] reported that Met34 of the heavy chain of an anti-respiratory syncytial virus human monoclonal antibody was partially oxidized. Shen et al. [26] studied the susceptibility of Met residues of humanized monoclonal antibody HER2 by incubating it with *t*BHP at room temperature for 20 h. They found that heavy chain Met255 was the most susceptible site, which was followed by heavy chain Met431. Light chain Met4 and heavy chain Met107 were oxidized very slowly. Heavy chain Met361 was slightly oxidized at 1% *t*BHP, while heavy chain Met83 was unaffected even at 10% *t*BHP. Lam et al. [27] reported that Met255 was the primary oxidation site, while Met431 was also oxidized when recombinant humanized monoclonal antibody HER2 in liquid formulation was incubated at elevated temperature (30 and 40 °C) for 2 weeks.

In this report, oxidation of Met residues of a fully human recombinant IgG_1 monoclonal antibody was investigated. This antibody has one Met residue on each of the light chains and four Met residues on each of the heavy chains (Fig. 1). Two of the four Met residues are located in the Fab region of the heavy chain, while the other two are located in the Fc region. Met oxidation of the fully human recombinant monoclonal antibody was investigated under both chemically stressed conditions and thermal stability conditions where the sample was incubated in formulation buffer at 25 °C for 12 months. A basic shift of the antibody on a weak cation exchange chromatography column was observed in the thermal stability sample. Analysis by mass spectrometry indicated that this basic shift was due to oxidation of the Met residues in the Fc region. The susceptibility of Met residues to oxidation in the thermal stability sample was successfully predicted from the chemically stressed samples. However, detailed analysis indicated that Met residues on the two heavy chains were almost randomly oxidized in *t*BHP treated samples, while in the thermal stability sample, Met residues on one heavy chain were preferred.

2. Experimental

The fully human monoclonal IgG_1 antibody was produced by transfected Chinese hamster ovary (CHO) cell lines and purified during development of the manufacturing process at Abbott Bioresearch Center (Worcester, MA). 30 mL sample at 50 mg/mL in formulation buffer (5.57 mM sodium phosphate monobasic, 8.69 mM sodium phosphate dibasic, 106.69 mM sodium chloride, 1.07 mM sodium citrate, 6.45 mM citric acid, 66.68 mM mannitol and 0.01% Tween) at pH 5.2 was incubated at 25 °C for 12 months in a 30 mL polyethylene terephthalate copolyester (PETG) bottle (NALGENE Labware, Rochester, NY). The same antibody at approximately 70 mg/mL in the same formulation buffer stored at −80 °C was used as a control.

For *t*BHP (70%, Sigma, St. Louis, MO) treatments, samples in the formulation buffer at approximately 70 mg/mL were diluted to 10 mg/mL using the same formulation buffer. *t*BHP was included in the sample preparations to a final concentration of 0, 0.1, 0.5, 1 and 5%. Samples (10 mL) were incubated at room temperature (25 °C) for 24 h in 15 mL Corning tubes (Corning Inc. Corning, NY). Samples were then buffer exchanged to phosphate buffered saline (PBS) using Econo-Pac 10 DG disposable chromatography columns (Bio-Rad, Hercules, CA). The columns were washed with 30 mL PBS and then 3 mL of each sample were loaded to the column. Liquid was allowed to flow through. 4 mL of PBS was then added to each column and 4 mL of each sample was collected.

A Shimadzu HPLC (Shimadzu) and a weak cation exchange WCX-10 column (250 mm × 4 mm) from Dionex (Sunnyvale, CA) were used for analyzing charge variants of this fully human recombinant antibody. Eluent A was 10 mM sodium phosphate, pH 7.5. Eluent B was 10 mM sodium phosphate, 500 mM sodium chloride, pH 5.5. Samples were diluted to 1 mg/mL using Milli-Q water. 100 μg of each sample was loaded at 6% eluent B, and

then eluent B was increased to 16% in 20 minutes. The column was washed at 100 % eluent B for 4 minutes and equilibrated at 6% eluent B for 7 minutes before the next injection. The flow-rate was set at 1 mL/min. Protein eluted from the column was monitored by UV at 214 and 280 nm.

Samples from the control, thermal stability sample, and *t*BHP treated samples were digested with papain (Roche, Indianapolis, IN). Samples were diluted to 2 mg/mL using 50 mM sodium phosphate and 4 mM EDTA (Sigma), pH 6.5. Papain was pre-activated by adding cysteine (Pierce, Rockford, IL) to a final concentration of 25 mM and incubating at 37 °C for 5 minutes. The activated papain was then added to 1 mL of each sample at a final concentration of 1:50 (w:w) papain: sample ratio. The mixtures were incubated at 37 °C for 30 minutes. Digestions were stopped by adding iodoacetamide (Sigma) to a final concentration of 10 mM. Fab and Fc were separated using protein A sepharose (Applied Biosystems, Framingham, MA). Protein A sepharose slurry was pelleted by centrifuge at 10,000 rpm using an Eppendoff tabletop centrifuge for 6 min. The liquid was discarded. The pellet was washed by mixing with 1 mL PBS and followed by centrifuge. The liquid was discarded again. The wash was repeated two more times. Papain digested samples were added to each 100 μL of protein A sepharose pellet. The samples with protein A sepharose were mixed on a shaker for 30 minutes at room temperature (25 °C). The sample and protein A sepharose mixture were centrifuged at 10, 000 rpm for 6 minutes. The supernatant was collected as Fab fractions. The pellets were washed two more times with 1 mL of PBS each time. The supernatant from the same sample was combined. Fc was eluted off protein A sepharose by adding 1 mL 0.1 M acetic acid, 150 mM sodium chloride, pH 2.8 to the protein A sepharose pellets. After mixing, the samples and protein A sepharose were centrifuged and the supernatant was collected as Fc. The same procedure was repeated two more times, and the supernatant from the same sample was combined. The sample pH was neutralized immediately using 1 M Tris, pH 8.5.

