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Rapid characterization of IgG1 conformation and conformational dynamics by hydrogen/deuterium exchange mass spectrometry

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Abstract

Protein function is dictated by protein conformation. For the protein biopharmaceutical industry, therefore, it is important to have analytical tools that can detect changes in protein conformation rapidly, accurately and with high sensitivity. In this paper we show that hydrogen/deuterium exchange mass spectrometry (H/DX-MS) can play an important role in fulfilling this need within the industry. H/DX-MS was used to assess both global and local conformational behavior of a recombinant monoclonal IgG1 antibody, a major class of biopharmaceuticals. Analysis of exchange into the intact, glycosylated IgG1 (and the Fab and Fc regions thereof) showed that the molecule was folded, highly stable and highly amenable to analysis by this method using less than a nanomole of material. With improved chromatographic methods, peptide identification algorithms and data-processing steps, the analysis of deuterium levels in peptic peptides produced after labeling was accomplished in 1–2 days. Based on peptic peptide data, exchange was localized to specific regions of the antibody. Changes to IgG1 conformation as a result of deglycosylation were determined by comparing exchange into the glycosylated and deglycosylated forms of the antibody. Two regions of the IgG1 (residues 236–253 and 292–308) were found to have altered exchange properties upon deglycosylation. These results are consistent with previous findings concerning the role of glycosylation in the interaction of IgG1 with Fc receptors. Moreover, the data clearly illustrate how H/DX-MS can provide important characterization information on the higher order structure of antibodies and conformational changes that these molecules may experience upon modification.

Keywords

antibody; glycosylation; biopharmaceutical; crystallography

INTRODUCTION

Monoclonal antibodies (mAb) represent a unique and large class of protein therapeutic agents^{1–3} that offer new hope in battling an array of different diseases. In particular, recombinant immunoglobulin gamma (IgG) mAbs constitute the largest percentage of molecules in the biopharmaceutical development pipeline⁴. With such therapeutic significance, the ability to quickly and effectively characterize these proteins is of great

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importance, as it will help to facilitate their transition through discovery into development, eventual commercialization and their ultimate intended therapeutic use. Achieving this goal is partially dependent on the availability of appropriate analytical tools to provide an array of chemical and physical information during all stages of discovery and development. As a result, there has been significant need and emphasis on developing new or improved analytical methods and tools for mAb characterization^{5–7}. Unfortunately, the size and overall complexity of mAbs (as well as other protein biopharmaceuticals in general) makes their characterization complicated and challenging especially for assessing higher order structure with the biophysical methodologies that are presently available. Since structure dictates function, the understanding of structure and its interplay with function is essential for developing effective, safe and cost effective protein biopharmaceuticals.

Although X-ray crystallography is capable of providing detailed structural information, it is dependent on generating appropriate crystals that will diffract well. At present, the crystal structures of entire IgGs are few in number^{8–11}. In addition, the insight provided in these cases is only that of a very stable structure sampled by the protein; information pertaining to dynamics or in-solution motion is mostly absent. In the case of NMR, whole IgGs (~150 kDa) are currently too large for conventional NMR experiments. Furthermore, protein NMR typically requires high sample concentrations, which may not only be difficult to obtain but which could distort the conformation of these molecules and lead to aggregation^{12, 13}. Further, many NMR experiments require the protein to be isotopically labeled (*e.g.* with ¹⁵N or ¹³C). Such requirements make the method inhibitory for routine sample analysis. Nevertheless, smaller pieces of IgGs have been successfully examined by NMR, generating useful information. Examples include the analysis of glycoforms of the Fc fragment of an anti-CCR4 IgG1 antibody¹⁴, the effects of fucosylation on the solution structure of the Fc fragment of anti-CCR4¹⁵, and Fab-antigen complexes¹⁶. However, the interpretation of the intact IgG structure from IgG fragments (Fab's and Fc) still requires modeling to reconstruct and piece together the whole antibody, which may or may not be an accurate representation of the entire native protein¹⁷.

