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Conformational Difference in Human IgG2 Disulfide Isoforms Revealed by Hydrogen/Deuterium Exchange Mass Spectrometry

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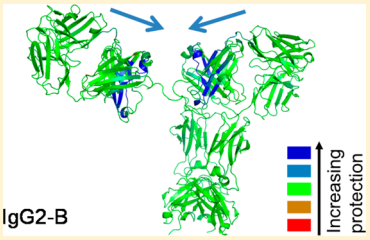
Supporting Information

ABSTRACT: Both recombinant and natural human IgG2 antibodies have several different disulfide bond isoforms, which possess different global structures, thermal stabilities, and biological activities. A detailed mapping of the structural difference among IgG2 disulfide isoforms, however, has not been established. In this work, we employed hydrogen/deuterium exchange mass spectrometry to study the conformation of three major IgG2 disulfide isoforms known as IgG2-B, IgG2-A1, and IgG2-A2 in two recombinant human IgG2 monoclonal antibodies. By comparing the protection factors between amino acid residues in isoforms B and A1 (the classical form), we successfully identified several local regions in which the IgG2-B isoform showed more solvent protection than the IgG2-A1 isoform. On the basis of three-dimensional structural models of IgG2, these identified regions were located on the Fab domains, close to the hinge, centered on the side where the two Fab arms faced each other in spatial proximity. We speculated that in the more solvent-protected B isoform, the two Fab arms were brought into contact by the nonclassical disulfide bonds, resulting in a more compact global structure. Loss of Fab domain flexibility in IgG2-B could limit its ability to access cell-surface epitopes, leading to reduced antigen binding potency. The A2 isoform was previously found to have disulfide linkages similar to those of the classical A1 isoform, but with different biophysical behaviors. Our data indicated that, compared to IgG2-A1, IgG2-A2 had less solvent protection in some heavy-chain Fab regions close the hinge, suggesting that the A2 isoform had more flexible Fab domains.



Monoclonal antibody (mAb) has become the most important class of therapeutic proteins in the biopharmaceutical industry. Of the four IgG antibody subclasses, IgG1 and IgG2 are the two major forms that have been mostly explored for their therapeutic applications. Compared to IgG1, the IgG2 subclass antibody has two additional disulfide bonds in the hinge region that cross-link the two heavy chains. The disulfide linkages in the IgG2 antibody were first established by Milstein and Frangione in 1971[1] as represented by the IgG2-A isoform in Figure 1. For more than three decades, it was considered as the only disulfide bond connectivity for IgG2 antibodies, until Wypych and co-workers reported two alternative disulfide bond linkage patterns. These alternative disulfide bond isoforms, as shown in Figure 1, differed from the classical isoform by the linkage in four of the six interchain disulfide bonds and were designated as IgG2-A/B and IgG-B disulfide isoforms.[2,3] Since then, much effort has been made to elucidate the detailed disulfide connections in these isoforms.[3−5]

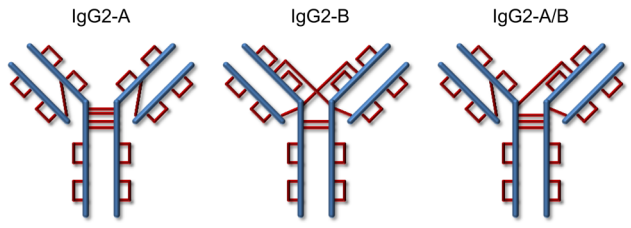


Figure 1. Disulfide structure of major IgG2 disulfide isoforms.

These IgG2 disulfide isoforms can be separated by cation exchange chromatography or reversed-phase chromatography.[2,3] An isoform that eluted later than IgG2-A via reversed-phase chromatography was later discovered to have a disulfide structure similar to that of the IgG2-A form,[6] but with different biophysical and biochemical properties.[7] The late-eluting isoform was originally named IgG2-A*.[6] In recent reports, it was renamed as IgG2-A2, while the original IgG2-A isoform was termed IgG2-A1.

