Published in final edited form as:

J Am Soc Mass Spectrom. 2013 June ; 24(6): 835–845. doi:10.1007/s13361-013-0582-4.

Complementary MS Methods Assist Conformational Characterization of Antibodies with Altered S–S Bonding Networks

Lisa M. Jones¹, Hao Zhang¹, Weidong Cui¹, Sandeep Kumar², Justin B. Sperry², James A. Carroll², and Michael L. Gross¹

¹Department of Chemistry, Washington University in St. Louis, One Brookings Drive, St. Louis, MO 63130, USA

²Analytical Research and Development, Biotherapeutics Pharmaceutical Sciences, Pfizer, Inc., Chesterfield, MO 63017, USA

Abstract

As therapeutic monoclonal antibodies (mAbs) become a major focus in biotechnology and a source of the next-generation drugs, new analytical methods or combination methods are needed for monitoring changes in higher order structure and effects of post-translational modifications. The complexity of these molecules and their vulnerability to structural change provide a serious challenge. We describe here the use of complementary mass spectrometry methods that not only characterize mutant mAbs but also may provide a general framework for characterizing higher order structure of other protein therapeutics and biosimilars. To frame the challenge, we selected members of the IgG2 subclass that have distinct disulfide isomeric structures as a model to

© American Society for Mass Spectrometry, 2013

Correspondence to: Michael L. Gross; mgross@wustl.edu.

Lisa M. Jones and Hao Zhang contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s13361-013-0582-4) contains supplementary material, which is available to authorized users.

evaluate an overall approach that uses ion mobility, top-down MS sequencing, and protein footprinting in the form of fast photochemical oxidation of proteins (FPOP). These three methods are rapid, sensitive, respond to subtle changes in conformation of Cys→Ser mutants of an IgG2, each representing a single disulfide isoform, and may be used in series to probe higher order structure. The outcome suggests that this approach of using various methods in combination can assist the development and quality control of protein therapeutics.

Keywords

Antibody characterization; Protein structure; Fast photochemical oxidation of proteins (FPOP); Top-down; Ion mobility; Antibody mutants; IgG2

Introduction

The development and quality assurance of protein therapeutics and biosimilars present a significant challenge because proteins, unlike small-molecule drugs, are highly vulnerable to conformation and oligomerization-state changes that may compromise efficacy and pose immune problems [1]. The issue is immediate because the worldwide sale of biologics already exceeds \$100 billion annually [1] and is growing by approximately 10 % per year. Biotherapeutics, which include recombinant blood products, monoclonal anti-bodies, and recombinant vaccines [2], provide treatment for a wide range of diseases [3, 4]. Specific analytical needs are evaluation of biosimilars and demonstration of similarity between innovator and biosimilar materials [1, 5], especially as production methods change and regulatory approval is sought (these issues are covered in recent reviews [1, 5–7]). The reviews emphasize approaches to determining primary structure and locating sites of glycosylation. The issue we address is higher order structure and conformation. The best approach may be a “totality-of-the-evidence” approach that uses multiple complementary methods to evaluate structure, providing “fingerprints” of products that are manufactured differently [8]. Here, we describe our response to this need by implementing a serial MS-based approach for characterizing antibody mutants.

We chose monoclonal antibodies (mAbs) because they are the largest class of developing biotherapeutics [9, 10]. Most are of the IgG1 subclass owing to its increased effector functions [11]. Interest in IgG2, however, is increasing in indication where effector functions are unnecessary [12] or even detrimental to the therapeutic effect [13]. A successful approach should be sensitive to small differences in primary and higher order structure from changes in manufacturing or storage.

IgG2 antibodies display disulfide-bond heterogeneity [14, 15]. Previously, three distinct disulfide isomers, IgG2-A, A/B, and B (Figure 1), were partially separated, and their disulfide-bonding patterns mapped [14]. Depending on redox environment, these isomers interconvert, possibly enabling IgG2 antibodies to regulate function [15]. To understand the role of isomers, Lightle et al. [16] constructed cysteine-to-serine substitutions on the IgG2 anti-CD44 antibody. The single and double substitutions force the antibody into a single isoform that displays no significant binding differences to the CD44 extracellular domain [16]. A large difference occurs, however, in the binding of a secondary antibody to the hinge region of the IgG2, suggesting that disulfide isomers have structural alterations in the hinge region. We take advantage of the availability of these substituted IgG2 antibodies to test the sensitivity of three mass-spectrometry methods used in combination to reflect structure of these closely related materials.

Elucidating conformational differences of IgG2 isomers should take advantage of X-ray crystallography, NMR, mass spectrometry (MS), and molecular modeling/dynamic simulations [17–21]. MS, although of lower resolution than NMR and X-ray, offers multiple protein-structure approaches [17–21], the sum of which is “greater than the parts” [22]. Approaches amenable to combination include ion mobility MS (IMMS) [23], top-down MS [24, 25], protein footprinting [26–30], peptide mapping [31], native ESI [32], and high mass resolving power FTICR MS [33], all of which have been used separately for antibody structure. IMMS reports on size and shape of macromolecules [34], whereas top-down MS provides structural information lost in bottom-up proteomics [20, 35, 36]. Footprinting reveals site-specific, conformation-sensitive solvent accessibility. Footprinting via fast photochemical oxidation of proteins (FPOP) labels on the μ s timescale [37, 38], preempting any labeling-induced structural changes and providing residue-level information [28, 39]. These methods, although powerful in their own right, have different turnaround times. IMMS and top-down MS experiments, for example, can be conducted in approximately 1 h, whereas footprinting is considerably slower for data processing but has considerably higher resolution at the peptide and even amino-acid levels. Thus, we first applied modeling, ion mobility, and top-down MS because they have faster turnaround. Differences seen by IMMS or top-down MS motivate carrying out the more time-demanding footprinting experiments (e.g., FPOP, hydrogen deuterium exchange (H/DX), or specific amino-acid labeling) to pinpoint the conformational differences between samples (here, disulfide isomers of the IgG2 antibodies [14]). This “toolkit” not only characterizes the structural differences of IgG2 isomers but also provides a framework for assessing other biotherapeutics.

