Conformational changes in oxidatively stressed monoclonal antibodies studied by hydrogen exchange mass spectrometry

William Burkitt,* Paula Domann, and Gavin O'Connor

Department of Mass Spectrometry, LGC, Queens Road, Teddington, London, TW11 0LY, United Kingdom

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Abstract: Oxidation of methionine residues in biopharmaceuticals is a common and often unwanted modification that frequently occurs during their manufacture and storage. It often results in a lack of stability and biological function of the product, necessitating continuous testing for the modification throughout the product shelf life. A major class of biopharmaceutical products are monoclonal antibodies (mAbs), however, techniques for their detailed structural analysis have until recently been limited. Hydrogen/deuterium exchange mass spectrometry (HXMS) has recently been successfully applied to the analysis of mAbs. Here we used HXMS to identify and localise the structural changes that occurred in a mAb (IgG1) after accelerated oxidative stress. Structural alterations in a number of segments of the Fc region were observed and these related to oxidation of methionine residues. These included a large change in the hydrogen exchange profile of residues 247–253 of the heavy chain, while smaller changes in hydrogen exchange profile were identified for peptides that contained residues in the interface of the C_H2 and C_H3 domains.

Keywords: hydrogen exchange; mass spectrometry; monoclonal antibody; oxidation and structural analysis

Introduction

Monoclonal antibodies (mAbs) and other recombinant proteins are increasingly appearing on the pharmaceutical market as therapeutic agents.¹ However, unlike small molecule pharmaceuticals they have a higher order structure, which is intimately linked to their biological activity. Oxidation of methionine is one of the most common modifications to occur to biopharmaceuticals during their manufacture and storage, and has been shown to affect the structure and biological function of a number of proteins.² Efforts to improve the physicochemical and pharmacological properties of such molecules are

currently impeded by a lack of techniques suitable for their detailed structural analysis.^{3,4}

Techniques, such as X-ray crystallography and nuclear magnetic resonance (NMR) offer the highest spatial resolution for the structural analysis of proteins and other biomolecules. However, both techniques are only applicable under a limited number of conditions, because of the restrictions placed upon them in terms of sample preparation. X-ray crystallography requires a crystal of the sample to have been prepared, which for some proteins can be difficult at best or impossible for others. The requirement for the sample to be crystallised also results in the analysis being undertaken with the sample in the solid phase, and consequently small dynamic changes in protein structure may not be easily detected. For analysis by NMR the high sample concentrations required can often not be achieved because of limited solubility of the sample, and

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: William Burkitt, Department of Mass Spectrometry, LGC, Queens Road, Teddington, London, TW11 0LY, United Kingdom. E-mail: william.burkitt@lgc.co.uk

analysis becomes increasingly difficult with increased molecular weight. Other techniques, such as circular dichroism (CD) and Fourier transform infra red spectroscopy (FTIR), are often used for the analysis of protein structure, however, the structural details provided are only of low resolution, limiting the value of the techniques. Diligent data analysis and careful choice of the buffers used is also required.

Hydrogen/deuterium exchange mass spectrometry (HXMS) is a medium resolution technique that is routinely applied to the analysis of protein structure and dynamics.^{5–9} HXMS has proved to be a powerful tool for determining and locating changes in protein structure,^{5,10,11} and is increasingly being applied to the analysis of biopharmaceuticals.^{5,12,13} It offers advantages over other techniques in terms of sensitivity and sample preparation, and provides a measure of the stability and solvent accessibility of a protein's backbone amide residues.

The HXMS experiment utilizes mass spectrometry to measure the change in a protein's mass that occurs when a hydrogen atom is exchanged for a deuterium. Proteins contain a number of hydrogens that exchange with the solvent, those attached to nitrogen, oxygen or sulfur exchange readily. The exchange reaction is catalysed by both hydronium and hydroxide ions, with the result that the rate of exchange is pH sensitive.^{14,15} The exchangeable hydrogens attached to residue side chains exchange extremely rapidly, while those of the backbone amides have a slower rate of exchange, as a result the latter are more commonly studied. The rate of exchange of a hydrogen atom is also greatly affected by its local structural environment. The rate of exchange is considerably reduced if the hydrogen forms a stable hydrogen bond, for example in a β -sheet or a α -helix, or if the hydrogen is shielded from the solvent by other parts of the protein.¹⁶ The sensitivity of the rate of hydrogen exchange to the local structure of the protein allows measurements of hydrogen exchange to be used as a probe of protein structure.