With the discovery of these nonclassical disulfide linkages in human IgG2 antibodies, increasing efforts have also been made in investigating their origin,[6,8] distribution,[6] and the relevant impact on the molecular structure and biological activities.[3,9] It is now well understood that these different disulfide isoforms exist at various abundances in both natural human IgG2 antibodies in vivo and recombinant IgG2 expressed from mammalian cells.[2] Using multiple biophysical technologies, Dillon et al. suggested that these different isoforms have different packing structures at the global molecular level, with IgG2-B, -A/B, -A1, and -A2 isoforms following a decreasing order of global structural compactness.[3] In another study, Bagal et al. showed that the IgG2-B isoform has a collision cross

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section larger than that of IgG2-A in the gas phase by using ion mobility mass spectrometry.[10] Because of the low-resolution biophysical techniques employed in these previous studies, the rationale for the contradictory observations has not been understood, and the understanding of the structural differences among the various isoforms has been limited.

In addition to the lack of knowledge of their structures, our understanding of the potential implications to the biological activities of various disulfide isoforms is quite limited, too. Though there has been one case of study to show that the enriched IgG2-A isoform has an antigen binding affinity for interleukin-1 receptor higher than that of the unfractionated mixture in a cell-based potency assay, followed by the enriched IgG2-B isoform,[3] the universal applicability of the observation to other IgG2 antibodies in terms of antigen binding is unclear. As for the Fc effector functions, the disulfide bond heterogeneity in the hinge region seems to have minimal impact on the binding activities of both FcγR and complement factor C1q.[9] The rationale for why the different disulfide bond linkages in the hinge region have a more profound impact on the spatially distant antigen binding rather than the Fc effector functions in the proximity is not clear, because of the lack of detailed structural elucidation of various disulfide isoforms. For therapeutic applications of any recombinant IgG2 mAbs in the biopharmaceutical industry, the presence of various disulfide isoforms becomes a major source of product heterogeneity. With limited knowledge of their structures and impact on biological activities, it can raise significant concerns about their potential implications for the therapeutic efficacy and safety of IgG2.

In this study, we employed the technology of hydrogen/deuterium exchange combined with mass spectrometry (HDX-MS)[11,12] to map the detailed structural difference between different IgG2 disulfide isoforms purified from two IgG2 mAbs. HDX-MS has been an increasingly popular technology for protein conformation analysis and provides both high resolution and sensitivity for the detection of minor local structural changes in different conformational states. Recently, an improved HDX-MS platform with sophisticated modeling of labeling and back-exchange kinetics in a fully automated data processing package was developed in our lab. Its application was demonstrated in monitoring the gradual unfolding of all domains in an IgG1 mAb with increasing guanidine concentrations.[13] In this work, we studied the conformational differences of three major purified IgG2 disulfide isoforms, including IgG2-B, IgG2-A1, and IgG2-A2, using the improved HDX-MS platform. The IgG2-A/B isoform was not examined in this study, taking into account its transient nature in vivo[6] and the fact that it is a hybrid of isoforms A and B.

MATERIALS AND METHODS

IgG2 Disulfide Isoform Purification. The two recombinant IgG2 κ mAbs (IgG2#1 and IgG2#2) were produced by Amgen. Disulfide isoforms IgG2-B, -A1, and -A2 were purified by cation exchange (CEX) chromatography followed by affinity chromatography utilizing antibodies against human IgG2 epitopes specific to the individual disulfide isoforms. The detailed purification procedure was published elsewhere.[14] Briefly, the IgG2 solution was loaded onto an XK 50/60 column (GE Healthcare) packed with the YMC Biopro S30 CEX resin (500 mL) operated on an AKTA FPLC system (GE Healthcare) at ambient temperature. Mobile phase A consisted of 20 mM sodium acetate (pH 5.2) and mobile phase B of 20 mM sodium acetate and 400 mM sodium chloride (pH 4.5). Proteins were eluted with a linear gradient, and timed fractions were collected. Pooled fractions were concentrated and buffer-exchanged into 20 mM sodium acetate buffer (pH 5.2) using a 30 kDa Centricon Plus-70 device (EMD Millipore, Billerica, MA) and then subsequently further purified by anti-human IgG affinity chromatography that bound specifically to the IgG2-A isoforms. The IgG2-B isoform was eluted by flow-through mode under phosphate-buffered saline, and the IgG2-A1 isoform was eluted with a linear gradient of acetic acid. The IgG2-A2 isoform was bound and eluted at different salt concentrations in acetate buffers. The purities of isolated isoforms were evaluated by reversed-phase chromatography on a C8 column operated at 75 °C.[3] The isolated IgG2-B, IgG2-A1, and IgG2-A2 samples were buffer-exchanged with the same 20 mM acetate buffer (pH 5.2) containing 5% sorbitol to minimize the matrix effect in HDX-MS analysis.