Experimental

Materials

Hydrogen peroxide (30 %), *L*-glutamine, *L*-methionine, trypsin, catalase, phosphate buffered saline, urea, *tris*-(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl), iodoacetamide, *DL*-dithiothreitol (DTT), acetonitrile, water, ammonium acetate, and cesium perfluoroheptanoate were from Sigma-Aldrich (St. Louis, MO, USA). Lys-C was from Roche Applied Science (Indianapolis, IN, USA). Silica capillary tubing (360 μ m o.d., 150 μ m i.d.) was from Polymicro Technologies (Phoenix, AZ, USA). The native ESI spray tip was custom-made by using a pulling machine (Sutter Instrument Co., Novato, CA, USA), as described elsewhere [40]. The protocols of cloning, expression, and purification for human IgG2 antibody (anti-CD44 monoclonal antibody) were reported previously [16]. Those protocols were adopted without modification for preparing the mAb samples used in these experiments.

Homology-Based Model Building

The homology model for full length CD44 mAb was made using ‘Homology Model’ module in MOE (Chemical Computing group, www.ccg.com). The crystal structure of a full length murine IgG2a antibody from Protein Data Bank (PDB) (www.pdb.org, 1IGT) was the template, after replacing its hinge with that found in human IgG2 mAbs [41]. The murine IgG2a structure (1IGT) was chosen over that of full-length human IgG1 antibody because its hinge region is most similar in sequence with the human hinge IgG2. One thousand (100 back-bone models and 10 side-chain models per backbone model) intermediate homology models were generated and optimized via energy minimization by using the homology model module in MOE2010.10 (all-atom AMBER99 force field was used). A cutoff of 12 Å with switching starting at 10 Å was applied to non-bonded interactions. Generalized Born implicit solvation was employed; interior and exterior dielectric values were 4 and 80, respectively. The 1000 intermediate models were ranked using Generalized Born Volume Integral (GB/VI) score, and the best ranked model was selected for further optimizations.

The final model was checked for stereochemical precision and was found to be of high quality.

Structural Models of Mutants

The initial A-form homology model of the CD44 IgG2 mAb was used to create models for Cys→Ser single and double mutants described here. The relevant Cys residue was mutated to Ser using 'Mutate' command in MOE. The side-chain rotamers for the mutated residue were explored to find the best rotamer. The conformations of mutated residue (or two residues in case of double mutants) and the neighboring residues were energy minimized while keeping fixed the remaining atoms. In the next step, all of the atoms were unfixed, and the whole model was energy minimized again by using the protocol described above.

Accessible Surface Area Calculations

Accessible surface area values for wild type (WT) and mutant antibodies were computed using 'Solvent Accessibility' command under Structure module in Discovery Studio 3.1.

Ion Mobility

Antibodies were buffer exchanged into 200 mM ammonium acetate pH6.8 with a Vivaspin 500 ultrafiltration device (Vivaproducts, Littleton, MA, USA). Antibody samples were delivered by NanoAcquity UPLC (Waters Inc., Milford, MA, USA) at flow rate of 100–400 nL/min. The sample solution was injected to a hybrid ion-mobility quadrupole time-of-flight (Q-IM-TOF, Synapt G2 HDMS; Waters Inc., Milford, MA, USA) by a custom nanospray tip. All samples were acquired under the same gentle instrument conditions (capillary voltage 1.5 kV, sampling cone 80 V, extraction cone 4 V, source temperature 80 °C, trap collision energy 6 V, and transfer collision energy 0 V). The pressure of the backing region was 5 mbar. For the IM, the helium cell and the IMS gas flows were 180 and 90 mL/min, respectively, the IMS wave velocity was 650 m/s, and the IMS wave height was 40 V. Nitrogen was the carrier gas. An IM spectrum was acquired from m/z 1500–7500 every 1 s, after external calibration to 8000 m/z with NaI solution. Peak picking and data processing were by Masslynx (ver. 4.1) and DriftScope software (Waters Inc., Milford, MA, USA). The ion-mobility signal was averaged for 2–3 min, and Gaussian fitting gave centroid drift time values (in triplicate).

Top-Down Mass Spectrometry

Antibody samples in the 200 mM NH_4OAc solution were infused into a Solarix 12 T FTICR mass spectrometer (Bruker Daltonics, Bremen, Germany) by nanospray at 100 nL/min. The capillary voltage was 1.1–1.5 kV. External calibration to m/z of 8500 was with cesium perfluoroheptanoate clusters. Parameter optimization gave a good native ESI spectrum, as described previously [20, 42]. For top-down MS, the voltage for in-source collision-induced dissociation (ISCID) was tuned from 0 to 100 V. Electron-capture dissociation (ECD) pulse length was 0.04 s, bias was 0.4 V, and lens voltage was 10 V. The ECD hollow cathode heater current was 1.6 A. Both ISCID and ECD were applied to obtain fragmentation. Spectra from 200 to 15000 m/z were an average of several hundred scans. Peak picking and spectral deconvolution were done by using Bruker Data Analysis software and Prosight PTM (V1.0, <https://prosigthptm.northwestern.edu>).