To initiate the exchange, the sample is put into an excess of D₂O, the increase in the mass of the protein that results from the exchange of hydrogens for deuteriums can then be determined by mass spectrometry. To increase the resolution of the measurements, the rate of exchange of the backbone amides can then be slowed to the extent that, the pattern of deuterium exchange can be retained, whilst the protein is enzymatically digested into smaller peptides and analysed by liquid chromatography and mass spectrometry.⁹ The peptides produced are typically 5 to 15 residues in length. Reducing the rate of exchange is achieved using a combination of pH control and temperature reduction. Under these conditions of "slow exchange" the rate of exchange of the backbone amides is at a minimum, while the rate of exchange of other exchange-

able hydrogens is much greater, resulting in these being "washed" from the protein during the liquid chromatography part of the analysis. Digesting the protein into smaller fragments under conditions of "slow exchange" allows greater resolution of the exchange measurements.

There have been a number of previous studies detailing the structural changes that occur to IgG1s as a result of methionine oxidation.^{17–19} Liu *et al* analysed oxidised recombinant human IgG1 Fc that had been expressed in *E. coli* using a variety of biophysical and biochemical techniques.¹⁷ Analysis by CD and NMR of an isotopically labelled Fc fragment showed that methionine oxidation resulted in detectable secondary and tertiary structural changes in the interface between the C_H2 and C_H3 domains. Differential scanning calorimetry (DSC) was used to measure the melting temperature (T_m) of the C_H2 and C_H3 domains. These were found to decrease in response to oxidation and to be dependent upon the extent of oxidation of the two methionines present in the Fc, which was confirmed using site-specific mutants lacking either of the methionines. The molecules investigated by D. Liu *et al* however, were only the Fc portion of IgG1 and lacked glycosylation, which has been shown to be important in the structure and function of IgG1s. Analysis of the whole IgG1 by NMR would have been very complex. H. Liu *et al* studied the effects of deglycosylation and methionine oxidation on a recombinant monoclonal antibody by limited tryptic and chymotryptic proteolysis.¹⁸ The structural changes that occurred as a result of deglycosylation or methionine oxidation were investigated using the indirect probe of susceptibility of the molecules to trypsin and chymotrypsin proteolysis. It was reported that oxidation of the methionines in the Fc region did not result in significant structural changes, while deglycosylation resulted in an increase in susceptibility of the molecules to proteolysis, which was interpreted as evidence for conformational changes. Zamani *et al* studied the rates of oxidation of methionine residues in an IgG1 in native and denaturing buffers, using liquid chromatography combined with mass spectrometry and multivariate data analysis. It was demonstrated that the rates of oxidation of the methionine residues were slower in native than denatured IgG1s.¹⁹

Houde *et al* characterised IgG1 conformation and conformational dynamics by hydrogen/deuterium exchange mass spectrometry.¹³ IgG1s were analysed with and without the glycans attached and changes in the exchange profiles resulting from the removal of the glycans identified. The regions of altered structure were residues 236–253 and 292–308 on the heavy chain. This demonstrated that HXMS could be successfully applied to biopharmaceuticals as large as IgG1s, something previously not thought possible. We have used these techniques

Figure 1. Stacked box plot of the measured hydrogen exchange for GSK-mAb. The sequence of GSK-mAb is shown with stacked boxes indicating exchange. The length of box indicates the peptide for which hydrogen exchange was measured. Each box in the stacks of seven boxes represents a different time of exchange, starting with 30 s for the top box and increasing to 30000s for the bottom box. The colour of the boxes represents the amount of exchange that was measured, as has been indicated. The domains of the protein are indicated above the sequence, as is the site of glycan attachment.

to analyse an IgG1 that had been subjected to accelerated oxidative stress.

Results and Discussion

HXMS was used to analyse three mAbs (IgG1s), however as the results presented here were very similar for all three of the mAbs, only the results for one have been shown (GSK-mAb, sequence shown in Fig. 1). The resolution of the exchange measurements was at the peptide level. Digestion of the mAbs, under conditions of slow exchange, was achieved using an immobilised pepsin column. Cleavage of peptide bonds by pepsin is somewhat nonspecific,²⁰ and consequently the peptide's mass alone could not be used for definitive identification. Therefore, identification of the peptides, produced by pepsin digestion of the proteins, was achieved using a combination of accurate mass (<1 ppm) from the FT-ICR measurements and MS/MS product ion spectra of selected ions using QToF mass spectrometry. Once positive identification was achieved, peptides were monitored using their masses and retention

times. For GSK-mAb 115 peptides were identified for which it was possible to measure their hydrogen exchange profiles. This allowed sequence coverage of 80% to be achieved. Coverage was not achieved for the heavy chain hinge region and the peptide at the site of glycan attachment, for the light chain coverage was partial for the variable region and parts of the chain containing disulfide bonds.