For peptide mapping, samples were denatured with 6 M guanidine hydrochloride (J.T. Baker, Phillipsburg, NJ) in 20 mM ammonium bicarbonate at room temperature for 15 min and then reduced with dithiothreitol (DTT) (Sigma) at a final concentration of 10 mM. After reduction at 37 °C for 30 min, samples were alkylated with iodoacetamide at a final concentration of 50 mM at 37 °C for 30 min. The samples were dialyzed against 20 mM ammonium bicarbonate at 4 °C overnight using Spectra/Por 6–8000 MW cut-off membrane tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA). Samples were digested with trypsin (Promega, Madison, WI) at a 1:20 (w:w) trypsin: antibody ratio at 37 °C for 4 h.

For the thermal stability sample, weak cation exchange chromatography fractions were collected using a WCX-10 Semi-preparative column (Dionex, 250 mm × 9 mm) using the same eluents and gradient as used for analytical WCX-10 column. The flow-rate was set at 5 mL/min. Samples were digested with carboxypeptidase B (CPB) (Roche) to remove C-terminal lysine residues to simplify the WCX-10 chromatograms. To do that, 20 μL of CPB was added to 1 mL of 10 mg/mL sample

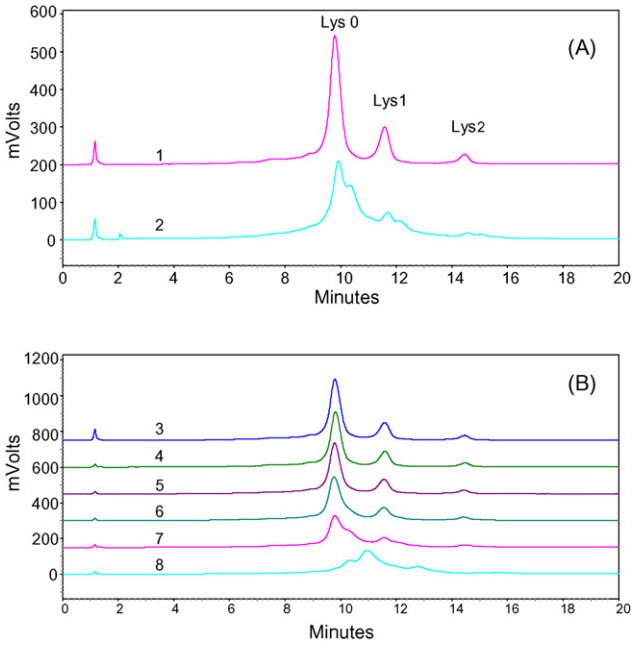


Fig. 2. WCX-10 chromatograms of the fully human recombinant monoclonal antibody after different treatments. (A) −80 °C sample (trace 1) and the 12 months, 25 °C thermal stability sample (trace 2); (B) −80 °C sample (trace 3), and samples treated with 0 % (trace 4), 0.1% (trace 5), 0.5 %(trace 6), 1% (trace 7) and 5% (trace 8) *t*BHP.

diluted using PBS and incubated at 37 °C for 6 h. Sample was diluted 10 times using Milli-Q water before loading. 500 μg of sample was loaded for each run. The collected fractions were concentrated using Amicon Ultra-4 centrifugal filter device (Millipore, Billerica, MA) of MW cut-off of 10 kDa. Fab and Fc of the concentrated fractions were prepared by following the same procedure as described previously.

An Agilent HPLC (Agilent, Santa Clara, CA) and a Q Star pulsar i LC-MS/MS mass spectrometer (Applied Biosystems) were used for Fab, Fc and peptide analyses of both chemically stressed and thermal stability samples. For molecular weight measurements of Fab and Fc with and without reduction, a C4 column (Vydac, 150 mm × 1 mm i.d., 5 μm particle size, 300 A pore size) was used. In order to achieve a better mass resolution for intact Fc, approximately 0.5–1 μg was injected. Fc was also reduced using 10 mM DTT at 37 °C for 30 min. For reduced Fc molecular weight measurements, 4–8 μg of protein was loaded onto the column. Samples were loaded at 95% eluent C (0.02% trifluoroacetic acid (TFA)(J.T. Baker), 0.08 % formic

Table 1
Percentage of oxidation of Met256 and Met432

Samples	Percentage of oxidation	
	Met256	Met432
0% *t*BHP	0.8	3.6
0.1% *t*BHP	6.7	8.4
0.5% *t*BHP	25.2	22.5
1% *t*BHP	46.5	39.4
5% *t*BHP	94.9	92.4
Accelerated stability samples	26.0	35.3

acid (Sigma) in Milli-Q water) and 5% eluent D (0.02% TFA, 0.08% formic acid in acetonitrile (EMD, Gibbstown, NJ), and then eluted by increasing the percentage of eluent D from 5% to 65% in 35 min. Mass spectrometer IonSpray voltage was set at 4500 Volts. Source temperature was set at 350 °C. Samples were scanned from *m/z* 800 to 2500. For peptide analyses, a C18 column (Vydac, 250 mm × 1 mm i.d., 5 μm particle size, 300 A pore size) was used. Approximately 10–20 μg sample was loaded at 98% eluent C and 2% eluent D and then eluted by increasing eluent D from 2 to 35% in 140 min. IonSpray voltage was set at 4200 Volts. Source temperature was set at 75 °C. *m/z* was scanned from 250 to 2000. For both Fc molecular weight measurements and peptide analyses, the flow-rate was set at 50 μL/min.

