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Investigating monoclonal antibody aggregation using a combination of H/DX-MS and other biophysical measurements

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Abstract

To determine how structural changes in antibodies are connected with aggregation, the structural areas of an antibody prone to and/or impacted by aggregation must be identified. In this work the higher-order structure and biophysical properties of two different monoclonal antibody (mAb) monomers was compared to their simplest aggregated form, i.e., dimers that naturally occurred during normal production and storage conditions. A combination of hydrogen/deuterium exchange mass spectrometry (H/DX-MS) and other biophysical measurements was used to make the comparison. The results show that the dimerization process for one of the mAb monomers (mAb1) displayed no differences in its deuterium uptake between monomer and dimer forms. However, the other mAb monomer (mAb2) showed subtle changes in hydrogen deuterium exchange compared to its dimer form. In this case, differences observed were located in specific functional regions of the C_H2 domain and the hinge region between C_H1 and C_H2 domains. The importance and the implications of these changes on the antibody structure and mechanism of aggregation are discussed.

Keywords

antibody dimerization; aggregation; size-exclusion chromatography (SEC); hydrogen-deuterium exchange mass spectrometry (H/DX-MS); differential scanning calorimetry (DSC); N-linked glycosylation; small-angle x-ray solution scattering (SAXS); domain swapping

Introduction

In the last two decades, antibodies have become one of the most important protein therapeutic agents in the pharmaceutical industry, with over 30 monoclonal antibodies (mAbs) approved for therapeutic use worldwide.^{1–3} Most of the marketed mAbs belong to the immunoglobulin (IgG) class, and consist of two heavy chains and two light chains linked by inter-molecular disulfide bonds.⁴ During the mAb development process, problems with chemical and physical stability and degradation (e.g., aggregation, deamidation or oxidation) can occur. Such changes are undesirable and can potentially lead to unwanted and adverse toxicological and immunological responses, which in extreme cases may be fatal to

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patients.^{5–7} Hence, degraded and unstable forms of mAbs need to be minimized. In terms of the many forms of instability and degradation associated with protein therapeutics, aggregation is by far the greatest concern to both the biopharmaceutical industry and the regulators who oversee it.⁸

The aggregation of a protein can occur from a variety of reasons and may involve both covalent and non-covalent interactions^{7,9,10} and lead to soluble or insoluble aggregates or a mixture of both, depending on the nature of the protein, its matrix and the environmental conditions.¹¹ A number of specific mechanisms¹⁰ have been discussed in the literature to explain aggregation. In general, the mechanisms are associated with two major properties of a protein. The first concerns the protein colloidal stability, which characterizes the intrinsic propensity of a protein to aggregate, given the protein's matrix and physical and chemical environment. This normally depends on the adventitious presence and arrangement of chemical groups on the surface of the protein that interacts with other chemical groups on either an identical or different surface of another protein molecule. The second property concerns the protein conformational stability, which characterizes the transient conformational changes (i.e., unfolding, or what Jahn and Radford¹² call 'partially unfolded forms', PUF) resulting from normal or abnormal conformational fluctuations in the proteins native structure. This later property causes the exposure of chemical groups, which are normally buried within the protein's interior, to be exposed to the hydrophilic bulk aqueous matrix. Hence, the surface exposure of these typically hydrophobic groups makes the protein prone to aggregate with other partially unfolded or native structures.¹³

Antibodies are proteins with domains rich in beta-sheets. These regions can unfold exposing "hot spots" that are prone to aggregation.¹⁴ Analysis of model peptides suggests that beta-sheet structures have a tendency to stabilize aggregates through a combination of inter-chain hydrogen bonding, hydrophobic associations and complementary packing of side chains.^{15,16} Another element of concern in antibody structure, is the glycosylation of the Fc region that can affect antibody aggregation and stability.^{14,17} In IgG1 molecules, there is a single N-glycosylation site at position N297 in each of the two heavy chains. This glycosylation plays an important role for complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) through modulating the binding to the Fc receptors.^{17,18} We, along with others, have previously shown that conformational changes occur in antibodies if the oligosaccharides present in the C_H2 domain of these molecules are removed or altered in terms of the presence or absence of various sugars.^{19–23}

In the present work, we used a combination of hydrogen/deuterium exchange mass spectrometry (H/DX-MS) and complementary biophysical measurements to study two mAb monomer/dimer systems, one with no glycans and the other with glycans (see next section for details). For each mAb, the higher-order structure (HOS) of the non-aggregated monomer was compared to that of the monomer in its simplest aggregated form, a dimer. The results demonstrate that dimerization proceeded differently for each mAb. The data are also consistent with the idea that dimerization of antibodies may actually proceed, in some cases, via three dimensional (3D) domain swapping (which in this report we simply refer to as domain swapping) around the hinge loop of the antibody.

Results

General description of the antibodies

The aggregation properties were studied between two different antibodies: mAb1 and mAb2. Both of these antibodies were expressed in CHO-cells and purified as described in the Materials and Methods section. mAb1 is expressed as a non-glycosylated antibody, while mAb2 is glycosylated (see Figure S1). These antibodies have high sequence homology

between the heavy and light chains (approximately 85 and 75%, respectively), nearly identical Fc regions, and contain the same number of cysteine residues and disulfide bonds. The starting concentration of both mAbs was approximately 50 mg/mL and after isolation of the monomer and dimer species (as described below), the concentration of both species was approximately 3 mg/mL. All antibodies studied here were formulated under the same buffer conditions (see Materials and Methods).

Purification and characterization of mAb monomer and dimer via SEC

For mAb1 and mAb2, no intentional stress factors were applied to produce the dimers. The dimers isolated and investigated in this work were “naturally” occurring; that is, they were the dimers inherently formed through the developed production, purification and storage conditions used to make each mAb. Analytical SEC was performed to both isolate monomers and dimers and assess their homogeneity. The percentage of dimer found in both starting mAb samples was approximately 1–3% as measured by SEC. These low levels of aggregation were independently confirmed using analytical ultracentrifugation (AUC) (data not shown). Multiple SEC separations of the original mAb1 and mAb2 samples allowed the collection of sufficient quantities of both monomer and dimer fractions, after pooling and concentration for further experiments. Re-analysis of the pooled monomer and dimer fractions by analytical SEC (Figure 1A) revealed that all mAb monomers antibodies eluted at ~ 12 min (± 2 min), whereas the dimers eluted at ~ 9 min (± 1 min). The purified, collected, non-aggregated mAb monomers were 98% pure, while the purified dimer isolated from mAb1 and mAb2 was 96% and 94% pure, respectively. No other higher MW species were observed in the chromatographic profiles of the pooled fractions. Purified monomer and dimer mAbs were stored at 2–8 °C before other analyses (i.e., DSC, H/DX-MS, SAXS, etc.). Periodic re-analysis of the mAb monomer and dimer preparations by SEC indicated that mAb1 and mAb2 were stable. Even after several months at 2–8 °C their SEC profiles remained unchanged and appeared as in Figure 1A. Given that the dimers of mAb1 and 2 were stable, further analysis (as described below) could be performed.