Other analytical tools that are available and commonly used for protein biophysical characterization include CD, fluorescence, DSC, ITC, AUC and various chromatographic techniques. Unfortunately these techniques are only capable of providing, in general, a global view of protein conformation and little if any information on protein conformational dynamics^{18–20}. Although global views can provide some useful information, particularly in detecting gross conformational changes, it provides little in the way of the site-specific and detailed conformational characterization(s) (*i.e.*, what regions are doing what) that are preferred. Detailed information would be very useful throughout all stages of protein drug development, from optimization of target binding and receptor interaction(s) in research to formulation, stability, and comparability studies in process development where information on changes in conformation could play an important role in the development and optimization of mAbs. Thus, there is a present need for additional analytical methods to characterize the conformation of mAbs in more detail in order to monitor subtle changes in conformation that could impact their quality and performance as a potential drug.

One analytical method that has the potential to address some of the deficiencies of classical biophysical methods is hydrogen/deuterium exchange mass spectrometry (H/DX-MS). This method has been shown to be very useful for exploring conformational dynamics and structural changes of proteins (recently reviewed by Maier & Deinzer²¹, Wales & Engen²², Hamuro et al.²³, and Tsutsui & Wintrode²⁴). Simply put, it monitors the exchange of backbone amide hydrogens in proteins with hydrogens in solution; if the normal all-H₂O solution is replaced with D₂O, deuteration of the protein occurs. The level of deuterium incorporation is an indication of solvent exposure and hydrogen bonding and can vary as much as 10⁸-fold as a

result of structure²⁵. By using a mass spectrometer to measure the mass increase of the protein as it becomes deuterated over time, a highly sensitive analytical tool can be realized that allows one to generate information about the conformational behavior of a protein in solution. More importantly, when this process is coupled with enzymatic digestion²⁶, deuterium incorporation information can be localized in a protein to stretches of 3-10 residues (note: this level of resolution is totally dependent on the number and nature of the overlapping peptides generated, which can theoretically reach a limit of 1 residue). Recent advances in this analytical tool, including the use of a newly described UPLC system²⁷, now allow much larger proteins to be studied as the large number of peptic peptides that are produced during digestion can be better resolved by UPLC. In addition, improved MS sensitivity and computational power allow detailed analysis to be performed and completed on proteins with less than a nanomole of material in a relatively short time (days).

In this work we have applied H/DX-MS to investigate the conformation and conformational dynamics of an intact mAb-IgG1 (hereafter referred to as IgG1) to demonstrate its capabilities as a powerful analytical tool to study much larger protein biopharmaceuticals than was reported previously²⁸. In particular we have focused our attention in this study on the conformational features of an intact glycosylated IgG1 relative to its deglycosylated version. In so doing this has enabled us to determine how glycosylation affects IgG1 conformation. The H/DX-MS results were consistent with previous findings^{14, 15, 29} concerning the role of glycosylation in the interaction of IgG1 with Fc receptors. In addition, the crystal structure of the Fab of the IgG1 was determined at 2.7 angstroms resolution (R 0.23, R_{free} 0.27) and used to create a model of the intact mAb that facilitated interpretation of the H/DX-MS results. Overall, we show that H/DX-MS is capable of rapidly and accurately providing useful new information about mAb conformation and dynamics in solution, data that are much more difficult or impossible to obtain with other analytical methods.

EXPERIMENTAL SECTION

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. The recombinant monoclonal IgG1 was manufactured and purified by Biogen Idec, Inc. N-glycanase (PNGaseF) was purchased from Prozyme (San Diego, CA, USA). Guanidine HCl and Tris(2-Carboxyethyl) phosphine Hydrochloride (TCEP) were purchased from Pierce (Rockford, IL, USA).

Deglycosylation

Glycosylated IgG1-mAb samples were buffer exchanged into 50 mM sodium phosphate, 100 mM sodium chloride, pH 6.0 to approximately 3 mg/mL using a 10K MWCO Amicon Biomax centrifugal membrane (Millipore, Billerica, MA, USA). Deglycosylated samples were prepared by incubating approximately 5 mg of the antibody with 100 mU of PNGase F in 50 mM sodium phosphate or Tris buffer at pH 7.5 for 18 to 24 hours at 37 °C. To remove PNGase F, deglycosylated samples were injected onto a size-exclusion column (TSKgel G3000SW_{XL}, TOSOH Bioscience, San Francisco, CA, USA) operating under isocratic conditions flowing at 0.5 mL/min with 50 mM sodium phosphate, 100 mM sodium chloride, pH 6.0. The antibody eluted at approximately 20 minutes while PNGase F eluted around 25 minutes. Fractions containing antibody were concentrated to approximately 3 mg/mL using a 10K MWCO Amicon Biomax centrifugal membrane. The control (glycosylated) antibody was prepared and purified similarly with the exception that no PNGase F was added. Deglycosylation was verified by mass spectrometry (see Supporting Information Figure S-1). Antibody concentrations were calculated from the absorbance measured at 278 nm and the theoretical extinction coefficient^{30, 31} of $\epsilon = 218,292 \text{ M}^{-1} \text{ cm}^{-1}$.