3. Results

The fully human recombinant monoclonal antibody used for this study has a typical IgG_1 structure as depicted in Fig. 1. It is composed of two light chains and two heavy chains. Glycosylation of a conserved asparagine (Asn) residue and partial removal of C-terminal lysine (Lys) are the two major posttranslational modifications. Each light chain has one Met residue. Each heavy chain has four Met residues with two in the Fab region and the other two in the Fc region.

Met residues of recombinant monoclonal antibodies can be oxidized using *t*BHP or by incubating the antibody in formulation buffers at elevated temperatures [27,28]. The same Met residues oxidized under the two different conditions

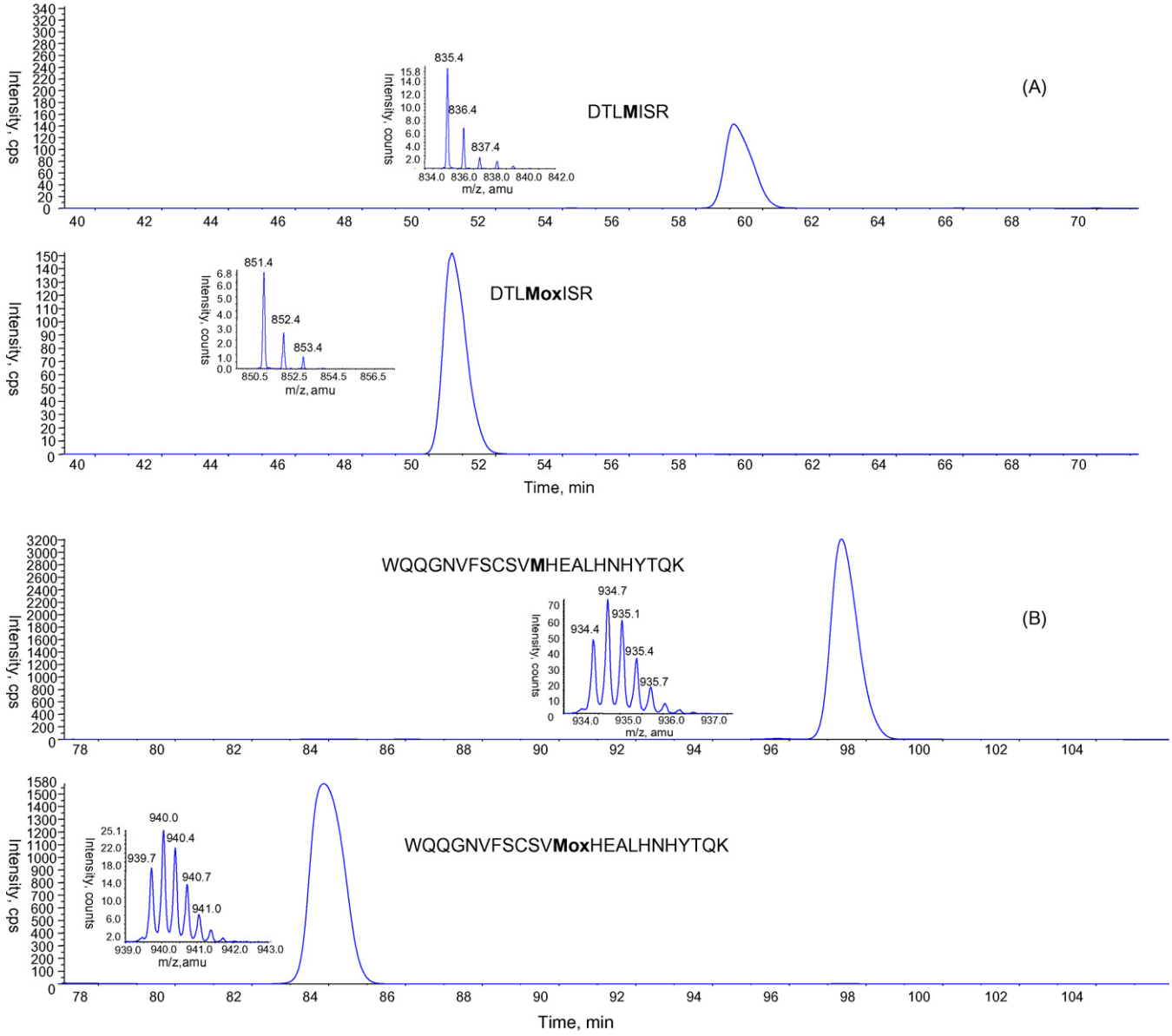


Fig. 3. Extracted ion chromatograms of oxidation of Met256 and Met432 from thermal stability sample. (A) extracted ion chromatogram of peptide containing Met256; (B) extracted ion chromatogram of peptide containing Met432. The calculated molecular weight $(M+H)^+$ of peptide containing Met256 is 835.4 Da. The observed molecular weights as shown in the insets of this peptide is 835.4 Da without oxidation and 851.4 Da with oxidation. The calculated molecular weight $(M+H)^+$ of peptide containing Met432 is 2801.3 Da with an alkylated Cys residue. The calculated molecular weights of this peptide based on the triplicate charged ions as shown as insets are 2801.2 Da without oxidation and 2817.1 Da with oxidation.

implied that susceptibility of Met residues of recombinant monoclonal antibodies in formulation buffer could be predicted from chemically stressed studies. However, Met residues on each heavy chain may not be equally susceptible under the same or different conditions. Also, it was interesting to determine the behavior of recombinant monoclonal antibodies after Met oxidation on ion exchange chromatography, which is by far the most common technique to monitor recombinant monoclonal antibody charge heterogeneity.