Characterization of dimers by SDS-PAGE

Non-reducing SDS-PAGE was performed on mAb1 and mAb2 monomer and dimer material obtained from appropriately pooled fractions isolated from SEC. SDS-PAGE (Figure 1B) revealed differences between the dimer species isolated from SEC for mAb1 and mAb2. The mAb2 dimer was very heterogeneous compared with the dimer of mAb1 and the most abundant population of mAb2 dimer migrated at approximately 300 kDa. In contrast, the mAb1 dimer migrated as a single 150 kDa band on the gel with only a slight band at 300 kDa. The isolated mAb2 SEC dimer material appeared to be composed of a mixed population including stable covalent dimers (displaying a MW of 300 kDa) with some non-covalent dimers. The mAb1 and mAb2 monomer and dimer samples were also analyzed by SDS-PAGE under reducing conditions (data not shown). The bands observed were those corresponding to the HC and LC, indicating that the mAb2 dimer is, at least in part, disulfide linked.

Disulfide mapping of mAb monomer and dimer samples

To determine if changes in disulfide bonding played any role in dimer formation, mAb1 and mAb2 monomers and dimers were subjected to LC/MS disulfide peptide mapping (see Figure 2). The chromatographic profiles of reduced and non-reduced mAb1 and mAb2 monomers and dimers were comparable, with no new peptides detected in the mAb dimer sample relative to their monomer counterparts. Some differences in peptide peak heights were observed but this is likely due to variability in digestion efficiency and chromatography. All expected peptides and disulfide linked peptides were detected and no evidence of unbound Cys residues and or scrambled disulfide bonds were observed (data not

shown). While non-reducing SDS-PAGE analysis of mAb1 dimer suggested that the sample was mainly non-covalent and mAb2 contained covalent dimer, disulfide mapping suggested that the dimers do not contain any inter or intra disulfide scrambling.

Thermal stability of mAb monomers and dimers using DSC

Differential scanning calorimetry (DSC) was used to analyze mAb1 and mAb2 monomers and dimers. Figure 1C shows that as expected for antibodies, the thermograms of the antibody monomers had multiple endothermic transitions.²⁴ The thermal transitions for all individual mAb domains (i.e., C_H2, C_H3, etc.) were unambiguously identified by isolating and analyzing the domains independently (data not shown).²⁵

The thermograms for the non-glycosylated antibody (mAb1) showed three distinct transitions, corresponding to different antibody domains, in both monomer and dimer forms (Figure 1C). The Fab-C_HL region and the Fc-C_H3 domain unfold at around 82 °C.^{25,26} The pattern of unfolding was very similar for both the monomer and the dimer. The Fab-V_HL domain unfolds at approximately 70 °C^{25,27} and again no difference in melting temperature was observed between the monomer and the dimer in mAb1. Thermal unfolding of the Fc-C_H2 domain occurs at lower temperatures^{24–26} and a difference was detected between mAb1 monomer and dimer in the Fc-C_H2 domain. The dimer unfolded at a temperature about 5 °C lower than that of mAb1 monomer (55 °C for the dimer vs. 60 °C for the monomer). Based on these DSC results, one would conclude that dimers of mAb1 had structural stability alterations in the Fc-C_H2 domain, relative to the monomer of mAb1.

On comparing the DSC thermograms for the mAb2 dimer relative to the mAb2 monomer, a very distinct difference was observed (Figure 1C). This difference is highlighted by what appears, in the dimer sample, as the merging of all domains (especially the Fc-C_H2) into one major and broad transition with an average melting temperature of ~75 °C. The DSC thermograms of the mAb2 antibody were considerably different than those of mAb1. In the case of the mAb2 monomer, the Fab melted at ~ 80 °C²⁵ and the Fc-C_H3 melted at ~ 86 °C, which appears as a shoulder on the main thermal transition at 80 °C.²² The Fc-C_H2 domain of mAb2 monomer melted at a higher temperature ($T_m \sim 70$ °C) relative to the Fc-C_H2 of mAb1 monomer ($T_m \sim 60$ °C), which indicates that mAb2 has a more stable Fc-C_H2 structure than mAb1 (Figure 1C) due to glycosylation, consistent with previous reports.²² DSC data thus indicates that there is a significant difference in the unfolding transitions between mAb1 and mAb2 dimers relative to their monomeric form.

Conformational assessment of the mAb monomers and dimers by SAXS

To determine how the dimers of mAb1 and mAb2 might differ in overall shape, small angle X-ray scattering (SAXS) was performed. SAXS is a solution-based technique that can provide structural information about a protein's size and shape. One important parameter derived from SAXS experiments is the radii of gyration (R_g). The R_g essentially is a measure of the distribution of a molecule's mass about its center of mass, and therefore provides information about molecule's size or dimensions. For the monomers of mAb1 and mAb2, the R_g were estimated to be 50.4 ± 0.75 Å and 47.3 ± 0.75 Å, respectively, while the R_g for mAb1 and mAb2 dimers were estimated at 80.0 ± 1.5 Å and 78.0 ± 1.5 Å, respectively. Circularly averaged scattered intensity from these proteins is shown in Figure 3. It was possible to obtain three-dimensional shape reconstructions of the monomer species (data not shown) and the results were found to be consistent with previous analyses of IgG1 monomers by SAXS.^{28–31} The nature of the dimer scattering - with no subsidiary maxima evident - suggested a very flexible molecule that gave rise to a broad distribution of conformations. Modeling of IgG1 dimers produced theoretical R_g values in the range of 69.2 to 80.5 Å; our observations were most consistent with the largest of the predicted values.

While the SAXS data describing the dimeric form of the mAbs were consistent with what would be expected, there were no obvious indications from SAXS that would explain the differences in the dimers as observed to this point by the other biophysical methods.

H/DX-MS analysis of the mAb monomers and dimers

H/DX-MS is an extremely useful method for the HOS characterization of mAbs because it can generate information on the locations of differences in conformation and conformational dynamics of proteins in solution.^{19,20,32–40} This information is acquired by monitoring the hydrogen/deuterium exchange (H/DX) of a protein's backbone amide hydrogen for deuterium. Because the level of deuterium incorporation is an indication of solvent exposure and hydrogen bonding,⁴¹ indirect information about the HOS of a protein and conformational dynamics can be obtained^{39,42}.