Each sample was analysed by measuring the exchange at seven time points, with four repeats of each measurement. The deuterium exchange has been expressed as the percentage of maximal expected deuterium exchange for the peptide analysed, see methods section for details on the calculation. For the mAbs the exchange of the peptides was between 0 and 75%, and the standard deviation of the four repeats of these measurements was on average 0.7%.

The results of the HXMS measurements of GSK-mAb, without oxidative stress, are shown in Figure 1. Selected peptides have been plotted onto the protein sequence as stacked bar plots. In these

Table I. Approximate Levels of Methionine Oxidation in the Native and Oxidised Samples of GSK-mAb.

Methionine	Native sample	Oxidised sample
HC 34	N.D.	<5%
HC 83	N.D.	<5%
HC 253	<5%	90%
HC 430	N.D.	70%
LC 4	N.D.	<5%

N.D. = Not detected.

plots the width of the stack of seven bars covers the sequence of the peptide that was identified. Each bar in the stack represents a time of exchange, from the shortest at the top of the stack to the longest time of exchange at the bottom of the stack. The colour of the bar represents the amount of exchange that was measured with blue colours representing lowest amounts of exchange to red colours the highest amounts of exchange. It should be noted that although the bars cover the whole of the peptide, the exchange of the first two residues are not measured, as the first residue does not have an amide, it forms the amine on the N-terminus, and the amide second residue from the N-terminus is lost from the analysis due to its rapid exchange. The hydrogen exchange at proline residues are not analysed either, as prolines do not have a backbone amide. Figure 1 shows that most of regions of the protein did not exchange more than 50%. The low amount of exchange is probably due to the extensive secondary

structure of IgGs, which contain a large amount of stable β -sheet structure. Regions of the protein chain that showed a higher extent of exchange were the C-terminus of the heavy chain and the C-terminal side of the C_H1 domain and the N-terminal section of the C_H2 domain.

GSK-mAb was oxidised using 100 mM H₂O₂ for 30 min. The level of methionine oxidation that was observed for each methionine in the oxidised and nonoxidised samples is indicated in Table I. The methionine at residue 253 in the heavy chain oxidised readily, a small amount of oxidation of this residue was found in the native sample, after exposure to H₂O₂ this residue was largely oxidised. The methionine at residue 430 was also extensively oxidised. The relative levels of oxidation of these two residues were in agreement with previous reports.^{18,21} The masses of the intact mAb were also measured for the oxidised and non-oxidised samples, shown in supporting information. By comparison of the weighted average of the two spectra it was calculated that an average of 3.0 methionine residues had been oxidised in each mAb during the accelerated oxidation.

The deuterium exchange that was measured for each peptide was compared between the oxidised and non-oxidised samples. This allowed peptides with different exchange profiles in the oxidised and non-oxidised samples to be identified. The excellent repeatability of the system allowed small differences

Table II. Measured Amount of Deuterium Incorporation for peptides in the Variable Region of the Heavy Chain of GSK-mAb