3.1. WCX-10 chromatography

As shown in Fig. 2A (trace 1), three distinct peaks were observed on a WCX-10 chromatogram of the fully human recombinant monoclonal antibody. These peaks were well characterized as antibody with zero, one or two C-terminal lysine residues referred to Lys 0, Lys 1 and Lys 2, respectively. Material eluted before Lys 0 is referred to as acidic species. When the thermal stability sample was analyzed by WCX-10 chromatography, shoulders on the descending sides (basic shoulders) were observed in Lys 0, Lys 1 and Lys 2 peaks (Fig. 2A, trace 2). To test the hypothesis that the shoulders observed on WCX-10 chromatograms were due to Met oxidation, antibody control was incubated with different percentage of *t*BHP and then analyzed by WCX-10 chromatography. Similar shoulders on the descending sides of Lys 0, Lys 1 and Lys 2 peaks were also observed with antibody treated with *t*BHP (Fig. 2B). A complete basic shift resulting in longer retention

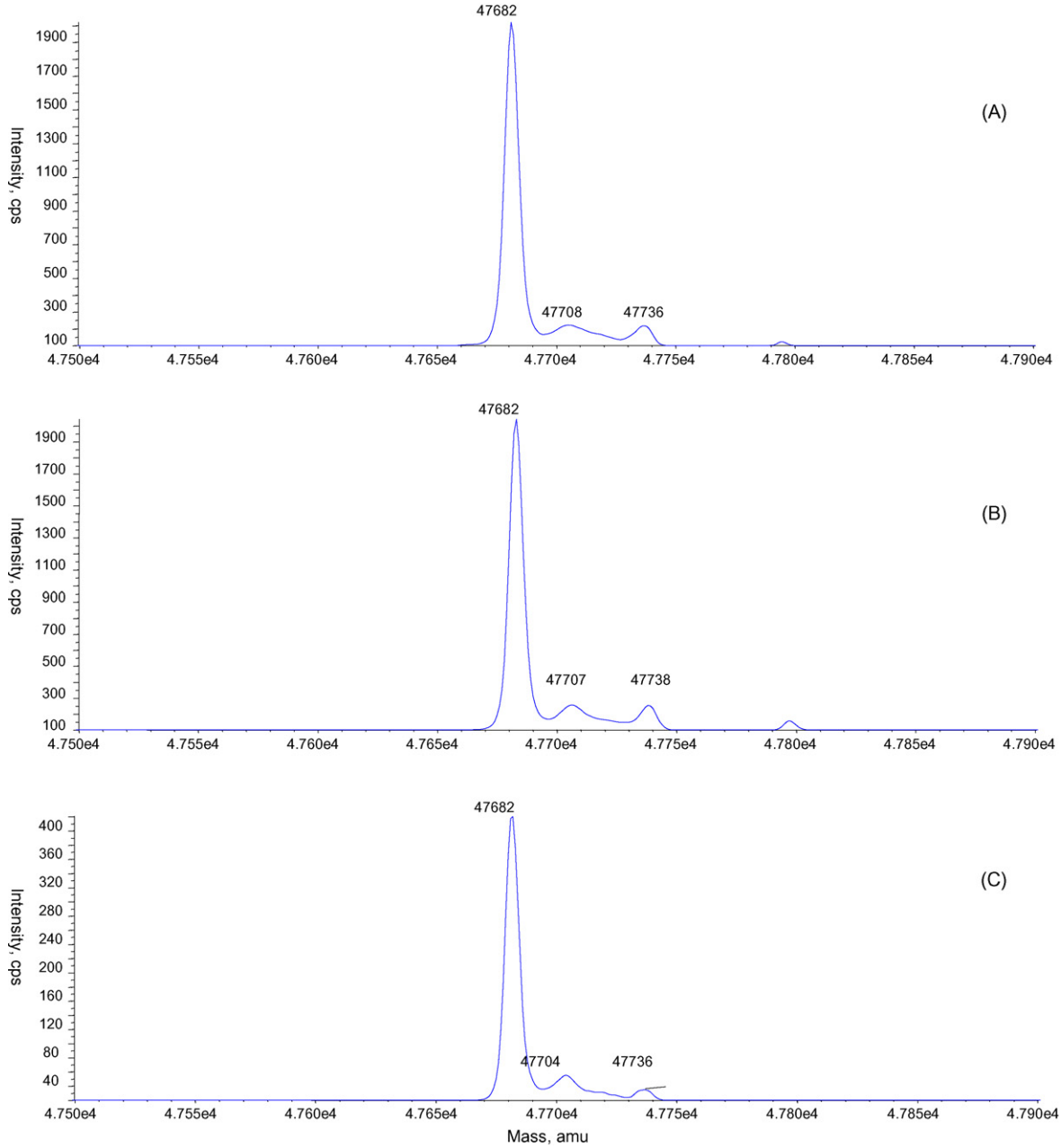


Fig. 4. Fab molecular weights. Spectra were acquired from: (A) −80 °C sample; (B) 5% *t*BHP treated sample; and (C) thermal stability sample (25 °C, 12 months).

time was observed in the sample treated with 5% *t*BHP (Fig. 2B, trace 8).