FPOP Labeling

Antibody samples were buffer exchanged into PBS buffer (10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4) and diluted to 10 μM in PBS. Glutamine (15 mM) was added to scavenge OH radicals, limiting their lifetime to $\sim 1 \mu\text{s}$ [37]. Immediately prior to labeling, 15 mM H_2O_2 was added, and samples were infused into silica tubing past a 2.45

cm window at a rate of 14 μ L/min with an exclusion fraction of 15%. The laser frequency was set to 5 Hz, and the power was 42 mJ. The FPOP device setup followed a previously published protocol without modification [39]. Samples were labeled and analyzed in triplicate. Samples were dried, resuspended in 8 M urea, reduced with 10 mM TCEP-HCl, alkylated with 20 mM iodoacetamide, and digested with LysC (1:100 ratio), trypsin (1:20 ratio), and desalted using C₁₈ Nu-Tip zip tips (Glygen, Columbia, MD, USA), as previously described [28]. The samples were loaded in a silica capillary column, custom-packed with C₁₈ reversed phase (Magic, 0.075 mm $\times$ 150 mm, 5 μ m, 120 Å; Michrom Bioresources, Inc., Auburn, CA, USA) with a custom-pulled tip. Samples were separated by using a 60 min gradient from 0%–80% acetonitrile at a flow rate of 260 nL/min pumped by an Ultra 1D +UPLC (Eksigent, Dublin, CA, USA). Product-ion mass spectra were collected on a LTQ-Orbitrap XL in data-dependent mode controlled by Xcalibur 2.0.1 software (Thermo-Fisher, San Jose, CA, USA) over m/z 350–2000 range at mass resolving power of 60,000 for m/z 400. The six most abundant ions, with a charge of at least +2 (+1 ions were rejected), with a minimum intensity of 1000 were subjected to CID in the linear ion trap.

FPOP Data Analysis

Data was analyzed as previously described [28]. Briefly, the raw MS files were aligned by using Rosetta Elucidator ver. 3.3.0.0.220 (Rosetta Biosoftware, Seattle, WA, USA) and then searched for modified and unmodified tryptic peptides by using MASCOT 2.2.06 (Matrix Science, London, UK). All known oxidative modifications [43–45] were added to the search. The extent of modification was calculated as described previously [28, 39]. Alkylation with iodoacetamide adds a carbamidomethyl group (MW=57.0214 Da) to cysteine-containing peptides. Additionally, the N-terminus of several peptides was carbamylated (increase of 43.0058 Da) by urea. These modifications were subtracted from the Net Mass Change column in Supplementary Information Table S1 and Table S2, and were not used for quantitative analysis of oxidative labeling.

Results

Modeling Shows Hinge-Region Changes

We built a homology-based model for the full-length WT by using the murine IgG2 mAb crystal structure (PDB entry, 1IGT) as a template to obtain models for the C135S, C223S, C224S, and C223S/C224S mutants (Figure 2). For C135S, where heavy-chain Cys is substituted by Ser, one possibility for its disulfide partner Cys214 in the light chain is to remain reduced. Alternatively, Cys214 can form a non-native S–S with either Cys223 or Cys224 in the upper hinge region, requiring breakage of inter-heavy chain S–S between Cys223 or Cys224. C135S (Figure 2) has a S–S between Cys214 (light chain) and Cys223 (hinge), inducing IgG1-like inter-chain S–S bonding (Figure 1). The C223S, C224S, and C223S/C224S mutants have an S–S between Cys135 (Fab, heavy chain) and Cys214 (Figure 2), as for IgG4 inter-chain S–S (Figure 1).

Solvent-accessible surface areas (SASA) from modeling show that mutants expose smaller surface areas than WT (based on the IgG2-A disulfide isoform), suggesting that mutants are more compact than WT disulfide isoform A (Table 1). The hinge regions have greater root mean square deviations (RMSD, Table 2) for C135S (light-chain Cys214 to heavy-chain Cys223), C223S, C224S, and C223S-C224S double mutant (Supplementary Information, Figure S1), forecasting large conformational changes in the hinge. The Fab arms of the mutants move towards each other and the Fc, and most conformational changes occur in the hinge, consistent with molecular dynamics of Hinge++ [41], as will be experimentally confirmed later.

Ion Mobility Reveals Conformational Differences

Given that IM is fast and gives a global view of conformational differences, it is a good first choice for experimentally testing differences of antibodies. Previous studies underscored the efficacy of IMMS for IgG2 disulfide isomers [23], permitting distinction of IgG2-A and IgG2-B. A single Cys→Ser substitution at 223 does not cause a change in drift compared with IgG2-A.

For conformational analysis of WT and disulfide mutants, we preserved structure by using native ESI (200 mM NH₄OAc) and saw the expected lower charging (Figure 3a) compared with denaturing ESI. The WT shows two peaks (Figure 3a), representing two conformers (Figure 3b), likely B and A/B, consistent with HPLC that shows WT is comprised of B (45%), A/B (35%), and A (15%) (Supplementary Information, Figure S2) [16, 46]. The drift times of the four mutants and that of one conformer of WT (Figure 3c) are similar, indicating the mutants are more compact than the slower drifting conformer of WT. Similarly, Bagal et al. [23] separated IgG2 into A (42%) and B (58%) by IMMS and demonstrated that A has the shorter drift time. We found that the hinge mutants, C223S, C224S, and C223S/C224S, have an A-like conformation because their drift times are similar to that of C135S that has an IgG1-like conformation. An A conformation is similar to that of IgG1, which was shown previously [23]. The faster drifting isoform in the WT sample is the A/B isoform, and the slower drifting, less-compact species is the B isoform. Conformer A may co-drift with the A/B, but this awaits confirmation by enrichment studies. Clearly, IMMS can reflect conformational states of IgG isoforms, but it does not provide high resolution.