Peptide	Native sample						
	Time of exchange (s)						
	30	100	300	1000	3000	10000	30000
HC[5–17]	20.1% $\pm$ 1.0%	29.3% $\pm$ 0.7%	35.8% $\pm$ 0.5%	43.9% $\pm$ 1.0%	52.1% $\pm$ 1.4%	56.2% $\pm$ 1.5%	59.2% $\pm$ 0.6%
HC[28–35]	13.7% $\pm$ 1.5%	23.9% $\pm$ 0.9%	29.0% $\pm$ 0.4%	32.9% $\pm$ 0.4%	36.8% $\pm$ 0.6%	41.4% $\pm$ 1.2%	45.9% $\pm$ 0.6%
HC[36–46]	24.1% $\pm$ 1.6%	29.7% $\pm$ 1.1%	29.7% $\pm$ 1.0%	32.0% $\pm$ 1.7%	36.2% $\pm$ 2.0%	42.7% $\pm$ 1.0%	47.6% $\pm$ 0.9%
HC[47–58]	9.1% $\pm$ 1.0%	14.3% $\pm$ 0.5%	19.2% $\pm$ 0.2%	21.4% $\pm$ 1.2%	22.2% $\pm$ 0.7%	22.4% $\pm$ 0.5%	24.6% $\pm$ 0.3%
HC[60–68]	19.4% $\pm$ 1.1%	21.5% $\pm$ 0.9%	25.0% $\pm$ 0.4%	34.1% $\pm$ 1.3%	42.3% $\pm$ 1.3%	44.9% $\pm$ 1.6%	49.5% $\pm$ 0.4%
HC[69–79]	21.2% $\pm$ 1.0%	27.4% $\pm$ 0.4%	31.0% $\pm$ 0.3%	33.6% $\pm$ 0.5%	34.6% $\pm$ 0.7%	36.7% $\pm$ 0.9%	41.3% $\pm$ 0.5%
HC[84–93]	13.5% $\pm$ 0.6%	13.5% $\pm$ 0.3%	12.6% $\pm$ 0.2%	13.0% $\pm$ 0.7%	13.5% $\pm$ 1.5%	15.1% $\pm$ 1.3%	18.4% $\pm$ 0.2%
HC[104–115]	21.4% $\pm$ 0.9%	23.9% $\pm$ 0.5%	24.2% $\pm$ 0.3%	26.5% $\pm$ 0.4%	29.3% $\pm$ 0.5%	33.0% $\pm$ 0.3%	38.8% $\pm$ 0.4%
Peptide	Oxidised sample						
	Time of exchange (s)						
	30	100	300	1000	3000	10000	30000
HC[5–17]	20.7% $\pm$ 1.7%	30.0% $\pm$ 0.9%	36.5% $\pm$ 0.2%	44.7% $\pm$ 0.4%	51.9% $\pm$ 0.3%	56.5% $\pm$ 1.3%	59.2% $\pm$ 0.2%
HC[28–35]	13.7% $\pm$ 1.8%	24.0% $\pm$ 0.3%	28.6% $\pm$ 0.3%	32.9% $\pm$ 0.2%	36.4% $\pm$ 0.4%	41.8% $\pm$ 0.6%	45.6% $\pm$ 0.2%
HC[36–46]	24.8% $\pm$ 0.9%	30.2% $\pm$ 1.1%	30.0% $\pm$ 1.1%	31.9% $\pm$ 1.3%	36.7% $\pm$ 0.5%	43.2% $\pm$ 1.6%	47.3% $\pm$ 0.3%
HC[47–58]	9.6% $\pm$ 0.8%	14.7% $\pm$ 0.2%	19.6% $\pm$ 0.4%	21.8% $\pm$ 0.3%	22.5% $\pm$ 0.4%	22.8% $\pm$ 1.0%	25.0% $\pm$ 0.5%
HC[60–68]	19.5% $\pm$ 1.7%	21.8% $\pm$ 1.0%	25.4% $\pm$ 1.0%	34.9% $\pm$ 0.4%	42.3% $\pm$ 1.3%	44.9% $\pm$ 2.6%	49.1% $\pm$ 2.2%
HC[69–79]	21.8% $\pm$ 0.9%	27.8% $\pm$ 0.5%	31.2% $\pm$ 0.5%	33.7% $\pm$ 0.1%	34.7% $\pm$ 0.5%	37.3% $\pm$ 1.1%	41.7% $\pm$ 0.1%
HC[84–93]	13.5% $\pm$ 0.5%	13.3% $\pm$ 0.1%	12.1% $\pm$ 0.4%	12.6% $\pm$ 0.1%	13.2% $\pm$ 0.3%	14.9% $\pm$ 1.0%	18.4% $\pm$ 0.1%
HC[104–115]	21.4% $\pm$ 0.9%	24.2% $\pm$ 0.2%	24.4% $\pm$ 0.6%	26.7% $\pm$ 0.6%	29.6% $\pm$ 0.5%	33.5% $\pm$ 0.3%	39.1% $\pm$ 0.4%

The percentage exchange was calculated as described in the experimental section. Measurements have been reported as the average of 4 repeats with the error reported as 2 standard deviations of the 4 repeats.