The deuterium incorporation as a function of time for each peptic peptide was determined in duplicate experiments for both the purified mAb monomers and for the dimers. For mAb1, and mAb2, H/DX was monitored in a total of 123 and 141 peptides, respectively, covering 96% and 99% of the heavy chain amino-acid sequence, respectively, and 95% and 94% of the light chain sequence, respectively (Figures S2 and S3). For each mAb, the deuterium levels for peptides that were common in both monomer and dimer forms were monitored and are presented in Figure S4 (mAb1) and Figure S5 (mAb2). Comparing the H/DX-MS data for the two antibodies, no differences in deuterium incorporation between monomer and dimer were observed in the light and heavy chains of mAb1. However, mAb2 showed some changes in deuteration in monomer relative to dimer.

In the mAb2 heavy chain, the majority of the peptides had identical deuteration between monomer and dimer (see Figure 4A). The difference plot (Figure 4B) revealed that a few peptides had altered deuterium incorporation above the statistically-determined significance threshold (dotted lines).³³ These differences, as shown in Figure 5, were located in the hinge region connecting the Fc-CH1 and Fc-CH2 domains and in the Fc-CH2 domain of the antibody.⁴³ The peptic peptide covering residues 206-242 was unambiguously identified via tandem mass spectrometry with MS^E and PLGS 2.5⁴⁴. Among the 30 exchangeable amide hydrogen's in peptide 206-242, only 4 backbone amide hydrogen's were exchanged for deuterium in the mAb2 monomer and ~3 were exchanged in the dimer (Figure 5A). Mass spectra of the deuterated mAb2 peptic fragment 206-242 (Figure 5B) show that the isotopic distributions exhibit a signature characteristic of multiple populations in both mAb2 monomer and dimer.³⁸ While only the +5 charge state is shown in Figure 5B, multiple charge states were observed and displayed the same exchange behavior. The hinge was the only segment in both the mAb2 monomer and the dimer to display this type of isotope distribution behavior. Peptide carryover between subsequent sample injections can sometimes be an issue, causing unusual isotope distributions that can lead to erroneous conclusions. Hence blank injections were run between sample injections to ensure that no peptides were carried over from one injection to the next.⁴⁵ The rates of deuteration of peptide 206-242 were different between monomer and dimer. For example, following 10 s of exposure to D₂O, the monomer was more deuterated than the dimer as indicated by the shift of the isotopic distribution to higher m/z (Figure 5B). In time, peptide 206-242 in the mAb2 dimer seemed to adopt a conformation that was protected from exchange (the higher mass population disappeared), indicating occlusion from exchange in this region upon dimerization; therefore the deuterium incorporation graph in Figure 5A shows a lower deuterium uptake at the later time points in the dimer. The peak width analysis is also indicative of cooperative unfolding (Figure 5B). A shorter, overlapping peptide (residues 206-217) did not display the multiple population behavior (data not shown), and there was no difference in deuterium uptake in 206-217 between the mAb2 monomer and dimer

(Figure 5A). Consequently, the residues involved in this exchange difference in mAb2 were refined to residues 218-242 of the hinge region (Figure 5C).

Interestingly, the corresponding hinge region in mAb1 did not exhibit the same deuterium uptake pattern, thus the conformational heterogeneity is likely different. Figure 5D show the deuterium incorporation graphs for three overlapping peptides that cover the hinge region of mAb1. (*Note:* because the amino acid sequence of the hinge region is different between mAb1 and mAb2, we were not able to identify a peptide fragment that corresponded exactly to amino acids 206-242 as in mAb2). Unlike mAb2, there was no difference in deuterium uptake between the non-aggregated mAb1 monomer and dimer in this region. As an example, the mass spectra of the non-aggregated mAb1 monomer and dimer for peptide 216-234 are illustrated in Figure 5E. We discuss the differences between mAb1 and mAb2 in this region in more detail below.

Another region in mAb2 which had different deuterium incorporation between the monomer and dimer was located at the beginning of the Fc-CH₂ domain. In the Fc-CH₂ domain, a series of overlapping peptides between amino acid 249-259, which are located at the N-terminus of the Fc-CH₂ domain, displayed increased deuterium incorporation in the mAb2 dimer compared to the mAb2 monomer (Figure 6A). Figure 6B shows that following 10 s of exposure to D₂O peptide 249-259 is at least 1 Da heavier in the dimer than in the free monomer. Increased deuterium uptake in the dimer was observed only after 10 s and 1 min of exchange. For longer incubation periods, the differences disappeared, most likely because in time (due to protein flexibility) this area eventually becomes exposed for deuteration. Unlike in mAb2, in the non-glycosylated mAb1 (Figure 6D–F), peptide 249-259 did not exhibit any significant deuteration difference between the monomer and the dimer. Figure 6D shows the deuterium uptake as a function of exchange incubation time for the same region of the Fc-CH₂ domain (249-259) in mAb1. The pattern of deuterium incorporation in mAb1 is different compared to mAb2 and the peptide 249-259 experienced less dynamic behavior with deuterium incorporation complete after 1 min (Figure 6E). This peptide behaved similarly in both the non-aggregated mAb1 monomer and dimer. The location of this peptide is highlighted on the crystal structure of both mAbs (Figure 6C, F). Other than the structural areas discussed above, no other conformational changes were observed upon dimerization, in either of the mAb1 or mAb2 antibodies (light chains, Fabs, CH₁, CH₂ or CH₃ domains). A summary of all H/DX-MS differences observed on mAb2 is shown in Figure S6.

Discussion

Aggregation of mAbs (or proteins in general) is an extremely complex process that appears to be influenced by a number of factors that include the conformational flexibility or dynamics exhibited by these molecules. As a result of the latter, it has been suggested that some mAbs aggregate through a pathway involving conformational stability, which leads to the attraction of exposed hydrophobic residues. However, if the intermolecular forces are not strong enough to attract and stabilize these partially unfolded antibodies, refolding of the mAb can occur (reversible unfolding) before aggregation can ensue. Therefore, this partial unfolding theory is essentially bound to the physical and chemical instabilities of the antibody. These instabilities, however, are linked to the amino acid sequence of these molecules and their physicochemical environment. The amino acid sequence and environment control how proteins fold and behave in solution, especially in terms of their dynamic structural properties, which in the end, controls the nature of the bonding forces (covalent, non-covalent, combination of both) involved in holding aggregates together.⁴⁶