Native ESI Top-Down MS Shows Flexible Regions

Antibodies under denatured conditions were studied by using ETD (electron transfer dissociation) [25] top-down analysis. Although 15 %–21 % sequence coverage can be achieved upon introduction by denaturing ESI, this coverage is not applicable here because the antibodies (Figure 4a) were introduced with native-like structures. Besides having fewer charges than when introduced by denaturing ESI, the antibody ions also give a narrower charge state span (+24 to +28) and higher *m/z*. ECD without preactivation cleaved a limited number of backbone sites in the light chain, consistent with considerable intramolecular interactions in this region. In our previous work [20, 42], we hypothesized that top-down fragmentation combined with native ESI of an intact protein assembly prefers to occur in flexible regions with high B-factor values, a measure of such in X-ray crystallography. ECD, following in-source collision-induced dissociation (ISCID) at 50 V, induces fragmentation (Figure 4a) to yield ions of *m/z* 500–4000 from the antibodies. Although no heavy-chain fragments are formed, the WT and mutants afford several light-chain c-type fragments (from residues 88–111, a region presumably of high flexibility [Figure 4b]). Fragmentation occurs close to the N terminus whereas the C-terminus (residues 126–214) is unreactive, presumably because this region is less flexible than the N-terminus (Figure 4b).

The fragmented, flexible regions of the protein, when mapped onto the homology model of the WT antibody, are in the loop secondary structure (Figure 4c) between Cys⁸⁸ and Cys¹³⁴, bounded by two disulfide bonds, Cys²³–Cys⁸⁸ and Cys¹³⁴–Cys¹⁹⁴. Presumably, these S–S bonds impart rigidity to the remaining part of the antibody. All mutant antibodies displayed ECD patterns that are similar to those of WT (Supplementary Information, Figure S3), indicating that disulfide isomers have minimal conformational differences in the light chain.

FPOP Correlates Flexibility and Solvent Accessibility

Another approach to distinguish macromolecules is footprinting. Hydrogen deuterium exchange (H/DX), which has been used for IgG1 antibodies [21, 27, 47], is arguably the

most widely used MS-based approach. H/DX of IgG2 antibodies is hampered, however, by difficulties in reducing the hinge disulfides and in digesting the hinge region, owing to the four closely spaced disulfide bonds in this region [48]. In contrast, IgG1 antibodies contain only two disulfide bonds in this region. Further, the hinge region of the IgG2 antibody used in this study contains several proline residues. Pepsin, the primary enzyme used in HDX studies, does not cleave well at proline sites [48, 49]. We invoked, alternatively, fast photochemical oxidation of proteins (FPOP) to probe differences between IgG2 isomers because results are readily obtained at the residue level, reflecting local solvent accessibility [45]. We expected those highly flexible regions identified by top-down MS to be solvent-accessible [50]. To test, we submitted the WT antibody and the four mutants to FPOP oxidative labeling, followed by trypsin digestion and LC/MS/MS analysis, to obtain detailed residue-level information [28].

Antibody digestion yielded 12 readily identified peptides from the light chain, 11 of which are oxidatively modified (Figure 5a, and Supplementary Information Tables S4–6). Although the N-terminal light chain (peptides 1–18 and 46–54) contains many FPOP-reactive residues (Leu, Ile, Val, and Pro), it shows minimal (<5 %) oxidative modification, indicating low solvent accessibility (Figure 5a). Further, the light-chain C-terminus (peptides 150–169 and 191–207) undergoes sparse modification, and peptide 170–183 exhibits no modifications at all (relative abundance with respect to unmodified peptide is <0.01%) despite containing a highly reactive tyrosine. Thus, the light-chain C-terminus is not solvent-accessible. Taken together, footprinting outcomes corroborate top-down MS results that also show minimal fragmentation in the N- and C-terminus of the light chain. The low flexibility in this region is due to structural constraints of the disulfide bond between residues 134 and 194 (Figure 4c).

In contrast to the N- and C-terminal peptides, those of 62–91 and 92–103 in the middle of light chain undergo higher levels of oxidative modification, pointing to higher solvent accessibility of reactive residues. Peptide 62–91 contains four oxidized residues (Cys⁸⁸, Gln⁹⁰, Asn⁹³, and Trp⁹⁴). Gln⁹⁰ is most reactive (>30 % oxidized, Figure 5a inset) even though Cys and Trp have higher reactivity with ·OH [44]. FPOP data agree with top-down MS results that show extensive fragmentation for residues 88–111 and 121–145 (Figure 4b). The outcomes underscore advantages of using tandem methods.

FPOP Identifies Local Conformation Changes

Since no discernible differences were observed in the FPOP modification in the light-chain region, our focus was shifted to the hinge region, where the major differences in the disulfide bonding pattern of IgG2 antibodies occur (Figures 1 and 2). The hinge region is represented by a single peptide, 223–248, which has modified Pro²²⁸, Pro²²⁹, and Pro²³¹ (Supplementary Information Tables S1–S3). These residues are not modified for the mutants, removing any analysis complications introduced by intrinsic hydroxyl radical reactivity. To demonstrate, we show a product-ion spectrum of the peptide, singly oxidized on Pro²²⁹ (Figure 5c). Although y₁₉, from cleavage at Cys²³⁰, is not modified, a mass shift of +16 Da occurs for y₂₀ (cleavage at Pro²²⁹), indicating modification on this site (Figure 5c). The y₂₀ ions, observed as 2+ and 2+ and 3+ each with loss of NH₃, give consistent results. The labeling extent for WT is >60% (Figure 5d), consistent with the high flexibility of the hinge [51]. The mutants all exhibit decreased oxidation (Figure 5d), especially for hinge mutants, C223S, C224S, and C223S/C224S, the oxidation levels of which are less than 10%. The modification extent of the C135S mutant is ~50%, only slightly less than that of WT. Although the substitution is not located in the hinge, the altered S–S bonding of Cys²¹⁴ (light chain) to Cys²²³ (heavy chain, Figure 2) must induce a structural change in the hinge. Given the hinge's decreased solvent accessibility, the mutant must be more compact than the WT (Table 1). Thus, both FPOP and ion-mobility results (B isoform has the longest

drift time) concur on the more open conformation, and FPOP localizes the responsible region to the hinge region.