Figure 2. Comparison of the hydrogen exchange measured for the peptide FLFPPKPKDTL (HC[242–252]) in native and oxidised samples of GSK-mAb. Plotted values are the average of 4 measurements, with the error bars showing 2 standard deviations of the measurement.

in exchange to be identified. For most of the peptides in the variable domain there was not a significant difference between their exchange in the native and oxidised samples. This is demonstrated in Table II where the exchange and two standard deviations of the measurement for eight peptides in the variable domain of the heavy chain are shown.

Differences in deuterium exchange were observed for several peptides in the Fc region. Peptides that were found to have differences in their exchange profiles were mostly found to have between 2–5% greater level of deuterium incorporation. Peptides that displayed this level of extra exchange were located in the following regions; HC[267–278], HC[280–295], HC[309–320], HC[336–360], HC[371–382], and HC[431–449]. Peptides between residues HC[242–253] were, however, found to have a much greater level of deuterium incorporation, in this case measured percentages of deuterium incorporation were up to 30% greater. Figure 2 shows the exchange that was measured for the peptide FLFPPKPKDTL, corresponding to residues 243–252 of the heavy chain. The peptide is located in the Fc region of the antibody adjacent to Met253, which was found to oxidise readily. Examination of the position of this peptide in the crystal structure of a homologous antibody (PDB entry 1HZH) showed it to be in contact with the glycan attached to that chain and also positioned close to Met430. The peptides in the Fc region for which differences in the rate of exchange had been identified, were placed into two groups, those with a large difference in exchange (>5%) and those for which a small difference in exchange (<5%). Figure 3 shows the crystal structure of the Fc region of a homologous antibody with the peptides that had larger differences in exchange coloured red and those with smaller differences coloured pink. The positions of the methionine residues and the glycans are highlighted in blue and green respectively. The peptides for which a change

in exchange profile was identified are located close to the two methionine residues in the Fc region and had residues in the interface between the C_H2 and C_H3 domains in the crystal structure. Comparison of the exchange profiles of the native and oxidised IgG1 samples enabled us to demonstrate that methionine oxidation resulted in structural changes to the IgG1s when the glycan is present, which is in contrast to the results reported by H. Liu *et al.*,¹⁸ we however, used a more direct measure of protein structure.

The non-specific nature of pepsin hydrolysis often results in overlapping peptides in the peptide map 22. This can be used to increase the resolution of the measured exchange.^{22,23} Three peptides that overlapped with HC[243–252] were found, these were HC[242–252], HC[242–253] and HC[243–253]. By comparison of the exchange of these peptides it was possible to establish that the amide on Phe244 on the heavy chain did not undergo hydrogen exchange, over the time course of the experiment. This also allows us to establish that the exchange that was observed for peptide HC[242–252] occurred on residues 247 or 249–252. The exchange of the two N-terminal residues of peptide HC[242–252] are not observed, the first residues does not contain an amide and the second is rapidly lost during analysis, it was also established that the third residue Phe244 did not exchange, residues 245, 246 and 248 are

Figure 3. Crystal structure of Fc region of an IgG (PDB entry 1HZH). Glycans are coloured green, residues coloured pink are peptides that exchanged faster in oxidised sample than in native sample of GSK-mAb. Residues coloured red are from peptides that exchanged much faster in the oxidised sample. Residue coloured dark blue is Met253. Residue coloured light blue is Met430. Sulfur atoms of methionine residues are coloured yellow. Residues 247–252 have been coloured a darker shade of red.

Figure 4. Hydrogen exchange measured for Met253 in native and oxidised samples of GSK-mAb. Standard deviations shown were calculated from a combination of those from the measurements used to calculate exchange of Met253.

prolines and do not have backbone amides, therefore the exchange must have occurred on the remaining residues of the peptide.

A second comparison was possible using the overlapping peptides. The exchange of residue Met253 was calculated and comparison made between the native and oxidised samples. Figure 4 shows the hydrogen exchange calculated for the amide of Met253 by subtraction of the rates of HC[243–253] and HC[243–252], which was calculated for the native and oxidised samples. The amide of this residue was found to exchange in both samples, with a rate of exchange that was different in the native and oxidised samples. The difference in rate of exchange of this residue between the two samples implied that there was a conformational change in this region of the protein, which did not result from oxidation of Met253 alone.