Remmele et al. investigated dimer aggregates of a “native” and thermally stressed IgG1 mAb (Epratuzumab).⁴⁷ They found that the apparent homogeneous dimer material was in fact heterogeneous, containing Fab:Fab, Fc:Fc and Fab:Fc linked dimers, of which 70% (of the native dimer) and 84% (of the thermally stressed dimer) were covalently linked. Further analysis of the covalent dimers revealed they were not all disulfide linked.⁴⁷ A different publication by Yoo et al. showed that IgG2 mAbs are prone to the formation of covalent dimers.⁴⁸ Since IgG2 antibodies have been found to contain a small level of free cysteine⁴⁹, Yoo et al. speculated that the small population of free cysteine residues may be involved in the dimerization.⁴⁸ Most recently, Huh et al. reported similar work in which they identified free cysteine residues and characterized aggregates that resulted from agitation stress.⁵⁰

Additional experiments involving the exposure of different IgG1 mAbs to freeze thawing, elevated temperatures, low pH and UV light to induce dimer formation indicated that the aggregate was primarily non-covalent, involving the monomer association between the Fab variable regions.⁴⁶ In this work, we characterized two different IgG1 mAb dimers with the goal of identifying areas prone to or impacted by aggregation.

Covalent versus non-covalent dimers

The mAb dimers examined in this report appear to aggregate through entirely different pathways. Our SEC data shows that dimers of mAbs 1 and 2 are stable, as indicated by the comparability of all of their SEC traces obtained from t=0 to t=12 months relative to their initial collection (data not shown). Characterization of mAb1 and mAb2 dimers by non-reducing SDS-PAGE allowed us to distinguish between covalent (which remain associated and appear at their oligomeric MW) and non-covalent oligomers (which dissociate and appear at their monomeric MW). It was evident from the gel, in Figure 1B, that the nature of the dimer is different between mAb1 and mAb2. Although the gel for mAb1 dimer displays a very faint band at 300 kDa and minor bands between 150 and 250 kDa, the majority of the mAb1 dimer molecules have the same MW as the monomer (i.e., 150 kDa). In contrast, the majority of the mAb2 dimer molecules migrate at 300 kDa with minor bands between 120 and 250 kDa. These results suggest that, in principle, mAb1 dimerizes through non-covalent interactions, which are disrupted during non-denaturing SDS-PAGE, while the majority of the aggregation of mAb2 occurs primarily via covalent bonds, which are not disrupted during non-denaturing SDS-PAGE.

Structural heterogeneity

The number of bands in the non-reducing SDS-PAGE of mAb2 indicates that the mAb2 dimer is heterogeneous. Structural heterogeneity is also evident in the SAXS and DSC data. SAXS analysis of mAb1 and mAb2 monomers revealed scattering intensity plots (Figure 3) that were expected and consistent with data obtained in previous experiments for other mAbs.^{29,31} However, the scattering plots generated for both mAb dimers were more characteristic of those obtained for random polymer chains (a pattern often associated with a denatured or disordered protein) than for globular proteins. Since SAXS data represents a sum of scattering from all conformational populations present in the mixture, for a molecule that is highly flexible, the structural ensemble will lead to scattering plots that are very broad, resembling that of a denatured protein. Thus the SAXS for mAb2 suggests that this sample, while a chemically homogeneous mixture, has a structurally heterogeneous population of dimers that are related to one another, perhaps by significant flexibility in the linkages between domains and possibly within the domains themselves. These data are not surprising given the heterogeneity observed in the SDS-PAGE analysis for the mAb2 dimer (Figure 1B). While SDS-PAGE analysis of the mAb1 dimer does not show the same heterogeneity (most likely due to the absence of covalent bonds that would maintain the different dimer structures during SDS-PAGE), SAXS data does suggest that the non-

covalent mAb1 dimer is also structurally heterogeneous and likely composed of many different dimer configurations associated non-covalently.

DSC of mAb2 dimer also supports the hypothesis concerning the structural heterogeneity of this material. A single broad transition was observed in the DSC thermogram of mAb2 dimer, most likely due to a high degree of structural heterogeneity, i.e., different populations of covalent and non-covalent dimers. In other words, the heterogeneous population of dimers gives rise to slightly different melting temperatures for each domain and for each population, making it practically impossible for DSC to resolve all the transitions.^{51,52} For mAb1, the DSC data only shows a change in the Fc-CH₂ domain, indicating that the hypothesized non-covalent association is coordinated primarily through the CH₂ domain. Since the SDS-PAGE gel indicates that the mAb1 dimer is likely non-covalent and the SAXS data suggests that the mAb1 dimer is heterogeneous, it is also possible that there are many other non-covalent dimer configurations in the mAb1 population which may not involve the CH₂ domain and do not affect mAb1 thermal stability.

More insight from location refinement

Results from the reduced and non-reduced peptide maps of the mAb dimers (Figure 2) indicate that the monomer and dimer samples were comparable in terms of disulfide bonds. Since mAb1 dimer is non-covalent (see non-reduced SDS-PAGE, Figure 1B) and because no new peaks appeared in the disulfide map (Figure 2A), the data suggests that the intra- and intermolecular disulfide bonds within mAb1 are correctly linked and internal disulfide scrambling is not the cause of dimer formation.

For mAb2, if the dimers were non-covalently linked, we would have not expected to see the large 300 kDa band by non-reducing SDS-PAGE. In the disulfide mapping of mAb2 (Figure 2B), the mAb2 dimer profile was comparable to the monomer and all expected disulfide linked peptides were found (data not shown), indicating that disulfide scrambling is not the cause of dimerization. Moreover, if the mAb2 dimer was covalently linked through primary chemical bonds that did not involve disulfide bonds we would have expected to see a new peak(s) in the reduced peptide map and none were detected (Figure 2B). Therefore, we hypothesize that the covalent dimerization of mAb2 occurs primarily through intermolecular disulfide bonds that have the same connectivity as those found in the monomer. In other words, the disulfide bond responsible for the formation of the mAb2 dimer connects the heavy chain hinge region or the heavy chain and the light chain of one monomer to their corresponding linkage in another monomer (i.e., a form of domain swapping, as shown in “FIG. 1C” by West et al.^{53,54}, see Discussion below).