Global hydrogen exchange analysis

Antibody (in 50 mM sodium phosphate, 100 mM sodium chloride H₂O, pH 6.0) was diluted 20-fold with 50 mM sodium phosphate, 100 mM sodium chloride, D₂O, pD 6.0 and incubated at room temperature for various amounts of time (10 seconds, 1, 10, 60 and 240 minutes). The exchange reaction was quenched by reducing the pH to 2.6 with a 1:1 dilution with 200 mM sodium phosphate, H₂O, pH 2.4. Quenched samples (~40 μ L) were immediately injected onto a Michrom protein microtrap (Michrom Bioresources, Auburn, CA, USA) or a Poros 20/R2 protein trap (Applied Biosystems, Framingham, MA, USA) at 0 °C. Injected samples were desalted online using a Shimadzu HPLC (Columbia, MD, USA) for 2 minutes with 10% acetonitrile, 90% water, 0.1% formic acid and 0.02% TFA and eluted in 2 minutes using a gradient to 90% acetonitrile, 10% water, 0.1% formic acid and 0.02% TFA. The eluate was sent into an LCT premier ESI-ToF mass spectrometer (Waters, Milford, MA, USA) and mass spectra obtained over the m/z range 1500–3500. The instrument was calibrated using multiple ions from cesium tridecafluoroheptanoate³². Mass spectra were transformed using MagTran³³. The mass of undeuterated protein was subtracted from the mass of the protein at each exchange-in time point and plotted. Each exchange point was analyzed with multiple replicates (see also Figure 1A· Table 1). No adjustment was made for deuterium back-exchange during analysis and therefore all results are reported as the relative deuterium level²².

Peptide-level hydrogen exchange analysis

Exchange was performed as indicated above and deuterated samples were quenched by reducing the pH to 2.6 with a 1:1 dilution of 200 mM sodium phosphate, 0.5 M TCEP and 4 M guanidine HCl, H₂O, pH 2.4. Quenched samples were digested, desalted and separated online using a Waters UPLC system based on a nanoACQUITY platform²⁷. Approximately 20 pmoles of exchanged and quenched antibody was injected into an immobilized pepsin column³⁴. The online digestion was performed over 2 minutes in water containing 0.05 % formic acid at 20 °C at a flow rate of 0.1 mL/min. The resulting peptic peptides were trapped on an ACQUITY UPLC BEH C18 1.7 μ m peptide trap (Waters, Milford, MA, USA) maintained at 0 °C and desalted with water, 0.1% formic acid. Flow was diverted by a switching valve and the trapped peptides eluted from the trap at 40 μ L/min onto a Waters ACQUITY UPLC BEH C18 1.7 μ m, 1mm $\times$ 100mm column held at 0 °C (average back-pressure was approximately 9500 psi). A 6 minute linear acetonitrile gradient (8–40%) with 0.1 % formic acid was used to separate the peptides. The eluate was directed into a Waters QToF premier mass spectrometer with electrospray ionization and lock-mass correction (using Glu-fibrinogen peptide). Mass spectra were acquired over the m/z range 50–1700. Pepsin fragments were identified using a combination of exact mass and MS/MS, aided by Waters Identity^E software³⁵. Peptide deuterium levels were determined as described by Weis et al. using the Excel based program HX-Express³⁶. Each peptide-level experiment was performed in triplicate (control was analyzed 5 times over several weeks). No adjustment was made for deuterium back-exchange during analysis and therefore all results are reported as relative deuterium level²².