The hydrogen exchange profile of peptide HC[242–253] in both its methionine and methionine

Figure 5. Comparison of the measured hydrogen exchange for peptides FLPPKPKDTLM and the same peptide containing oxidised methionine FLPPKPKDTLMox (HC[242–253]) in native and oxidised samples of GSK-mAb. Plotted values are the average of 4 measurements, with the error bars showing 2 standard deviations of the measurement.

sulfoxide form in the native and oxidatively stressed samples are plotted in Figure 5. The measured exchange for this peptide in the methionine form was different in the native and oxidatively stressed sample, and the exchange of this peptide in the methionine sulfoxide form was different in the native and oxidatively stressed samples. Figure 5 also shows that the magnitude of the difference in measured exchanges between the two solutions was greatest for the methionine peptide. This was taken to indicate that the greatest effect on the destabilisation of peptide HC[242–253] resulted from oxidation of Met253, however, the structure was also destabilised by another factor or factors. From the data on the exchange of this peptide described so far it was concluded that the oxidation of Met253 resulted in a change in the protein's structure within residues HC[247–253], however, even when the methionine in this peptide was not oxidised there was a change in the structure of this region of the protein following oxidative stress. Two hypotheses for the cause of the change in structure of this region of the protein were considered. Firstly, that the structural change observed resulted from oxidation of Met430 or alternatively, that the structure of the glycans was affected by the oxidation of the antibody and resulted in a change in structure of this region of the protein. We deemed it to be important that the possibility that the glycan was influencing this region of the protein was investigated, as Houde *et al* had demonstrated that the structure of this same region was affected by the removal of the glycans.¹³

To further investigate the possibility that the glycan groups were involved, we first established that the oxidation had not resulted in chemical modification of the glycan. This was achieved using PNGase F to enzymatically cleave the glycans from the protein followed by analysis by CGE-LIF and MALDI-TOF as shown in Figures 6 and 7, respectively. For the oxidised and unoxidised samples, the profiles show no significant differences using both

Figure 6. CGE-LIF analysis of APTS labelled GSK-mAb glycans from the oxidised and unoxidised samples released using the PNGase F digestion protocol. Capillary temperature was maintained at 20°C.

Figure 7. Positive ion MALDI mass spectra of glycans from oxidised and unoxidised GSK-mAb samples released using the PNGase F digestion protocol.

techniques and are consistent with the core fucosylated biantennary structures expected. The exact structural confirmation was not verified, as only a comparative profile was required to establish the absence of chemical modification to the glycan groups during the oxidation process. There was no covalent modification of the glycans. Therefore, for the glycans to have been involved in the structural change observed in peptide HC[243–253] a structural rearrangement of the glycans would have been required. From the results obtained it was not possible to establish whether or not the glycans had altered structure, we can only speculate as to a possible mechanism by which the glycan structure may have been altered and resulted in destabilisation of the peptide HC[243–253]. IgG1s contain two identical heavy chains, we were able to measure the hydrogen exchange of peptide HC[242–253] in its methionine and methionine sulfoxide forms, however, we were not able to determine that these peptides originated from IgG1s that contained oxidised or unoxidised Met253 in both chains. Consequently, although we established that residues HC[247–253] are destabilised in the oxidised sample, even when the methionine in the peptide is not oxidised, we were unable to establish whether this had or had not resulted from oxidation of Met253 on the opposite heavy

chain. We therefore cannot eliminate the possibility that as oxidation of Met253 resulted in the destabilisation of residues HC[247–253], which in turn destabilises the glycan structure, resulting in destabilisation of HC[247–253] on the second heavy chain. Though this appears to be a somewhat convoluted mechanism to explain the observations, it has been shown that there is contact between the two glycans through the two core mannose residues and that in the absence of this interaction the structure of the CH₂ domains are perturbed, highlighting the interaction between the glycans and this region of the Fc.^{24–26}

Now we consider the possibility that the effect observed was due to oxidation of Met430. Residues HC[247–253] have been shaded dark red in Figure 3, it can be seen that in the crystal structure these residues form an α -helix, which is located adjacent to methionines 253 and 430. Therefore it appears likely that this α -helix could be destabilised by oxidation of methionine 430 or 253. D. Liu *et al* reported analysis by DSC of mutants of Human IgG1 Fc that lacked either methionine residue analysis of Human IgG1 Fc demonstrated that oxidation of either methionine resulted in structural changes to the C_H2 and C_H3 domains, the results described here add further weight to this conclusion.¹⁷ It may

envisaged that measurement of the hydrogen exchange at the level of the intact mAb¹³ would allow clarification of which of the two hypothesis outlined above is correct. However, such experiments would be extremely challenging, due to the dispersity of the mAb masses, which results from the combined effect of the non-uniform glycosylation and oxidation. Mass resolution of the oxidised mAbs would be inherently difficult due to the overlapping isotope distributions of the oxidised mAbs, as illustrated in as Supplementary Material. The situation is complicated further if the widening of the isotope distributions, which results from the hydrogen exchange is also considered. It should also be noted that as oxidation was not induced at specific methionine residues, therefore although it may be possible to resolve species containing specific numbers of oxidations, the specific locations of these would not be known.