From a different perspective, perhaps the non-covalent associations holding the dimer together appear to originate in the Fc-CH₂ domain. Previous studies^{55,56} using DSC analysis on a non-glycosylated IgG murine CH₂ domain indicated that the Fc-CH₂ domain is unstable relative to the other antibody domains and that methionine oxidation at the Fc-CH₂ and Fc-CH₃ interface will diminish the stabilization energy of the Fc-CH₂ domain and/or Fc-CH₂ and Fc-CH₃ interactions.²⁶ Zheng et al. have shown that IgG1 aggregation (derived from Fc-CH₂) can be eliminated if the IgG1 interacts with protein A/G fragments.⁵⁶ In our current studies, the CH₂ domain from the non-glycosylated mAb1 had lower overall thermal stability, which may be correlated to an increase in structural flexibility. Based on our DSC thermograms generated for mAb1, the Fc-CH₂ domain seems to be affected by dimerization (see Figure 1C) and these data support previous results that the Fc-CH₂ is sensitive to changes in an IgG1 mAb. Consequently for mAb1, the non-covalent associations holding the dimer together appear to originate in the Fc-CH₂ domain. For our glycosylated mAb2, other domains appear to be sensitized upon dimerization (see Figure 1C) since the entire

thermogram is altered. However, it was not very clear based solely on DSC or SDS-PAGE which mAb2 domains (or parts of them) were more affected by aggregation.

The techniques highlighted above (i.e. SEC, SDS-PAGE, SAXS, DSC) provide useful information on the mAbs behavior and some insight into their dimerization from a global perspective. However, even in the best case scenario where information about individual domains could be obtained, they lacked the level of detail that H/DX-MS can provide. H/DX-MS is especially suited for analysis of “difficult and otherwise incompatible” proteins that cannot be studied with other methods like crystallography, NMR and cryoEM. The locations of conformational and long-range dynamic changes in proteins can be obtained with just small amounts of protein (low pmol range)^{35–39} as illustrated in H/DX-MS work conducted by Zhang et al. on the mechanisms of aggregation of a mAb under thermal and freeze-thaw stress conditions.⁵⁷ In our current work, we used H/DX-MS to help elucidate the dimerization pathways in mAbs that did not undergo intentionally-induced stress. Our focus was to investigate the conformation and conformational dynamics of “hot-spots” in the monomer mAbs that are susceptible throughout the normal manufacturing, purification and storage process. The following paragraphs elucidate the most important conclusions derived from our H/DX-MS analysis, which is further supported by our other biophysical techniques.

The hinge-loop region is sensitive to dimerization

Based on the H/DX-MS data, the peptide 206-217 (corresponding to a beta-hairpin loop like structure) overlapping the 206-242 region is more protected from deuteration at earlier time points, and in time, it becomes more solvent accessible, implying only partial flexibility of this region (Figure 5 A). The deuterium uptake curve for the entire peptide 206-242 indicates that this region is not very flexible and is protected from the solvent, as indicated by the low number of hydrogen atoms that are exchanged with deuterium. While the representative crystal structure (PDB: 1HZH) indicates that this peptide is located within an unstructured and looped region (particularly amino acids 218-242), our data suggests that this region is in a conformation that is more occluded from the solvent. From these data, there is a correlation with the published literature⁵⁸ indicating that sections of the hinge region are more rigid than others. The lack of shorter overlapping peptides covering this area does not allow us to clearly identify these regions, and we are only able to identify an average level of H/DX from this area.

Heavy chain peptide 206-242 on mAb2 contains four cysteine residues that are involved in disulfide bonds in the intact molecule. One of the cysteine residues (C208) is located in peptide 206-217 in which no differences in deuterium uptake were observed between mAb2 monomer and dimer. Disulfide mapping has shown that this cysteine residue is involved in an intramolecular disulfide bond within the heavy chain. Consequently, C208 is likely not involved in dimerization and the H/DX-MS data suggests that one or more of the other three cysteine residues C228, C234 and C237 may be responsible for the aggregation. In the monomeric form, cysteine residue C228 in the extended hinge region (residues 206-242) forms a disulfide bond with another cysteine residue in the light chain. One possible explanation for dimerization would be formation of a disulfide linkage between C228 from one heavy chain molecule and the same cysteine residue of another molecule. In this case, the resulting aggregate would lack two disulfide linked light chains and have a molecular weight of ~250 kDa. The non-reduced SDS-PAGE gel shows a minor band at 250 kDa for mAb2 dimer and supports this hypothesis. However, this disulfide linkage was not observed in the disulfide map. Since the disulfide map should be able to detect low level species (< 1%), the population of dimers associated with the 250 kDa band is likely heterogeneous and if different linkages are present, they are below the level of detection. Disulfide mapping did confirm that cysteine residues C234 and C237 form a pair of

disulfide bonds between two heavy chains at the hinge region.⁵⁴ These residues belong also to peptide 206-242 and might be involved in the creation of a 300 kDa dimer aggregate as evidenced by the most prominent SDS-PAGE band observed for mAb2 (Figure 1B).

The hinge region in an antibody is thought to play an important role in modulating some effector functions. The human IgG hinge sequences are only 50–70% homologous.⁴⁸ mAb1 antibody shares the amino acid hinge region corresponding to that of a human $\gamma 4$ isotype whereas mAb2 shares that of a human $\gamma 1$ isotype.⁴⁸ Reducing the length of the hinge region generally results in a decrease in complement fixation⁵⁹ or activation.^{60,61} Mutations or deletions in the upper (E224-T233) and middle (C234-P238) hinge have been shown to decrease rigidity and can have great effects on the overall antibody stability and complement dependent cytotoxicity.^{48,54,58} It was previously shown in an anti-HIV neutralizing antibody that if certain amino acids are removed from the upper hinge region (especially T231 and H232), the dimer/ monomer ratio increases. Furthermore, removal of D229 and K230 in addition to T231 and H232 affects antibody dimerization too, but to a lesser extent than the removal of the T231 and H232 only.⁵⁴ Interestingly, six amino acids (C228, D229, K230, T231, H232, T233) are missing from the middle hinge region of mAb1 where the four important amino acids residues discussed above are located (D229, K230, H232 and T233) (see Figure 7A, B). The H/DX-MS results show no differences in deuterium incorporation in mAb1, which is missing key pieces of the hinge. Whereas mAb2 has an intact hinge and contains differences in H/DX between monomer and dimer, which correlates well with the prior data and supports the hypothesis that mutations/deletions in the hinge region cause variation in the dimerization patterns of different mAbs. Unlike mAb2 whose dimerization pathway could occur through covalent and to a lesser extent non-covalent interactions, mAb1 aggregation may primarily result from non-covalent interactions. Since C228 is missing from the mAb1 hinge, and consistent with the observations just noted, dimerization of mAb1 through hinge regions effects would be diminished.