3.2. Peptide map

Tryptic peptides were separated by a C18 column and analyzed by a Qstar mass spectrometer. Oxidation of Met to form sulfoxide caused a molecular weight increase of 16 Da. Only Met256 and Met432 were found to be oxidized in the thermal stability sample and in *t*BHP treated samples. Very low levels of oxidation were observed in the control samples (−80 °C and 0% *t*BHP treated sample), which probably occurred during sample preparation. Oxidation of these two Met residues was confirmed by MS/MS experiments (data not shown). Extracted ion chromatograms of tryptic peptides containing Met256 and Met432 from the thermal stability sample are shown in Fig. 3. Mass spectra of the observed molecular weights of the two peptides with and without Met oxidation are shown as insets. Oxidation of Met residues resulted in an earlier elution of the peptides due to increased polarity. From the peak area of extracted ion chromatograms, the relative percentage of oxidation of Met256 and Met432 was calculated (Table 1). Met432 was slightly more susceptible to oxidation during sample preparation for peptide map. Met256 had a slightly higher oxidation rate in *t*BHP treated samples. However, in thermal stability sample, higher percentage of Met432 was oxidized.

3.3. Qstar analysis of Fab and Fc

The thermal stability sample and *t*BHP treated samples were digested using papain and then analyzed using a Qstar mass spectrometer. Fab of control, 5% *t*BHP treated and thermal stability samples had a molecular weight of 47,682 Da (Fig. 4), which is in good agreement with the predicted molecular weight of 47,681 Da. This data further confirmed that Met residues on Fab were not oxidized. The deconvoluted mass spectra of the Fc from 0%, 0.5%, 1%, and 5% *t*BHP treated samples and the thermal stability sample are shown in Fig. 5. The predicted molecular weight of Fc without C-terminal Lys and with NGA2F, which is the major oligosaccharide species of this antibody, is 52,755 Da. After reduction, the predicted molecular weight of Fc is 26,379 taking into account the reduction of two inter heavy chain disulfide bonds. A 16 Da increment in molecular weight was observed in the mass spectra of both non-reduced and reduced samples treated with 0.5%, 1% and 5% *t*BHP. However, in the thermal stability sample, a mass increase of approximately 32 Da was observed in both non-reduced and reduced Fc, which indicated that Met256 and Met432 on one heavy chain were preferentially oxidized. The data from 0.1% *t*BHP treated sample was not shown because at Fc level, no significant difference was observed from 0.5% *t*BHP treated sample. The calculated and observed molecular weights of the *t*BHP treated and the thermal stability samples are summarized in Table 2. Based on this data, the distribution of oxidation of Met256 and Met432 on the two heavy chains is summarized in Table 3.

Table 2
Calculated and observed molecular weights of Fc before and after reduction

Samples	Treatments	Calculated mass (Da)	Observed mass (Da)	Number of oxidation
Reduced Fc	0%	26,379	26,380	0
	0.5%	26,379	26,380	0
		26,395	26,396	1
		26,411	26,412	2
	1%	26,379	26,380	0
		26,395	26,396	1
		26,411	26,412	2
	5%	26,395	26,396	1
		26,411	26,412	2
	Stability	26,379	26,380	0
		26,411	26,412	2
Non-reduced Fc	0%	52,755	52,756	0
	0.5%	52,755	52,756	0
		52,771	52,770	1
		52,787	52,787	2
	1%	52,755	52,758	0
		52,771	52,772	1
		52,787	52,787	2
		52,803	52,802	3
	5%	52,803	52,801	3
		52,819	52,817	4
	Stability	52,755	52,756	0
		52,787	52,787	2
		52,819	52,815	4

Only molecular weights of Fc with NGA2F and without C-terminal Lys are shown in this table.

Table 3
Different oxidation forms of Fc observed in *t*BHP treated samples and in the thermal stability sample

Samples	Assignment	
	Reduced Fc	Intact Fc
0 % tBHP		
0.5 % tBHP		
1 % tBHP		
5 % tBHP		
stability		

1. "●" represents oxidation of Met256 (upper) and Met432 (lower) in the diagram.
2. Considering oxidation of Met on one heavy chain and oxidation of the same Met on the other heavy chain to be oxidation isoforms, only one isoform of each oxidation sate is shown in the Table.
3. Only molecular weights of Fc with NGA2F and without C-terminal Lys are shown in this table.