We have not been able to definitely determine which of the two hypotheses is correct from the data available, however, it would appear more appropriate to choose a simple mechanism involving oxidation of a local methionine over an elaborate mechanism involving the glycans.

Materials and Methods

Materials

Acetonitrile was Optigrade from LGC-Promochem (Teddington, UK), the water used was 18.2 M Ω cm⁻¹, 99.9% D₂O was from Fluorchem (Old Glossop, UK). The quench solution was made from a 7 M guanidine hydrochloride solution and 50 mM TCEP (tris(2-carboxyethyl)phosphine), to which NaOH had been added so that the pH of a 1:1 mixture of the quench and sample solutions was 2.5. Solutions of mAbs were provided by GSK (Beckenham, UK) in formulation buffers. PNGase F release kit and Ludger clean EB10 cartridges were purchased from Ludger. "Carbohydrate Labelling and Analysis Kit" as supplied (Beckman Coulter, Fullerton, CA), containing separation gel buffer, neutral coated capillary, APTS labelling solutions. 2,5-dihydroxybenzoic acid (DHB) and Pep Mix II calibration were from Bruker Daltonics.

Automated hydrogen/deuterium exchange analysis

The system used to perform the HXMS analysis has been described previously.²⁷ Briefly, the system used a HTS-PAL robotic autosampler to perform solvent manipulations, columns and switching valves were maintained at 0°C using a cooled water bath. Samples were exchanged in D₂O for 30 s, 100 s, 300 s, 1000 s, 3000 s, 10,000 s or 30,000 s (10 μ L of sample of concentration 10 mg ml⁻¹ was mixed with 190 μ L of D₂O for each analysis). After the desired amount

of time had elapsed for each sample, 45 μ L was taken and mixed with 45 μ L of quench solution, 20 μ L of this was then taken and injected on to the "dual column" HPLC apparatus for digestion with immobilised pepsin and separation of the resulting peptides, under conditions of slow exchange on reverse phase columns. Four repeats of each time point were performed, with samples being analysed in a semirandom order. A digestion time of 13 min was used.

Mass spectrometry

Mass spectrometry measurements for the HXMS were performed on a 4.7 T Bruker (Billerica, USA) Apex III Fourier transform ion cyclotron resonance (FTICR) mass spectrometer using positive mode electrospray ionisation (ESI). Bruker Daltonics ApexControl (version 1.0) and Bruker Daltonics HyStar (version 3.2) were used to control the instrument. Data acquisitions were initiated by contact closure, one spectrum was collected approximately once every three seconds over a ten-minute period, during which the peptides were eluting into the ion source of the mass spectrometer. Peptide identification was achieved using a combination of accurate mass from the FTICR measurements and tandem mass spectrometry (MS/MS) measurements made with a quadrupole time-of-flight (QToF) mass spectrometer, a QSTAR from Applied Biosystems (Foster City, USA). Matrix-assisted laser desorption/ionisation (MALDI) experiments were carried out using an Ultraflex II TOF TOF, Bruker (Billerica, USA) with a Smartbeam laser, operated in positive ion, reflectron mode with delayed extraction. Spectra were acquired using the flexControl software and processed using flexAnalysis software.

Preparation of oxidatively stressed samples

Samples were dialysed into a 10 mM sodium phosphate buffer (pH 7.4). Oxidation reactions were then performed at 37 °C, by addition of hydrogen peroxide to a concentration of 100 mM to each sample. After 30 minutes the samples were dialysed back into formulation buffer (pH = 5.5, 4 °C).