Crystal structure determination

The IgG1 Fab was crystallized by the nanodroplet vapor diffusion method using an Innovadyne Screenmaker crystallization robot (Santa Rosa, CA, USA) and placed at a temperature of 292 K. Plate-shaped crystals of diffraction quality grew in 1–3 days in a crystallization solution that contained 38% PEG 400, 200 mM sodium chloride, and 100 mM Tris at pH 8.5. Crystals (0.2 $\times$ 0.2 $\times$ 0.02 mm³) were harvested as is and flash frozen in liquid nitrogen. Diffraction data were collected at the Advanced Photon Source (APS) on beam line SGX-CAT. The data set was collected at 100 K using an ADSC q315 CCD detector, whereas APS data set used a MAR 165 detector. Data were integrated, reduced, and scaled using HKL2000 (HKL Research,

Charlottesville, VA, USA). The crystal was indexed in the monoclinic space group C2; data statistics are summarized in Table S-2 in the Supporting Information. The structure was determined by molecular replacement using the Campath-1h Fab structure³⁷ (PDB code 1CE1 with 93% sequence identity) as the search model with the program MolRep³⁸. The model was manually built with Coot³⁹. Structure refinement was performed using REFMAC5 [CCP4 1994]⁴⁰. The progress of the model refinement was monitored by cross-validation R_{free} , which was computed from a randomly assigned test set comprising 5% of the data. Refinement statistics are summarized in Table S-1 in the Supporting Information. The final model included two Fab molecules and 54 water molecules. No electron density was observed for residues 136-137 of the heavy chain. Analysis of the stereochemical quality of the model was accomplished using the AutoDepInput tool (<http://deposit.pdb.org/adit/>). Atomic coordinates and experimental structure factors of the Fab have been deposited with the PDB (code: 3FZU). The intact IgG1 model was obtained by docking the Fab structure to the Fc region of the crystal structure of the intact human IgG1 B12 (PDB code 1hzh⁸) through superpositioning their variable regions with the program Coot.

RESULTS AND DISCUSSION

For proteins that have not been interrogated by H/DX-MS before, it is often desirable to determine the exchange profile of the intact (undigested) protein²². These measurements are relatively straightforward and the data analysis is not very complicated. Such an experiment is useful because it provides information about the overall solvent protection of the protein and importantly, indicates if the protein is amenable to H/DX-MS and further investigation with digestion experiments. If multiple populations of conformationally distinct molecules were to exist in the solution, they would easily be revealed in this experiment (see below). Further, perturbations to the overall structure of the intact protein can be diagnostic for misfolding if correlated with functional data. In this study, therefore, we first measured deuterium exchange into an intact IgG1. These experiments were followed by analysis of exchange into isolated Fab/Fc fragments. Finally, we labeled the intact protein and digested it (after quenching the deuterium labeling reaction) into small pieces with pepsin. Digestion allowed for detailed measurement and interpretation of exactly where the deuterium was exchanging into the IgG1 and to what magnitude. Digestion experiments were performed for both glycosylated and deglycosylated forms of the IgG1.

H/DX-MS of intact IgG1

Deuterium incorporation was assessed for intact glycosylated IgG1 by incubating the protein in D₂O buffer for various amounts of time and measuring the resulting mass increase (see Experimental Section for details). Five independent experiments were performed (Figure 1A), each containing an undeuterated sample and five labeling times (see also Table 1). The error associated with each time-point ranged from ± 3.4 Da to ± 7.4 Da. This value is quite small considering that the intact IgG1 contains a total of 1322 residues with 1228 backbone amide hydrogens available for exchange (Maximum number of backbone amide hydrogens available for exchange = Total number of residues, minus prolines, minus one for N-terminus²²). None of the data presented here were adjusted for back exchange²⁶ because a totally deuterated control standard could not be prepared for IgG1; therefore, all results are reported as the relative deuterium level²². Totally deuterated standard proteins indicated that back exchange under the experimental conditions used here was approximately 18–22% (data not shown). Note that mass increases report only on backbone amide deuteration because side-chain deuterium was washed away during the chromatography step²². The results of global exchange on IgG1 showed that approximately 157 residues exchanged within 10 seconds (~13%, relatively) and ~565 residues (less than 50% of the available NHs) exchanged after 4 hours. These data are

consistent with the known high beta-sheet content in IgGs; highly hydrogen bonded beta-sheets can be quite protected from exchange⁴¹.