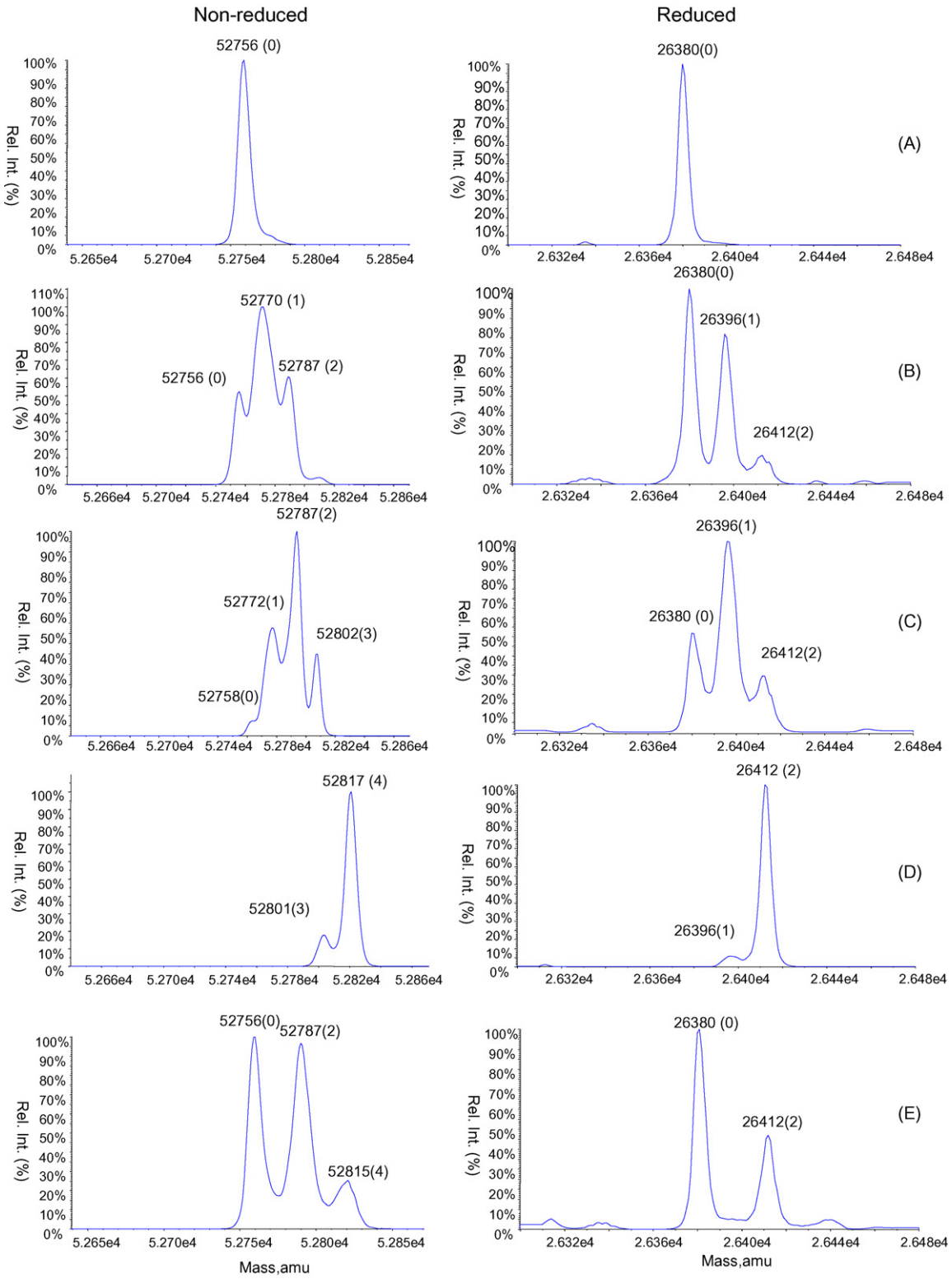
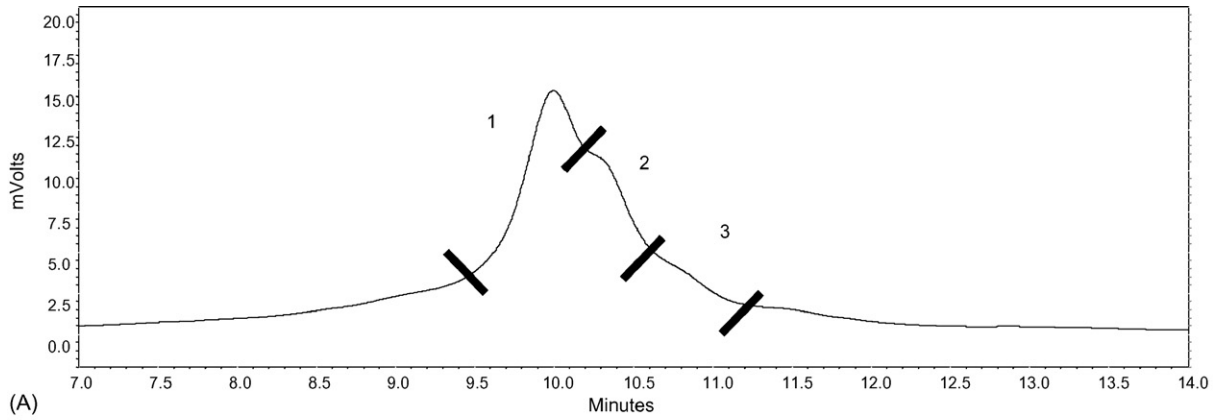


Fig. 5. Fc molecular weights of *t*BHP treated samples and the thermal stability sample. Spectra from non-reduced Fc are shown on the left. Spectra from reduced Fc are shown on the right. The spectra were acquired from: (A) 0% *t*BHP; (B) 0.5% *t*BHP; (C) 1% *t*BHP; (D) 5% *t*BHP; and (E) the thermal stability sample. The numbers after molecular weight indicate numbers of methionine oxidation.