Calculation of exchange data

For each sample and for each peptide at the seven time points and four repeat measurements the average mass of the peptide was calculated from the charge state of the peptide with the intensities and m/z of the peaks in the peptide's isotopic distribution. This mass was used to calculate the number of deuteriums that had been incorporated into the peptide. Where the percentage deuterium exchange has been used it was calculated using the measured number of deuteriums that had exchanged for that peptide and the maximum number of amide hydrogens for which the exchange was expected to have

been measurable using following formula, $N-2P$ where N is the number of amino acid residues, P is the number of proline residues but not including those present in the first two N-terminal residues. This formula assumes that the amide on the second residue exchanges rapidly and is not observed in the measurements.¹⁵

Glycan analysis

Glycans were released from 35 μL aliquots of oxidised and unoxidised samples at a concentration of approximately $10\text{ }\mu\text{g mL}^{-1}$ using the PNGase F kit as directed, with the exception that the reduction reagent was replaced with a TCEP solution. This reduced the sample whilst maintaining the sample at a lower pH, which avoided precipitation of the protein. All samples were incubated at $37\text{ }^{\circ}\text{C}$ overnight, and glycans isolated using the EB10 cartridges as described in the manufacturer's instructions. The samples were then split into two equal aliquots and the solvent removed prior to analysis by capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) and MALDI mass spectrometry. For CGE-LIF the amino pyrene trisulfonic acid (APTS) labelling of samples was undertaken using the "ProteomeLab carbohydrate labelling and analysis" kit as supplied. APTS labelled samples were allowed to react overnight in the dark. A dextran ladder was prepared as a system suitability check. Maltose was used as an internal standard in every sample. For MALDI-TOF analysis the dried glycan sample was dissolved in $10\text{ }\mu\text{L}$ of water ($18\text{ M}\Omega\text{ cm}^{-1}$). $1\text{ }\mu\text{L}$ aliquots of each sample were then cleaned using a Nafion 117 membrane²⁸ prepared using nitric acid. This was then transferred directly onto a stainless steel target plate. $0.2\text{ }\mu\text{L}$ of DHB solution (saturated solution in acetonitrile) was added and allowed to dry followed by $0.2\text{ }\mu\text{L}$ of ethanol. Three replicate analyses were carried out for every sample.

Glycan profiling was performed by CGE-LIF on a ProteomeLab PA800 system (Beckman Coulter, Fullerton, CA, USA) using LIF detection with an excitation wavelength of 488 nm and an emission band pass filter of $520\text{ nm} \pm 10\text{ nm}$. A neutral coated capillary was employed, i.d. of $50\text{ }\mu\text{m}$, total length of 52 cm and effective length 40 cm . The new capillary was pre-rinsed with carbohydrate separation gel buffer for 10 minutes at 30 psi. Three replicate analysis carried out for every sample. All data was collected analysed using 32 Karat software V 7.

Conclusions

Structural changes in mAbs that resulted from oxidative stress have been studied using HXMS, and the technique has been demonstrated to be appropriate for the analysis of chemically stressed mAbs. Regions of the antibodies with altered hydrogen

exchange profile were determined, these were located in the Fc and contained residues in the interface between the $\text{C}_\text{H}2$ and $\text{C}_\text{H}3$ domains. The $\text{C}_\text{H}2$ - $\text{C}_\text{H}3$ region of the molecule has important receptor binding properties, and it has previously been suggested that a loss of stability in this region may result in a reduced half-life of an antibody *in vivo*.²¹ Furthermore it was also shown that the change in the hydrogen exchange of residues HC[247–253] was much greater than in other regions. The hydrogen exchange profiles of peptides from this region were studied in greater detail, which revealed that the oxidation of Met253 resulted in a reduction in the stability of this region of the protein. By comparison to the crystal structure of a homologous antibody revealed that these residues formed an α -helix. Further analysis of the exchange profiles of the peptides was undertaken, peptides containing Met253 or the same peptides that contained oxidised Met253 were analysed in solutions that contained an excess of either non-oxidised or oxidised antibody. This revealed that residues HC[247–253] were destabilised even when Met253 had not been oxidised. Two hypotheses for the mechanism for the destabilisation of this α -helix, which did not involve the oxidation of Met253, were considered. The preferred mechanism to explain the results was that of oxidation of Met430 also destabilised the α -helix of residues HC[247–253]. This indicated that oxidation of either Met253 or Met430 resulted in destabilisation of this α -helix. We were able to demonstrate this for the first time without using a mutant form of the antibody that lacked either methionine residue and we were able to demonstrate this effect for a glycosylated IgG rather than a deglycosylated IgG Fc and the use of isotopically labelled molecules was not required.

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