H/DX-MS of IgG1 Fc and Fab fragments

To further characterize this IgG1, deuterium incorporation into the Fc and Fab regions alone was investigated. This experiment was aimed at determining if the domains behaved differently when connected versus in isolation (*i.e.*, were there detectable domain-domain interactions). The IgG1 was cleaved using lysyl endopeptidase limited proteolysis⁴² to generate Fab and Fc fragments which were then purified by weak cation-exchange chromatography (Fab eluted at 12.1 mins and Fc at 21.4 mins, data not shown). The purified fragments were buffer exchanged into the same buffer as the intact IgG1 (50 mM Na phosphate, 100 mM NaCl, pH 6.0) and labeled with deuterium as described for the intact IgG1. Figure 1B shows the deuterium levels in the Fab (solid trace) and Fc (dotted trace) fragments, respectively. Here, an undeuterated control and eight labeling times were analyzed (each in duplicate, see Table 1), and the error ranged from ± 3.0 to ± 7.0 Da. The exchange data from the IgG1 Fab/Fc experiments correlated well with the intact IgG1 experiment. For example, after 10 minutes of labeling, the intact IgG1 incorporated 370 deuterium atoms while the fragments incorporated 358 (Fab was +125 and Fc was +108. $2\text{Fab} + 1\text{Fc} = \text{intact IgG1} = 358$ deuteriums). Such results (which are the same within the error of these experiments) indicated that the conformation of the IgG1 in the intact form was not significantly altered in the Fab and Fc regions as a result of the limited proteolysis, nor were there any significant domain-domain interactions. Overall, the intact protein experiments resulted in a few basic but important conclusions. First, some parts of the IgG1 were heavily protected from hydrogen exchange; less than 50% of the molecule was deuterated after 4 hours of labeling. Second, parts of the IgG1 were dynamic and flexible enough in solution to incorporate deuterium with time, as indicated by the slow but steady increase in deuterium incorporation during the labeling time course (if no conformational movement was observed, the deuterium level at 10 seconds would be equal to the deuterium level at 4 hours). Third, the IgG1 appears to exist in one conformation. Had it existed in other conformations that were vastly different at equilibrium, multiple and distinct labeled populations would have been present in the H/DX-MS data⁴³. Finally, the experiments with intact protein or Fc/Fab fragments indicated that the IgG1 was suitable for H/DX-MS experiments under the labeling conditions described.

Pepsin digestion experiments

Global exchange information presented in the preceding sections, although useful, did not characterize or identify how much deuterium exchanged in particular regions. To acquire such information we applied pepsin digestion methodology to the labeled and quenched samples, as originally described by Zhang and Smith²⁶. Briefly, the glycosylated IgG1 was exchanged at various time points in deuterated buffer just as for the intact protein analyses described above. Labeling was quenched and the protein was digested online with an immobilized pepsin column³⁴. As digestion was performed after the labeling reaction had been quenched, the deuteration information that was later measured reflected the tertiary structure of the protein at the exchange pH (in this case, 6.0). Figure 2A shows the UPLC separations obtained for the peptic peptides of IgG1. All of the identified IgG1 pepsin peptides eluted between 2.8 and 7.3 minutes. In total, over 110 peptic peptides were unambiguously identified (well over 200 were detected and eluted in approximately 4 minutes) constituting over 90% coverage of the peptide backbone of the intact IgG1 with multiple overlapping peptides in both heavy and light chains (see Table S-2 and Figure S-2 in the Supporting Information). Each digestion and mass spectral analysis was completed in thirteen minutes (2 minutes for sample injection and pepsin digestion, 2 minutes of desalting, separation lasting 6 minutes, and column wash/re-equilibration for 3 minutes). Twenty pmoles (or less than 2 μg) of protein was injected on column for each time point; all six analyses consumed a total of 120 pmoles (or less than 12 μg) of protein. The entire

LC/MS experiment occupied the instrument for approximately 90 minutes. Table 1 summarizes the time requirements and protein consumption for each of the experiments discussed.