FPOP Reveals Solvent Accessibility in the CDR

The region of the antibody where the antigen binds is called the complementarity-determining region (CDR). In total, antibodies have six CDRs, three on the light chain (CDR-L1, L2, and L3) and three on the heavy chain (CDR-H1, H2, and H3). The three light-chain and the first two heavy-chain CDRs can be identified from their amino acid sequence and have a limited number of canonical structures. The third heavy-chain CDR, CDR-H3, which is important in antigen recognition, lies in the center of the antigen-binding site and is substantially diverse in length, structure, and sequence [52]. CDR3-H3 determines affinity and specificity for antigen binding [52]. Because a specified antibody from a different source, for example, should not show changes in this region, we analyzed the FPOP labeling data to determine whether FPOP can inform conformation differences among the mutant antibodies in the CDR.

The light-chain CDR has greater solvent accessibility than the heavy chain because it gives more oxidative modification. In CDR-L1, for example, H1 undergoes nearly 6-fold more modification than does L1 even though L1 contains an expectedly reactive Tyr, which does not detectably react. The oxidative modification levels of both WT and the C135S mutant are less in the CDR-L1 and H2 compared with the C223S, C224S, and C223S/C224S (Figure 5b). The C135S mutant also displays decreased modification in the CDR-H1. The modifications of CDR-L2 and -L3 are fairly constant among the antibodies. Notably, CDR-H3, which is responsible for antigen specificity and affinity [52], displays no significant FPOP differences for mutants versus WT, suggesting antigen-binding affinity of the mutants and WT should be similar [16].

Discussion

The use of multiple complementary MS-based methods provides rapid structural characterization of the antibody mutants, even in the absence of high resolution structures. MS-based methods provide, for each mutant, a structural “fingerprint” that can establish the presence of conformational differences. Although IM provides information only on the global level, its rapid throughput is advantageous as a first step. In this case, IM verifies that the WT is composed of two major isomers and that each of the mutants is more compact than the dominant WT component. The lack of high resolution, however, remains an obstacle in full characterization. For example, although differences in mobility are conclusive, identical mobilities may not mean identical conformations.

Top-down MS and FPOP provide more site-specific information for these conformational changes. Moreover, top-down MS shows that flexibility occurs in the light chain for all mutants. Although the top-down data at this level of coverage do not verify those regions that are changing, it does quickly probe certain regions of the antibody for which a conclusion can be drawn. FPOP, on the other hand, provides residue-level information for the entire antibody, locating the conformation differences determined by IMMS, identifying the hinge as the site of these differences, verifying the flexible regions in the light chain, and demonstrating that few solvent-accessibility differences occur in the CDRs. When IM, as a starting point, establishes the presence of conformational differences, a more detailed and demanding FPOP (or H/DX) analysis is motivated.

All the data provide a consistent picture for interrogating structural changes in an antibody and its mutants. Previous MD simulations on model human IgG2 mAbs [41] indicate that the absence of disulfide bonds causes the two heavy chains to pack against one another in

the hinge via noncovalent interactions. When the disulfide bonds are removed in the hinge, as for mutants C223S, C224S, and C223S/C224S, the structure in these regions become more compact. IM shows that the B isoform has the longest drift time and, thus, the most open conformation. Further, FPOP shows only a slight decrease in modification in the C135S mutants, which represents an IgG1-like structure, confirming this protein is not as compact as the C223S, C224S, and C223S/C224S mutants. IMMS is unable to confirm this because it is of lower resolution. The lack of significant oxidative modification differences of the anti-CD44 IgG2 CDR region indicates that the structural differences between the isoforms do not affect the antigen binding affinity. This was also demonstrated by ELISA [16].

Conclusion

The outcomes from molecular modeling and biophysical MS afford a more complete picture than from any method alone. This particular approach, which probes protein native structure, consists of IMMS, top-down fragmentation, and protein footprinting. Although the approaches are at an early stage of development and do not yet afford complete answers, the results and the importance of the problem provide incentive for improvement and expansion. Future advances in the resolution and precision of IMMS will permit finer distinctions than can be made today. Similarly, improvements in the dynamic range of top-down sequencing will allow discrimination of regions having different and lower flexibility, but, even with these improvements, the most flexible regions will still be identified as they are in this study. Both the ion mobility and top-down MS require native electrospray to maintain as much as possible the native structures in the gas phase, and improvements should increase sensitivity by introducing more ions to the instrument. It may also be possible to break apart the antibody into various components, as was mentioned in a recent review [6], and use a middle-down approach, provided that native structure can be maintained.

Improvements in FPOP will see the development of new reagents to complement •OH radicals and give different views of the antibodies, but the major improvement needed is more efficient data processing to permit faster turnaround. The use of multiple MS approaches is sufficiently advantageous that they may become standard practice for characterizing novel biologics and biosimilars and for minimizing the need for animal and human testing. We certainly do not rule out other combinations but rather encourage exploring them based on the premise that efficiency and information content are increased by using combinations of methods. We acknowledge that although mass spectrometers are expensive, the throughput, information output, and commercial impact in drug development justify the investment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported by grants from the National Institute of General Medical Sciences (8 P41 GM103422-35) of the NIH, by a Pfizer Global Research and Development postdoctoral fellowship for L.M.J., and by the NSF (IBDR 0964199 to M.L.G.). Partial salary support for H.Z. and instrument support for the Waters Synapt G2 mass spectrometer were from the Photosynthetic Antenna Research Center, an Energy Frontier Research Center funded by the US DOE, Office of Basic Energy Sciences (grant no. DE-SC 0001035 to Robert E. Blankenship). The authors thank Professor Blankenship for providing instrument access, M. Martinez, Waters, for help with nanoESI, B. Ruotolo, University of Michigan, M. Plasencia, and H. Rohrs for helpful discussions, and B. Gau for help with data processing.