3.4. Mass spectrometry analysis of WCX-10 fractions

To further confirm that the basic shift of the thermal stability sample on WCX-10 column was due to oxidation of Met residues, WCX-10 fractions were collected and analyzed by MS. C-terminal Lys residues were removed to simplify the chromatograms and fraction collection. As shown in Fig. 6A, three fractions were collected and analyzed. The data is summarized



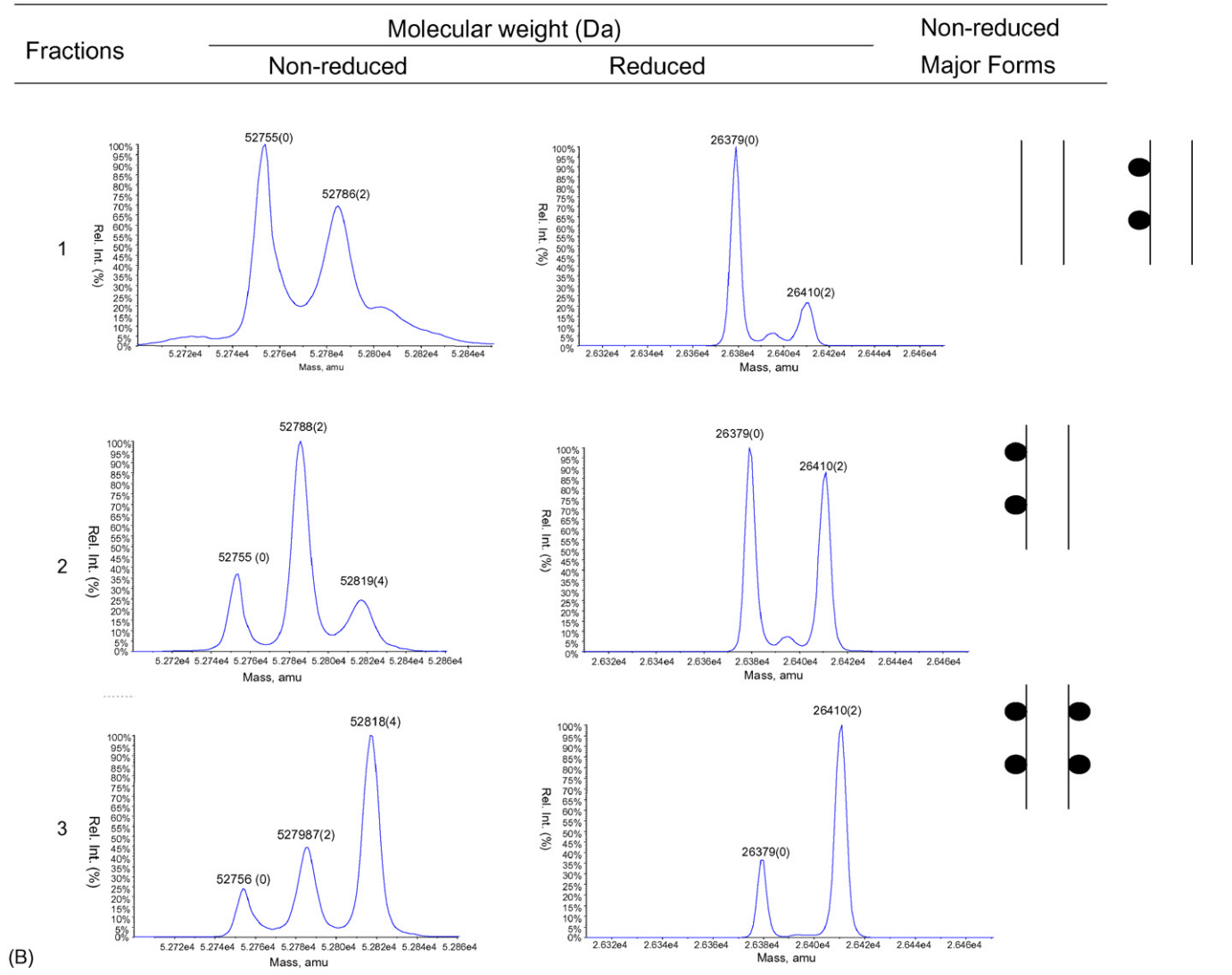


Fig. 6. (A) WCX-10 chromatogram of the thermal stability sample after CPB treatment. Fractions (1, 2 and 3) were collected as indicated on the chromatogram. (B) different oxidation forms of Fc. "●" represents oxidation of Met256 (upper) and Met432 (lower) in the diagram. Considering oxidation of Met on one heavy chain and oxidation of the same Met on the other heavy chain to be oxidation isoforms, only one isoform of each oxidation state is shown in the figure. Only molecular weights of Fc with NGA2F and without C-terminal Lys are shown in the figure.

in Fig. 6B. It can be seen that antibody with more oxidation eluted later.

4. Discussion

Recombinant monoclonal antibodies, as large protein therapeutics, possess a lot of features that can cause instability of the molecules through many different degradation pathways including oxidation [2]. Among the twenty amino acids, Met is one of the most susceptible residues to oxidation. It can be oxidized by exposing samples to oxidizing reagents such as H_2O_2 and *t*BHP and in formulation buffer without the addition of oxidizing reagents [24,27,28]. As the susceptibility of Met residues has commonly been assayed using chemicals, it was interesting to determine its relationship to Met oxidation in formulation buffer under normal or thermal stability condition. It was also interesting to study the effect of Met oxidation on ion exchange chromatography, which has been widely used for charge variant monitoring of recombinant monoclonal antibodies.