An illustration of the quality of the mass spectral data obtained for the IgG1 is shown in Figure 2B. IgG1 heavy chain peptic peptide covering residues 412-421 (sequence: TVDKSRWQQG) eluted at approximately 4.5 minutes (arrow in Figure 2A). Mass spectra of the +2 charge state of this peptide (Figure 2B) showed an increase in mass upon deuteration and the typical signal to noise levels that were obtained for most of the peptides. These data and that of all the other peptides were processed (see Experimental Section) to generate deuterium uptake curves for all 110 IgG1 pepsin fragments that were routinely monitored (see Supporting Information Figure S-3, blue traces, and Table S-2).

While all the raw data are shown in the Supporting Information, in order to make this amount of data easier to interpret, the changes were plotted on the tertiary structure of the IgG1. To do this, the relative percent exchanged was calculated for each peptide by dividing the experimentally determined value of deuterium incorporation for a particular peptide by the number of backbone amide hydrogens in that peptide (note no correction for back exchange has been made here and the values are therefore noted as relative percent deuteration). The interpretation was specific for this particular IgG1 because in addition to obtaining the H/DX-MS data for the protein in solution, the Fab portion was crystallized and the structure solved using X-ray diffraction (coordinates have been deposited in the Protein Data Bank, code 3FZU; see also Table S-1). Because it was not possible to obtain the crystal structure of the entire IgG1, the structure determined for the Fab fragment of this IgG1 was fused *in silico* to an existing crystal structure of a mAb Fc region⁸ (PDB code 1hzh). The structure of the Fc fragment from PDB 1hzh⁸ had 100% sequence homology in the CH1, CH2 and CH3 domains to that of the IgG1 used in our study. The combined Fab-Fc coordinates were minimized to create a model of the entire IgG1. This final model was used in illustrating and interpreting deuterium incorporation (Figure 3).

The results of the H/DX-MS analysis of IgG1 deuterium incorporation at the peptide level lead to a significant enhancement in the understanding of the solution dynamics of the intact IgG1. Figure 3B–3F depicts the deuterium incorporation profiles of the IgG1 at 10 seconds, 1, 10, 60 and 240 minutes exchange. An animation of the exchange can be found in the Supporting Information (Animation S-1). At 10 seconds (Figure 3B) the protein was mostly protected from exchange with a major portion of the molecule incorporating 10% or less deuterium (purple segments). A few regions showed higher levels of exchange particularly in loops in the CH2 and CH3 domains. After 1 and then 10 minutes (Figures 3C and 3D, respectively), the deuterium incorporation slowly increased as evident by the increased green and yellow colors. A few regions remained very well protected from exchange, mostly β -strands buried within the domains of the IgG1 (black segments). At 1 and 4 hours of exchange (Figures 3E and 3F, respectively), the IgG1 took on more deuterium (increased yellow and orange color) but remained at most only about 50–60% deuterated. Overall and as suspected, based on the data from the intact IgG1 analyses, it is a quite stable molecule.

A more detailed interpretation of the data will be illustrated here for a few segments; such interpretations could be extended to any other regions of the protein. The residues mentioned above in Figure 2B (heavy chain peptide 412-421) form part of an alpha helix located within the CH3 domain (see Figure 3B for location). This structured region appeared solvent exposed in the crystallography derived model and this was reflected in the rate of deuterium incorporation. As the time the IgG1 spent in deuterium increased, a steady increase in the deuterium level was observed for this peptide. The region exhibited an initial ~11% relative deuterium incorporation (teal color) in 10 seconds but took on more deuterium later in the time course: in 1 minute ~20% deuterated (green), in 10 minutes ~30% deuterated (yellow), in 1

hour ~40% deuterated (orange) and after 4 hours >40% deuterated (orange). This gradual increase in the level of deuterium (see also Figure 2B for the spectra and Figure S-3 for the uptake curve) over time with an isotopic distribution of uniform width is indicative of an EX2 exchange regime⁴⁴, typical for exchange into most parts of most proteins²². In another example, pepsin fragment 181–186 (see Figure 3B for peptide location) located in a β -strand in the CH1 domain of the heavy chain did not show incorporation of any deuterium over all exchange time points sampled (black color). This is indicative of a very protected region of the IgG1 that is likely a highly hydrogen bonded structure whose non-covalent forces are not easily perturbed and are strong enough such that no backbone amide hydrogen's became exchange competent⁴⁵ during the time-course of labeling. These data are a small illustration of the diversity of deuterium incorporation results occupied by a single protein. Again, it should be noted that if multiple conformations of this IgG1 simultaneously existed in solution, the data would have revealed more complex deuterium distribution profiles for some peptides. Such complex distributions were not detected in any peptide spectra and we therefore conclude that there was only one population of molecules that were all in a similar conformational state in solution.