References

1. Berkowitz SA, Engen JR, Mazzeo JR, Jones GB. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. *Nat Rev Drug Discov.* 2012; 11:527–540. [PubMed: 22743980]
2. Giezen TJ, Mantel-Teeuwisse AK, Straus SM, Schellekens H, Leufkens HG, Egberts AC. Safety-related regulatory actions for biologicals approved in the United States and the European Union. *JAMA.* 2008; 300:1887–1896. [PubMed: 18940975]
3. Aggarwal S. What's fueling the biotech engine? *Nat Biotechnol.* 2007; 25:1097–1104. [PubMed: 17921989]
4. Kling J. Jama study casts cloud over biologic safety. *Nat Biotechnol.* 2009; 27:11–12. [PubMed: 19131983]
5. Woodcock J, Griffin J, Behrman R, Cherney B, Crescenzi T, Fraser B, Hixon D, Joneckis C, Kozlowski S, Rosenberg A, Schrager L, Shacter E, Temple R, Webber K, Winkle H. The FDA's assessment of follow-on protein products: a historical perspective. *Nat Rev Drug Discov.* 2007; 6:437–442. [PubMed: 17633790]
6. Beck A, Wagner-Rousset E, Ayoub D, Van DA, Sanglier-Cianferani S. Characterization of therapeutic antibodies and related products. *Anal Chem.* 2013; 85:715–736. [PubMed: 23134362]
7. Beck A, Sanglier C, Van DA. Biosimilar, biobetter, and next generation antibody characterization by mass spectrometry. *Anal Chem.* 2012; 84:4637–4646. [PubMed: 22510259]
8. Kozlowski S, Woodcock J, Midthun K, Sherman RB. Developing the nation's biosimilars program. *N Engl J Med.* 2011; 365:385–388. [PubMed: 21812668]
9. Aggarwal S. What's fueling the biotech engine-2010 to 2011. *Nat Biotechnol.* 2011; 29:1083–1089. [PubMed: 22158359]
10. Scolnik PA. Mabs: A business perspective. *MAbs.* 2009; 1:179–184. [PubMed: 20061824]
11. Reichert JM, Valge-Archer VE. Development trends for monoclonal antibody cancer therapeutics. *Nat Rev Drug Discov.* 2007; 6:349–356. [PubMed: 17431406]
12. Jefferis R. Antibody therapeutics: Isotype and glycoform selection. *Expert Opin Biol Ther.* 2007; 7:1401–1413. [PubMed: 17727329]
13. Beenhouwer DO, Yoo EM, Lai CW, Rocha MA, Morrison SL. Human immunoglobulin G2 (IgG2) and IgG4, but not IgG1 or IgG3, protect mice against *Cryptococcus neoformans* infection. *Infect Immun.* 2007; 75:1424–1435. [PubMed: 17220317]
14. Wypych J, Li M, Guo A, Zhang Z, Martinez T, Allen MJ, Fodor S, Kelner DN, Flynn GC, Liu YD, Bondarenko PV, Ricci MS, Dillon TM, Balland A. Human IgG2 antibodies display disulfide-mediated structural isoforms. *J Biol Chem.* 2008; 283:16194–16205. [PubMed: 18339624]
15. Dillon TM, Ricci MS, Vezina C, Flynn GC, Liu YD, Rehder DS, Plant M, Henkle B, Li Y, Deechongkit S, Varnum B, Wypych J, Balland A, Bondarenko PV. Structural and functional characterization of disulfide isoforms of the human IgG2 subclass. *J Biol Chem.* 2008; 283:16206–16215. [PubMed: 18339626]
16. Lightle S, Aykent S, Lacher N, Mitaksov V, Wells K, Zobel J, Oliphant T. Mutations within a human IgG2 antibody form distinct and homogeneous disulfide isomers but do not affect fc gamma receptor or c1q binding. *Protein Sci.* 2010; 19:753–762. [PubMed: 20120022]
17. Ruotolo BT, Giles K, Campuzano I, Sandercock AM, Bateman RH, Robinson CV. Evidence for macromolecular protein rings in the absence of bulk water. *Science.* 2005; 310:1658–1661. [PubMed: 16293722]
18. Benesch JL, Ruotolo BT, Simmons DA, Robinson CV. Protein complexes in the gas phase: Technology for structural genomics and proteomics. *Chem Rev.* 2007; 107:3544–3567. [PubMed: 17649985]
19. Uetrecht C, Versluis C, Watts NR, Roos WH, Wuite GJ, Wingfield PT, Steven AC, Heck AJ. High-resolution mass spectrometry of viral assemblies: Molecular composition and stability of dimorphic hepatitis b virus capsids. *Proc Natl Acad Sci USA.* 2008; 105:9216–9220. [PubMed: 18587050]