C_H2 domain is more easily deuterated upon dimerization

The H/DX-MS data for the mAb2 antibody are in good agreement with common knowledge that the Fc-C_H2 domain unfolds faster and therefore is less stable than other domains. For instance, peptide 249-259 from mAb2 incorporated more deuterium upon dimerization; therefore, this region of the Fc-C_H2 domain becomes more solvent exposed, i.e., less stable. We previously showed by H/DX-MS that partial or complete removal of the glycans from an antibody will affect its conformation in the exact same Fc-C_H2 region.^{19,20} It was shown that the backbone of K254 makes contact with a galactose residue (this would occur in mAb2), leading to an increase in structural rigidity.²⁰ This contact will be lost when the sugars are not present, thus making this region (249-259) more solvent accessible. Additionally, by DSC and PNGaseF digestion, Zheng et al.⁵⁶ showed that three IgG1 antibodies exhibited higher aggregation rates after glycans were removed and they suggested that the Fc-C_H2 domain is involved in aggregate formation. More recently Majumdar et al. showed how salts affect the backbone flexibility of an IgG1, and in particular C_H2 residues 241-252.^{62,63} These data point to the overall sensitivity of this region to its environment and storage conditions. Because differences in dimer-related deuterium incorporation were only detectable in the glycosylated antibody, mAb2, the glycans and their location in close proximity with peptide 249-259 may indirectly affect the dimerization of glycosylated antibodies. In mAb1, residues 249-259 are more rapidly deuterated due to a conformation that is more solvent accessible in the absence of glycans compared with the same residues in mAb2 (Figure 6A, 6D). This protection must be related to the presence of the glycans and the structural stability the glycans impart on the mAb2 C_H2 domain.

Domain Swapping

To explain aggregation at the molecular level, several reports have invoked the concept of domain swapping.^{64–66} Domain swapping occurs when the internal contacts within a given protein domain are disrupted and interactions are swapped with the corresponding contacts from another molecule's identical domain. The result is a dimer or a higher-order oligomer.⁶⁴ Proteins that undergo domain swapping adopt two conformations: a “closed” interface (in the monomeric form) which is preserved in the newly formed dimer, and an “open” conformation in the oligomeric form.⁶⁷ Interestingly the two conformations are almost the same differing in only one area: a loop or a hinge element that links the swapped domain with the rest of the polypeptide chain, whose movement/flexibility enables the swapping to occur.⁶⁷ In other words, the HOS of the swapped domains in each monomer is maintained in the dimer form. The only change that occurs, results from the entanglement of the random coil of the two aggregated mAb hinge regions. Such an entangled structure may yield H/DX-MS results that differ little, in terms of measurable H/DX behavior.

Based on our data we hypothesize that mAb2 dimerization occurs through domain swapping at the hinge region. The H/DX-MS data show that the hinge region linker is affected by dimerization, which may result from the switch between the “closed” to the “open” conformations⁵³ (see also “FIG. 1C” by West et al.⁵⁴). Even if the monomer is in a “closed” configuration, the H/DX-MS data reveal different conformational states that can lead to different levels of deuteration (Figure 5B). Upon dimerization when the monomer would transition into the “open” conformation, the hinge remains more solvent protected, as can be seen in the differences between the bimodal distributions observed for peptide 206-242 in both mAb2 monomer and dimer (Figure 5B). Concurrent to this, conformational changes detected at the hinge cause peptide 249-259 of the Fc-CH₂ region to become more solvent accessible in mAb2 dimer relative to the mAb2 monomer.

Domain swapping is not always accompanied by detectable H/DX-MS conformational changes. It is possible that domains swap without causing solvent protection/exposure or inducing allosteric changes and secondary structure disruption. For example, in the heavy chain portion of Fabs that were interchangeable between two monomers⁵⁴, it is possible that no direct conformational changes could be detected by H/DX-MS. Moreover, if most of the interactions involved in dimerization occur through side-chain interactions, H/DX-MS may reveal nothing as it only monitors backbone amide exchange. This latter situation might explain the results observed for mAb1 where no significant differences in deuterium uptake were observed upon dimerization. If the dimer of mAb1 were driven by non-covalent forces that involved mostly side-chain association, and no backbone amide hydrogen environments were affected, no changes would be observed by H/DX-MS. Finally, because the mAb1 hinge is shorter than that of mAb2, we suggest that (without discrediting the possibility of domain swapping) due to spatial constraints mAb1 is less likely to undergo dimerization through this process.

Conclusions

Using several orthogonal techniques, the HOS of two mAb monomers and dimers was investigated. Our results show that the aggregation mechanism between the two mAbs is different, even though they share high sequence similarity. H/DX-MS revealed no conformational changes between mAb1 monomers and its corresponding dimer. However, DSC suggested that mAb1 dimerization affects the CH₂ domain, while SAXS indicated that the dimer is structurally heterogeneous. We propose that the aggregation of mAb1 predominantly involves the amino acid side chains of the CH₂ domain, whose exchange rates are not measurable by H/DX-MS.

On the other hand, differences in the H/DX-MS of mAb2 were observed between monomer and its dimeric form in the hinge and Fc C_H2 domain. SDS-PAGE and SAXS demonstrated that the dimer contains structurally heterogeneous covalent and non-covalent forms. Therefore, mAb2 aggregation most likely proceeds through a collection of mechanisms, including: domain swapping, disulfide scrambling and surface interactions.

In this paper, we have shown that by combining H/DX-MS results with those obtained from other biophysical methods (i.e., SEC, DSC, SDS-PAGE, disulfide mapping and SAXS), we were able to place constraints on speculating and interpreting H/DX-MS data. Although H/DX-MS affords the highest spatial resolution among these techniques, and should play a key role in revealing the smallest structural elements affected by aggregation, this may not always be realized. Hence a broad approach of engaging different methodologies, as presented in this paper, builds a better foundation for obtaining important information for understanding those properties that are responsible for causing aggregates. Such knowledge should be of significant importance in assessing the risks associated with aggregation encountered with different protein drugs.

Materials and Methods

Reagents

96% formic acid was purchased from Sigma-Aldrich. TCEP ((tris (2-carboxyethyl) phosphine) was purchased from Thermo Scientific. Sodium chloride, guanidine hydrochloride (GnHCl), sodium phosphate monobasic, sodium phosphate di-basic was purchased from Research Products International Corp. Deuterium oxide (99.9%) was purchased from Cambridge Isotopes. Acetonitrile and water (W5–4 HPLC grade) were purchased from Fisher Scientific. The pepsin column was purchased from Applied Biosystems. All chemicals were used without further purification unless otherwise specified.

Antibodies

The antibodies were expressed in Chinese Hamster Ovary (CHO) cells and purified by ion-exchange and size-exclusion chromatography at Biogen Idec (Cambridge, MA). The final buffer composition for all antibodies was: 100 mM Na phosphate and 200 mM sodium chloride (NaCl), pH 6.8.