The effects of deglycosylation

Previous work with NMR spectroscopy¹⁴ has revealed spectral shifts in IgG1 Fc regions upon deglycosylation, specifically in amino acids spanning the hinge and into CH2 and CH3 domains (roughly residue positions 240 through 263 and 298 through 338, depending on the sequence). As a measure of the potential for H/DX-MS to assess conformational changes in an IgG1, we compared the deuterium exchange, at the peptide level, of glycosylated vs. deglycosylated IgG1. The IgG1 used in our work contained two N-linked glycosylations (one per heavy chain) at Asp 298 and was primarily composed of BiNA0-1Gal (Man3GlcNAc4Gal1Fuc1) species glycans. To prepare the deglycosylated form, the glycans were removed with N-glycanase (PNGase F), the deglycosylated IgG1 was purified by size exclusion chromatography (see Experimental Details); removal of the sugars was confirmed by mass spectrometry (see Supporting Information Figure S-1). Deuterium exchange was performed on the glycosylated and deglycosylated IgG1 and the quenched samples were analyzed under identical experimental conditions. Figure 4A summarizes the significant differences observed as a result of deglycosylation, mapped again onto the model of the structure of this IgG1 (see also description of Figure 3). Colored regions highlight the regions with specific changes: blue indicates where the IgG1 was overall more protected (less deuterium incorporation) as a result of deglycosylation and the regions colored in red indicate where the IgG1 was overall less protected (more deuterium incorporation) as a result of deglycosylation. Figure 4B illustrates representative exchange profiles of three segments that had statistically significant increases or decreases in deuterium levels as a result of deglycosylation. CH2 domain heavy chain residues 292-308 underwent a decrease in deuterium incorporation while residues 236-242 and 242-253 in the CH2 domain experienced an increase in deuterium levels in the deglycosylated form. This region was recently identified by X-ray crystallography as an anchoring point of Fc γ RIII receptor binding to antibody Fc²⁹ and glycosylation seems to play a role in receptor recognition. NMR experiments on IgG1 glycosylation^{14, 15} also indicated conformational disturbances in this area, although far more material and time were consumed in those experiments. Overall, H/DX-MS showed that changes in conformation as a result of deglycosylation were in areas critical for Fc receptor binding. Such experiments indicate the value of using H/DX-MS in the study of protein conformation: now that the regions of alteration have been identified, comparison H/DX-MS experiments with and without Fc receptor will help unravel how conformational disturbance in the identified areas contributes to changes in Fc receptor binding.

CONCLUSIONS

Hydrogen/deuterium exchange mass spectrometry was used to study the conformation and dynamics of an intact IgG1 antibody (average MW for the IgG1 discussed is 144,569 Da deglycosylated). Using instrumentation designed for H/DX-MS work (a custom Waters nanoAQUITY²⁷) and developing software tools (Waters Identity^E) for peptide identification, the experimental analysis of proteins the size of IgG1 can be accomplished by H/DX-MS in a relatively short amount of time. Rather than the typical month-long H/DX-MS experiments one would expect for a molecule of this complexity²², these experiments were completed within days (see Table 1). In addition, the sample requirements were only ~20 pmoles per injection (120 pmoles per experiment) for the pepsin digestion experiments. These sample consumption requirements are one of the most attractive features of using this analytical method for these kinds of conformational characterizations because other methods typically require a minimum of 10-fold or more material. Presently, as we and others are aware, data analysis remains the largest involvement of time. With continued software developments that are in progress in multiple places (*e.g.*, Pascal *et al.* 46), along with hardware development including the addition of robotics⁴⁷, the overall time required for conformational assessment of any protein will become even shorter.