20. Zhang H, Cui W, Wen J, Blankenship RE, Gross ML. Native electrospray and electron-capture dissociation in fticr mass spectrometry provide top-down sequencing of a protein component in an intact protein assembly. *J Am Soc Mass Spectrom.* 2010; 21:1966–1968. [PubMed: 20843701]
21. Kaltashov IA, Bobst CE, Abzalimov RR, Berkowitz SA, Houde D. Conformation and dynamics of biopharmaceuticals: Transition of mass spectrometry-based tools from academe to industry. *J Am Soc Mass Spectrom.* 2010; 21:323–337. [PubMed: 19963397]
22. Banci L, Baumeister W, Enfedaque J, Heinemann U, Schneider G, Silman I, Sussman JL. Structural proteomics: From the molecule to the system. *Nat Struct Mol Biol.* 2007; 14:3–4. [PubMed: 17203065]
23. Bagal D, Valliere-Douglass JF, Balland A, Schnier PD. Resolving disulfide structural isoforms of IgG2 monoclonal antibodies by ion mobility mass spectrometry. *Anal Chem.* 2010; 82:6751–6755. [PubMed: 20704363]
24. Bondarenko PV, Second TP, Zabrouskov V, Makarov AA, Zhang Z. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer. *J Am Soc Mass Spectrom.* 2009; 20:1415–1424. [PubMed: 19409810]
25. Tsybin YO, Fornelli L, Stoermer C, Luebeck M, Parra J, Nallet S, Wurm FM, Hartmer R. Structural analysis of intact monoclonal antibodies by electron transfer dissociation mass spectrometry. *Anal Chem.* 2011; 83:8919–8927. [PubMed: 22017162]
26. Baerga-Ortiz A, Hughes CA, Mandell JG, Komives EA. Epitope mapping of a monoclonal antibody against human thrombin by H/D-exchange mass spectrometry reveals selection of a diverse sequence in a highly conserved protein. *Protein Sci.* 2002; 11:1300–1308. [PubMed: 12021429]
27. Houde D, Arndt J, Domeier W, Berkowitz S, Engen JR. Characterization of IgG1 conformation and conformational dynamics by hydrogen/deuterium exchange mass spectrometry. *Anal Chem.* 2009; 81:5966. [PubMed: 19606834]
28. Jones LM, Sperry JB, Carroll JA, Gross ML. Fast photochemical oxidation of proteins for epitope mapping. *Anal Chem.* 2011; 83:7657–7661. [PubMed: 21894996]
29. Zhang L, Lilyestrom W, Li C, Scherer T, van Reis R, Zhang B. Revealing a positive charge patch on a recombinant monoclonal antibody by chemical labeling and mass spectrometry. *Anal Chem.* 2011; 83:8501–8508. [PubMed: 22004540]
30. Watson C, Sharp JS. Conformational analysis of therapeutic proteins by hydroxyl radical protein footprinting. *AAPS J.* 2012; 14:206–217. [PubMed: 22382679]
31. Bongers J, Devincents J, Fu J, Huang P, Kirkley DH, Leister K, Liu P, Ludwig R, Rumney K, Tao L, Wu W, Russell RJ. Characterization of glycosylation sites for a recombinant IgG1 monoclonal antibody and a ctla4-ig fusion protein by liquid chromatography-mass spectrometry peptide mapping. *J Chromatogr A.* 2011; 1218:8140–8149. [PubMed: 21978954]
32. Kukrer B, Filipe V, van Duijn E, Kasper PT, Vreeken RJ, Heck AJ, Jiskoot W. Mass spectrometric analysis of intact human monoclonal antibody aggregates fractionated by size-exclusion chromatography. *Pharm Res.* 2010; 27:2197–2204. [PubMed: 20680668]
33. Valeja SG, Kaiser NK, Xian F, Hendrickson CL, Rouse JC, Marshall AG. Unit mass baseline resolution for an intact 148 kDa therapeutic monoclonal antibody by fourier transform ion cyclotron resonance mass spectrometry. *Anal Chem.* 2011; 83:8391–8395. [PubMed: 22011246]
34. Ruotolo BT, Hyung SJ, Robinson PM, Giles K, Bateman RH, Robinson CV. Ion mobility-mass spectrometry reveals long-lived, unfolded intermediates in the dissociation of protein complexes. *Angew Chem Int Ed Engl.* 2007; 46:8001–8004. [PubMed: 17854106]
35. Tran JC, Zamdborg L, Ahlf DR, Lee JE, Catherman AD, Durbin KR, Tipton JD, Vellaichamy A, Kellie JF, Li M, Wu C, Sweet SM, Early BP, Siuti N, Leduc RD, Compton PD, Thomas PM, Kelleher NL. Mapping intact protein isoforms in discovery mode using top-down proteomics. *Nature.* 2011; 480:254–258. [PubMed: 22037311]
36. Oh H, Breuker K, Sze SK, Ge Y, Carpenter BK, McLafferty FW. Secondary and tertiary structures of gaseous protein ions characterized by electron capture dissociation mass spectrometry and photofragment spectroscopy. *Proc Natl Acad Sci USA.* 2002; 99:15863–15868. [PubMed: 12444260]