Oxidation of Met residues caused a shift to basic side on WCX-10 chromatography (Fig. 2). Compared to the side chain of Met residue, methionine sulfoxide, which is the stable product of Met oxidation, is more basic. Therefore, antibody with oxidized Met was retained longer on the column than antibody without oxidized Met residues. Retention time shift can also be caused by the indirect effect of Met oxidation. It has been shown that separation of antibody variants by ion exchange chromatography is not only based on charge or overall charge of the molecule [29,30]. Instead, separation of recombinant monoclonal antibody variants may be more dependent on local charge differences of the molecules. Thus, if Met oxidation caused conformation changes, this can be reflected in local charge redistribution and resulted in a retention time shift. Certainly it was also possible that retention time shift was caused by a combination of the direct and indirect effects of Met oxidation.

Different Met residues in this fully recombinant monoclonal antibody had varying levels of susceptibility to oxidation. Data obtained from this study is in agreement with what has been reported for recombinant monoclonal antibody, HER2 [26,27]. Met5 is the only Met residue in each light chain of this fully human monoclonal antibody (Fig. 1). It is close to the N-terminus of the light chain and is not in the β-sheet structure [31]. No detectable oxidation of this residue was observed in the thermal stability sample and in the *t*BHP treated samples, which indicated that this Met residue is well protected by the three dimensional structure. Met34 and Met83 are located in the variable region of heavy chain, with Met34 in complementarity determining region 1 (CDR1) and Met83 in the framework between CDR2 and CDR3. No detectable oxidation was observed for Met34 and Met83. Based on the crystal structure of human Fc [32], a Met residue equivalent to Met256 is located in the β-turn structure of the CH2 domain, and a Met residue equivalent to Met432 is located in the CH3 domain. Both Met residues reside close to the CH2 and CH3 domain interface and are probably exposed to solvent. Therefore, it is not surprising that Met256 and Met432 were the two most susceptible residues to oxidation.

It was very interesting to note that Met256 and Met432 on one heavy chain were preferentially oxidized in the thermal stability sample, and randomly oxidized on both heavy chains in the samples exposed to *t*BHP. Preferential oxidation of the two Met residues on one heavy chain in the thermal stability could be due to several possibilities. The first is that the two heavy chains of Fc are not symmetrical. The two halves of human IgG_1 antibody are not identical in crystal structures [33]. While the two CH3 domains are symmetrical [33,34], the CH2 domains can "tilt" around the CH2 and CH3 interface. In the crystal structure of a murine IgG_1 antibody, the two CH2 domains had dramatically different mobility [35]. In the crystal structure of human Fc, one CH2 domain of one Fc interacts with the CH2 domain of the neighboring antibody [32], thus making the two halves of IgG Fc nonsymmetrical which would lead to different exposure to solvents. Thus, Met256 and Met432 on one side could be protected better than on the other side. The second possibility is oxidation of one Met residue can promote oxidation of the other Met residue on the same side. For example, photooxidation of Met can form persulfoxide, which is unstable and can further react with a second Met to form two Met sulfoxide [36]. Close localization makes this hypothesis possible. However, for this hypothesis to be correct, it is a prerequisite that oxidation of the first Met to be preferred over the same Met on the other heavy chain. Based on the relative susceptibility of the two Met residues, oxidation of Met256 is likely to occur first, which located in the less symmetric CH2 domains. The third possibility is antibody–antibody interaction can protect Met residues on the side of interaction, thus preventing oxidation from occurring. As discussed earlier, in the crystal structure of human Fc, it was found that only one chain of the CH2 domain interacts with one chain of the CH2 domain of the neighboring antibody [32]. The argument against this possibility is similar asymmetric oxidation was not found in *t*BHP treated samples. It is possible that even at very low *t*BHP concentration, the oxidation process was still too fast compared to the condition of the thermal stability sample. Thus, the *t*BHP process cannot offer a high "resolution" as in the thermal stability sample. The last and most likely possibility is related to both the first and the second hypotheses. It has been shown that metal-catalyzed oxidation is site-specific [36], so it is possible that due to the asymmetry of the two heavy chains, metals at low stoichemistry level preferentially bind to one heavy chain and catalyze oxidation of the Met residues. It has been shown that copper was able to bind to Fab region of IgG_1 from a myeloma patient [37] and Fc region of humanized murine and murine IgG_1 monoclonal antibodies[38]. Copper was also able to cleave a humanized monoclonal IgG_1 antibody around hinge region [39], which suggested a potential binding site around this region. Therefore, if preferential oxidation of Met residues on one heavy chain was indeed a metal-catalyzed reaction, it may suggest that there was a potential metal binding site around CH2 and CH3 interface.

In summary, Met256 and Met432 are the two most susceptible residues in the fully human recombinant monoclonal antibody in the thermal stability sample and in samples exposed to *t*BHP. The susceptibility of Met residues in the stability sample can be predicted from the samples incubated with *t*BHP. However, the

two Met residues on the two heavy chains in the samples incubated with *t*BHP were randomly oxidized, while in the stability sample, Met residues on one heavy chain were preferentially oxidized. Thus, caution should be taken for protein therapeutics stability assessment under thermal stability or normal storage condition using data from chemically stressed samples.

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