SEC

Size exclusion chromatography (SEC) of all antibody monomer and dimer samples was performed on a Waters 2690 HPLC system (Waters, Corp.) with a Tosoh Bioscience G3000SWxl column at ambient temperature in 100 mM Na phosphate, 200 mM NaCl, pH 6.8 under isocratic conditions with a flow rate of 0.5 mL/min. 100 µg of mAb was injected for each analytical separation. The collection of the dimer species was performed with injections of 200 to 500 µg of mAb. Protein elution profiles were recorded by monitoring absorption at 280 nm.

SDS-PAGE

Non-reducing Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on a Novex 4–12% Tris-Glycine precast gel from Invitrogen – Life Technologies (Grand Island, NY). 1.5 µg of each monomer and dimer antibody sample, as well as a molecular weight marker, were loaded onto each lane and the separation proceeded for 1.3 hours at 30 mA and 220V. The gel was stained in Coomassie blue to visualize the protein bands.

Reduced and Non-Reduced LC/MS Peptide Mapping

mAb samples were digested with Lys-C by first diluting 100 μ g of protein in 90 μ L 8 M guanidine hydrochloride and 1 μ L 10 mM N-ethylmaleimide (NEM) with 50 mM sodium phosphate buffer, pH 6.5 to a final volume of 125 μ L. The sample was kept at 25 °C for 1 hour for denaturation. The mixture was then diluted using 50 mM sodium phosphate buffer (pH 6.5) to a final volume of 370 μ L. The digestion was performed with the addition of 10 μ g of Lys-C (10 μ L of 1 μ g/ μ L Lys-C in water) (enzyme/substrate 1:10) at 25 °C for 18–20 hours. For reduced maps, 10 μ L of 100 mM dithiothreitol (DTT) was added to 100 μ L of the non-reduced protein digest and incubated at 25 °C for 1 hour. All Lys-C protein digests (reduced and non-reduced) were analyzed using an Agilent 1100 HPLC with an ion-trap mass spectrometer (Deca XP, Thermo Fisher). Approximately 150 pmol of protein digest was injected onto an YMC C18 column and detected by mass spec between 400–2000 m/z. Mobile phase A was 0.1% (v/v) trifluoroacetic acid (TFA) in water and mobile phase B was 0.1% (v/v) TFA in 90% acetonitrile. Peptides were eluted from the column with a linear gradient of 0% B to 65% B in 60 minutes. The column was then washed with 100% B for 10 minutes and equilibrated with 0% B for 10 minutes. Lys-C digestion of mAb1 and 2 resulted in over 97 and 95% amino acid sequence coverage, respectively. The unidentified sequence, which corresponds to the approximate 5% are very small di- and tri-peptides that likely elute in the initial aqueous phase of the gradient.

DSC

All DSC measurements were performed using a MicroCal capillary VP-DSC system with Origin VPViewer2000 version 2.0.64 controlling software (GE Healthcare). Sample measurements in the DSC were made at a concentration of 0.5 mg/mL (total protein concentration) in 100 mM Na phosphate, 200 mM NaCl pH 6.8. The thermograms were generated by scanning the temperature increase from 30 to 110 °C at a rate of 2 °C / minute. Buffer blank runs were also measured under the same experimental conditions to correct all DSC sample runs. All data was processed using Origin 7SR2 software and Microsoft Excel.

SAXS

SAXS data were collected using the undulator-based beam line X9 at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory using two detectors to collect both SAXS and WAXS data simultaneously, to collect scattered intensity in the range of $0.006 < q < 2.0 \text{ \AA}^{-1}$.⁶⁸ X-ray wavelength was 0.9184 Å. A Photonic Science CCD detector operated as the WAXS detector and a Mar 165 CCD as the SAXS detector. The SAXS detector was located 3.4 m from the sample. Samples were loaded into a 96-well plate and aspirated into the 1.5 mm diameter, thin-walled sample tube using an automated system as described.⁶⁸ Data processing was carried out using the X9 software package to produce circularly averaged intensity profiles combining data from the two detectors and extending over the entire q range.

H/DX-MS

H/DX-MS experiments were performed essentially as described in Houde et al.¹⁹ where deuterium labeling in solution was performed for purified forms of a known monomeric or dimeric version of the same IgG1 antibody. All the antibody concentrations (for monomer and dimer) were adjusted to 11.6 μ M in 100 mM Na phosphate, 200 mM NaCl pH 6.8. For each labeling time point, 3 μ L of antibody (monomer or dimer) was diluted with 15-fold 100 mM sodium phosphate, 200 mM NaCl, (pD 6.8) D₂O buffer at room temperature. At each deuterium exchange time point (from 10 sec to 4 hrs) an aliquot from the exchange reaction was removed and labeling was quenched by adjusting the pH to ~2.5 with an equal volume of quench buffer [4 M GnHCl, 200 mM phosphate buffer, 0.5 M TCEP ((tris (2-

carboxyethyl) phosphine), H₂O]. Quenched samples were immediately frozen on dry ice and stored at -80 °C until analysis.

Each flash frozen sample was rapidly thawed and injected into a Waters nanoACQUITY with HDX Technology (Waters Corp.).⁶⁹ The protein samples were digested online using a Poroszyme immobilized pepsin cartridge 2.1mm × 30 mm (Applied Biosystems). The digestion temperature was set to 15 °C and the digestion was performed for 30 sec. The cooling chamber of the UPLC system, which housed all the chromatographic elements were held at 0.0 ± 0.1 °C for the entire time of the measurements. The injected peptides were trapped and desalted for 3 min at 100 µL/min and then separated in 14 min by an 5% – 40% acetonitrile:water gradient at 40 µL/min. The separation column was a 1.0×100.0 mm ACQUITY UPLC C18 BEH (Waters Corp.) containing 1.7 µm particles and the back pressure averaged 8800 psi at 0.1 °C. The average amount of back-exchange using this experimental setup was 18% to 25%, based on analysis of highly deuterated peptide standards. Deuterium levels were not corrected for back-exchange and are therefore reported as relative³⁸; however, all comparison experiments were done under identical experimental conditions thus negating the need for back exchange correction.³⁸ The UPLC step was performed with protonated solvents, thereby allowing deuterium to be replaced with hydrogen from side chains and the amino/carboxyl terminus that exchange much faster than amide linkages.⁴¹ All experiments were performed in duplicate. The error of determining the deuterium levels was ± 0.2 Da in this experimental setup consistent with previously obtained values.⁴⁰ In order to eliminate peptide carryover, a wash solution of 1.5 M GnHCl, 0.8% formic acid and 4% acetonitrile was injected after each run.