37. Hamblly DM, Gross ML. Laser flash photolysis of hydrogen peroxide to oxidize protein solvent-accessible residues on the microsecond timescale. *J Am Soc Mass Spectrom.* 2005; 16:2057–2063. [PubMed: 16263307]
38. Gau BC, Sharp JS, Rempel DL, Gross ML. Fast photochemical oxidation of protein footprints faster than protein unfolding. *Anal Chem.* 2009; 81:6563–6571. [PubMed: 20337372]
39. Zhang H, Gau BC, Jones LM, Vidavsky I, Gross ML. Fast photochemical oxidation of proteins for comparing structures of protein-ligand complexes: the calmodulin-peptide model system. *Anal Chem.* 2010; 83:311–318. [PubMed: 21142124]
40. Wen J, Zhang H, Gross ML, Blankenship RE. Native electrospray mass spectrometry reveals the nature and stoichiometry of pigments in the fmo photosynthetic antenna protein. *Biochemistry.* 2011; 50:3502–3511. [PubMed: 21449539]
41. Wang X, Kumar S, Singh SK. Disulfide scrambling in IgG2 monoclonal antibodies: Insights from molecular dynamics simulations. *Pharm Res.* 2011; 28:3128–3144. [PubMed: 21671135]
42. Zhang H, Cui W, Wen J, Blankenship RE, Gross ML. Native electrospray and electron-capture dissociation ftr mass spectrometry for top-down studies of protein assemblies. *Anal Chem.* 2011; 83:5598–5606. [PubMed: 21612283]
43. Xu G, Chance MR. Radiolytic modification of acidic amino acid residues in peptides: Probes for examining protein–protein interactions. *Anal Chem.* 2004; 76:1213–1221. [PubMed: 14987073]
44. Xu G, Chance MR. Hydroxyl radical-mediated modification of proteins as probes for structural proteomics. *Chem Rev.* 2007; 107:3514–3543. [PubMed: 17683160]
45. Gau BC, Chen H, Zhang Y, Gross ML. Sulfate radical anion as a new reagent for fast photochemical oxidation of proteins. *Anal Chem.* 2010; 82:7821–7827. [PubMed: 20738105]
46. Wang Q, Lacher NA, Muralidhara BK, Schlittler MR, Aykent S, Demarest CW. Rapid and refined separation of human IgG2 disulfide isomers using superficially porous particles. *J Sep Sci.* 2010; 33:2671–2680. [PubMed: 20730829]
47. Houde D, Peng Y, Berkowitz SA, Engen JR. Post-translational modifications differentially affect IgG1 conformation and receptor binding. *Mol Cell Proteom.* 2010; 9:1716–1728.
48. Allen, G. Sequencing of proteins and peptides. 2. Elsevier/North-Holland; Amsterdam; The Netherlands: 1981. revised ed
49. Hamuro Y, Coales SJ, Molnar KS, Tuske SJ, Morrow JA. Specificity of immobilized porcine pepsin in h/d exchange compatible conditions. *Rapid Commun Mass Spectrom.* 2008; 22:1041–1046. [PubMed: 18327892]
50. Zhang H, Zhang T, Chen K, Shen S, Ruan J, Kurgan L. On the relation between residue flexibility and local solvent accessibility in proteins. *Proteins.* 2009; 76:617–636. [PubMed: 19274736]
51. Brekke OH, Michaelsen TE, Sandlie I. The structural requirements for complement activation by IgG: Does it hinge on the hinge? *Immunol Today.* 1995; 16:85–90. [PubMed: 7888072]
52. Kuroda D, Shirai H, Kobori M, Nakamura H. Structural classification of cdr-h3 revisited: a lesson in antibody modeling. *Proteins.* 2008; 73:608–620. [PubMed: 18473362]

Figure 1.

Disulfide isomers of IgG2. IgG2 antibodies have three different disulfide isoforms, IgG2-A (left), IgG2-A/B (middle), and IgG2-B (right). The relevant cysteine residues are labeled in the inset. The disulfide bond between C135 and C214 in IgG2-A is similar to disulfide bonding pattern in IgG4 antibodies. The disulfide bond between C214 and C223, observed in IgG2-B, is similar to that of a IgG1 antibody

Figure 2.

Structural models of the Cys→Ser mutants. Homology model of the wild type IgG2 with light chain 1 in blue, light chain 2 in red, heavy chain 1 in magenta, and heavy chain 2 in green. The mutations force the antibody into single isoforms. The WT displays the classic A-conformation with a disulfide bond between Cys135 and Cys214. The C135S mutant has an IgG1-like conformation with a disulfide bond between Cys214 and Cys223. The hinge mutants C224S, C223S, and C223S/C224S have an IgG4-like conformation

Figure 3.

IM of wild type (WT) and mutants. **(a)** The spectrum of WT sample, plotted in two dimensions, m/z and drift time. The IM spectrum of the most abundant charge state was extracted from the 2-D spectrum and plotted on the left side to show two conformers. **(b)** 3-D (m/z , drift time, and ion counts) spectrum of WT sample showing two conformers for +23 and +24 charge states. **(c)** Mass and IM spectra of WT and mutants ionized by native ESI afford a narrow charge distribution from m/z 5250 to 6750. The drift times (centroid value) of +24 charge states were plotted on the right. The precision is $\sim \pm 0.1$ ms

Figure 4.

Top-down MS reveals regions of flexibility within the antibody. (a) Native spray mass spectrum of WT IgG2 (top) with increasing amounts of in-source collision-induced dissociation. Charged reduced species are observed over the m/z range of 6000–14,000. (b) Sequence of the light chain with cleavage sites to give fragment ions (highlighted in red). (c) The regions of flexibility identified by top-down MS in yellow for the light chain structure alone and in context with the rest of the antibody structure (inset). The locations of disulfide bonds are highlighted with stick representation

Figure 5.

(a) FPOP labeling of the WT light chain (dark grey bars), C135S (grey bars), C223S (light colored bars), C224S (diagonally striped bars), and C223S/C224S (checkered stripes) on the peptide level and residue level (inset) showing regions of high solvent accessibility between residues 62–103. (b) FPOP labeling of the complementarity determining region. (c) Production spectrum of peptide 222–234 showing oxidative modification of Pro229. (d) FPOP labeling of the hinge region

Table 1Solvent-accessible surface area calculations ($\text{\AA}^2$) for wild type and mutant antibody structures

Molecular structure	Solvent accessibility ($\text{\AA}^2$)
Wild type antibody	52,530
C135S mutant (LC Cys 214 and HC Cys 223 disulfide bonded)	51,880
C135S mutant (LC Cys 214 and HC Cys 224 disulfide bonded)	51,450
C135S mutant (LC Cys 214 unpaired)	51,600
C223S mutant	52,230
C224S mutant	52,310
C223S–C224S double mutant	52,420

Table 2

Backbone atom root mean square deviation values ($\text{\AA}$) obtained from simultaneous superposition of different regions from WT antibody and the mutants

Molecular region	Backbone atom root mean square deviation ($\text{\AA}$)
Full length mAbs	7.54
Fab 1 (Heavy chain: Gln 1–Val 219, Light chain: Glu 1–Cys 214)	1.30 (0.92 for Fab2)
Hinge region (heavy chains: Glu 220–Pro 231)	3.34
Fc region (heavy chains: Val 235–Lys 447)	1.00