As we have demonstrated here for an IgG1, H/DX-MS analysis can now be routinely applied to very large biopharmaceuticals (which were initially thought to be too big for practical analysis by this method) to obtain detailed information about their conformation and conformational dynamics. The ability to obtain such results with other analytical methodologies is quite challenging since most of these other methods report only global conformation, not localized conformational information. H/DX-MS methodology can clearly detect subtle changes in conformation and can do so with very little material, with high reproducibility, and within a relatively short time. As a result, we are hopeful that this type of analysis will become a mainstream technique for routine characterization of protein conformation and conformational dynamics of protein biopharmaceutical products in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CD	Circular Dichroism
DSC	Differential Scanning Calorimetry
ITC	Isothermal Titration Calorimetry
AUC	Analytical Ultracentrifugation

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Figure 1.

HX MS data for intact IgG1, Fab and Fc fragments. (A) Intact IgG1 exchange analyzed at five different deuterium exchange time points between 0 min and 240 min. The entire exchange experiment was repeated 5 times (each open circle represents an independent determination) and the error range was ± 3.4 to ± 7.4 Da. The total number of backbone amide hydrogens in intact IgG1 is 1228. (B) Deuterium levels in intact Fab (solid symbols, solid line) and intact Fc (open symbols, dotted line) fragments of the IgG1, produced by limited proteolysis of IgG1^{42, 48} at eight different exchange time points between 0 min and 240 minutes. The exchange experiment was repeated twice and the error range was ± 3.0 Da to ± 7.0 Da. The errors bars

shown indicate the maximum observed error of ± 7.0 Da. The total number of backbone amide hydrogens in Fab and Fc is 413 and 402, respectively.

Figure 2.

Representative separations (A) and mass spectra (B) obtained during peptic peptide analyses. The reversed-phase UPLC separation of on-line pepsin digested IgG1 at 0 °C is shown in panel A. The total ion chromatograms are shown for the unlabeled sample (UN, top trace) and the five deuterium exchange time points, as indicated, over the elution window from 2.5 to 7.5 minutes. Mass spectra of the +2 charge state of heavy chain residues 412-421 (sequence TVDKSRWQQG) during the time course of exchange are shown in panel B. The arrow at ~4.5 minutes in panel A indicates the retention time of this peptide.

Figure 3.

Deuterium incorporation information modeled to the structure of IgG1. (A) Model structure of IgG1 illustrating the location of CH1, CH2 and CH3 domains. This structure is a model of the solved crystal structure of IgG1 Fab studied (PDB code: 3FZU) merged with the known IgG1 B12 structure (PDB 1hzh)⁸ of the Fc region (100% sequence homology). The relative percent deuterium incorporation is shown at 10 seconds, 1 minute, 10 minutes, 60 minutes and 240 minutes (B–F, respectively). In panel B, heavy chain residues 181-186 and 412-421 located in the CH1 and CH3 domains, respectively are indicated. The data used to create this figure are found in Supporting Information Table S-2, Figure S-3. An animation of these data is provided in Supporting Information Animation S-1.

Figure 4.

Comparison of deuterium levels in IgG1 with and without glycosylation. (A) The model structure of IgG1 as shown in Figure 3, with the glycosylation indicated in black sticks. Parts colored blue indicate regions where the deglycosylated form had, over all time points, less deuterium (more protection from exchange). Parts colored red indicate regions where the deglycosylated form had, over all time points, more deuterium (less protection from exchange). The data used to make this summary as provided in Supporting Information Table S-2 and Figure S-3. (B) Representative deuterium incorporation profiles comparing exchange in heavy chain residues 292-308 (PREEQYNSTYRVVSVLT), 236-242 (LGGPSVF) and 242-253

(FLFPPKPKDTLM). The black line (solid symbol) represents data from the glycosylated form and the dotted line (open symbol) represent data from the deglycosylated form.

Summary of the experiments performed, the number of replicates for each experiment, the amount of sample required for each, and the overall time involved.

Table 1

Experiment	Exchange time points per experiment [*]	Protein load per injection	Protein consumption per experiment	Independent experiments	LC/MS time per experiment	Data processing time per experiment
Intact IgG	6	400 pmoles	2.4 nmoles	5	1.5 hours	2–4 hours
Intact Fc or Fab	9	200 pmoles	1.8 nmoles	2	2.3 hours	4–6 hours
IgG digestion	6	20 pmoles	0.12 nmoles	3	1.5 hours	40–50 hours

^{*} Exchange time points per experiment include the undeuterated or zero time point.