Mass spectra were obtained with a Waters XEVO G2 TOF or Synapt G1 equipped with standard ESI source (Waters Corp.). The instrument configuration was the following: capillary was 3.2 kV, trap collision energy at 6 V, sampling cone at 35 V, source temperature of 80 °C and desolvation temperature of 175 °C. Mass spectra were acquired over an m/z range of 100 to 2000. Mass accuracy was ensured by calibration with 500 fmol/µL GFP, and was less than 10 ppm throughout all experiments. The mass spectra were processed with the software DynamXTM (Waters Corp.) by centroiding an isotopic distribution corresponding to the +2, +3, or +4 charge state of each peptide. Deuteration levels were calculated by subtracting the centroid of the isotopic distribution for peptide ions of undeuterated protein from the centroid of the isotopic distribution for peptide ions from the deuterium labeled sample. The resulting relative deuterium levels were automatically plotted versus the exchange-in time. Identification of the peptic fragments was accomplished through a combination of exact mass analysis and MS^E using Identity Software (Waters Corp.). MS^E was performed by a series of low-high collision energies ramping from 5–30 V, therefore ensuring proper fragmentation of all the peptic peptides eluting from the LC system.⁷⁰ Peptic maps were obtained with the help of the on-line MS Tools application⁷¹ and DynamX software from Waters Corp. Pymol software⁷² was used to map the conformational changes on the crystal structure of an IgG1 antibody (PDB: 1hzh).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Biophysical characterization of mAb1 and mAb2 monomers and dimers. (A) Normalized analytical SEC traces obtained after several rounds (>20 injections per mAb) of collection and reinjection. Each injection contained 100 μ g of mAb and SEC was monitored at 280 nm. The black traces correspond to the mAb1 monomer (solid line) and dimer (dotted line). The red traces correspond to the mAb2 monomer (solid line) and dimer (dotted line). (B) Coomassie blue stained non-reducing SDS-PAGE. 1.5 μ g of each sample was loaded on the gel. From left to right: the first lane corresponds to the molecular weight standard; next two lanes correspond to the mAb1 monomer and dimer; next two correspond to the mAb2 monomer and dimer. (C) Thermally induced unfolding of mAbs monitored by DSC. All DSC measurements were performed in 100 mM sodium phosphate, 200 mM NaCl pH 6.8 and at fixed concentration of 0.5 mg/mL. Thermograms of the non-glycosylated antibody mAb1 are represented by the solid black (monomer) and dotted black (dimer) lines. Thermograms of the glycosylated antibody mAb2 are shown with solid red (monomer) and dotted red (dimer) lines.

Figure 2. Disulfide mapping of mAb1 and mAb2 monomer and dimer samples. (A) LC/MS traces of reduced and non-reduced mAb1 monomer and dimer samples. (B) LC/MS traces of reduced and non-reduced mAb2 monomer and dimer samples.

Figure 3. SAXS scattering intensity and Guinier plots for mAb1 and mAb2 monomers and dimers. (A) SAXS scattering profile plotted as a function of the momentum transfer q for the monomer and dimer of both antibodies. The mAb1 and mAb2 monomers are black and red solid lines, respectively, while the mAb1 and mAb2 dimers are black and red dotted lines, respectively. (B) Guinier analysis of the low q data from solutions containing mAb1 and mAb2 monomer (red and black solid lines, respectively), mAb2 monomer (red solid line), mAb1 and mAb2 dimer (red and black dotted line, respectively).

Figure 4. H/DX-MS comparability profile³³ of mAb2 monomer heavy chain versus the dimer. (A) Mirror or butterfly plot of the average relative fractional exchange data for monomer (top) versus the dimer (bottom), as a function of peptide “ i ”. The x -axis is the calculated peptide midpoint, i , position of each of the 84 peptides compared. The y -axis is the average calculated relative fractional exchange. Each point is an average of two separate and independent H/DX-MS comparison experiments. The orange, red, cyan, blue, and black lines correspond to data acquired at 10 s, and 1, 10, 60, and 240 min of deuteration, respectively, for both samples. (B) Plot of the H/DX-MS difference data from panel A for mAb2 monomer and dimer. The average error between two measurements in a peptide was ± 0.2 Da. Significant changes above the black horizontal lines are considered as described in reference.³³

Figure 5.

The hinge region is sensitive to dimerization in glycosylated **mAb2** (A–C). (A) Amino acid sequence of the peptide 206-242 and deuterium uptake curves corresponding to overlapping peptides 206-217 and 206-242. These data show the average uptake for three charge states in duplicate experiments. For peptide 206-242, the maximum deuteration is 30 Da but for ease of visualization, we show only up to 12 Da on the y-axis. All the other deuterium incorporation graphs presented for the representative peptic peptides show the maximum theoretical deuteration number, which is calculated by subtracting the first amino acid in the peptide and the proline residues. (B) Mass spectra during deuterium incorporation for the (+5) ion of peptide 206-242. (C) Physical representation on the crystal structure (PDB: 1hzh) of the two overlapping peptides across the hinge region: residues 206-217 are shown in green, whereas the remaining residues (218-242) are represented in purple. The hinge region is not affected by dimerization in non-glycosylated **mAb1** (D–E). (D) Amino acid sequence of the peptides between residues 199-242 overlapping the hinge region in mAb1 and deuterium uptake curves corresponding to overlapping peptides 199-221, 216-234 and 201-234. (E) The mass spectra during deuterium incorporation is showing the (+4) ion for the peptide covering 216-234.

Figure 6.

The Fc-CH₂ domain is more rapidly deuterated in the dimer. (A–C) **mAb2**: Deuterium uptake curves corresponding to overlapping peptides 249-259 and 250-259. The mass spectra corresponding to the (+3) ion of peptide 249-259 is shown here. Cartoon representation on the crystal structure (PDB: 1hzh) of the peptide 249-259 in the Fc-CH₂ domain (red). Glycans attached to position N297 are shown in black sticks. (D–F) **mAb1**: Deuterium uptake curves and mass spectra of the (+3) ion corresponding to peptide 249-259. Cartoon representation on the crystal structure (PDB: 1hzh) of the peptide 249-259 in the Fc-CH₂ domain (red).

Figure 7.

Important amino acids are missing from the mAb1 hinge region. (A) Sequence alignments for the hinge region in both antibodies. The amino acids that are different between the two monoclonal antibodies samples are indicated in orange in mAb1 sequence and in cyan in mAb2 sequence. The amino acids missing from mAb1 but present in mAb2 are shown in a black box. (B) Cartoon representation on the crystal structure (PDB: 1hzh) of the two peptides across the hinge region: the peptide 206-242 is represented in purple and the different amino acids are shown in either orange or cyan as described above.