HDX-MS Setup. The detailed experimental setup has been described previously.[13] Briefly, deuterium labeling, quenching, and peptic digestion were performed on a Leap PAL HD-X (Leap Technologies, Carrboro, NC) system. Deuterium labeling was initiated by diluting the IgG2 sample 10-fold into D_2O buffer. After different time intervals, the labeling was quenched by diluting the labeling solution 4-fold into a quench buffer [0.45 M glycine (pH 2.7)] containing 0.625 M tris(2-carboxyethyl)phosphine and 7.25 M urea at 1 °C. The quenched solution was then diluted 4-fold into a pepsin solution for digestion (6 min at 1 °C), followed by liquid chromatography/mass spectrometry (LC−MS) analyses on an Agilent (Santa Clara, CA) 1290 Infinity system coupled to a Thermo Scientific (San Jose, CA) LTQ-Orbitrap high-resolution mass spectrometer with an electrospray ionization interface. The proteolytic peptides were separated on either a Waters (Milford, MA) BEH C18 column (2.1 mm × 50 mm) at a flow rate of 0.36 mL/min or a Waters BEH C18 column (1.0 mm × 50 mm) at a flow rate of 0.09 mL/min. The column temperature was set at 1 °C, and peptides were eluted with an acetonitrile gradient, and 0.02% trifluoroacetic acid and 0.1% formic acid in each mobile phase. Mass spectrometric data were acquired in the Orbitrap instrument with a resolution of 60000 (at m/z 400) in centroid mode. Prior to the HDX experiment, an unlabeled protein sample was analyzed three times by data-dependent LC−MS/MS for the purpose of peptide identification. Two data-dependent MS/MS scans were performed in the linear ion trap after each full MS scan. For accurate HDX modeling to derive possible protection factors, both non-deuterated and fully deuterated controls were analyzed as external back-exchange standards. The nondeuterated control was obtained by direct quenching of an unlabeled protein solution, and the fully deuterated control was obtained by performing deuterium labeling in the presence of 6.9 M guanidine for 8 h. Additionally, an equal concentration of a peptide mixture (bradykinin, angiotensin I, and leucine enkephalin) was added to the unlabeled protein solution as well as the D_2O buffer as internal back-exchange standards. A tetrapeptide Pro-Pro-Pro-Ile (synthesized by AnaSpec, Fremont, CA) was mixed into the protein solution at half of the protein molar concentration as an internal standard for modeling the intrinsic HDX rates, so that the slight HDX difference caused by the sample matrices will be monitored and corrected.

H/D exchange was performed at three different conditions, including a 100 mM phosphate buffer (pH 7), a 100 mM

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phosphate buffer with 2 M urea (pH 7), and a 100 mM acetate buffer (pH 5). All pH values were directly read from a pH meter.

Data Processing. All HDX-MS data were processed on MassAnalyzer[15] using the HDX modeling function. MassAnalyzer receives the raw HDX-MS data and the protein sequence as input and generates possible protection factors of individual residues as output in a fully automated fashion. Details of the HDX model have been described elsewhere.[13] Briefly, the model contains the protection factors of each residue as parameters. Optimizing the model with all experimental data generates protection factors of all residues. The HDX model is an underdetermined system, meaning more than one set of protection factors fit the experimental data equally well. To gain a more accurate and complete representation of the protein conformation, a Monte Carlo method is applied to obtain 20 possible sets of protection factors. For the sake of visual clarity, the average values of the 20 sets of protection factors are presented.

Homology models of IgG2-A1 were generated on Molecular Operating Environment (Chemical Computing Group, Montreal, QC), using a human IgG1 crystal structure as a template [Protein Data Bank (PDB) entry 1HZH].[16] To create the homology models, the IgG1 template was first mutated by replacing several appropriate residues with cysteine residues, and then the appropriate sulfur atoms were manually linked to create the disulfide linkages that mimic those in the IgG2-A1 isoform. The resulting structure was then refined by energy minimization using the Amber10:EHT force field with reaction-field solvation. This newly created IgG2-A1-like structure was then used as a template for the homology modeling of IgG2-A1 isoforms.

RESULTS

Isolation of IgG2 Disulfide Isoforms. The disulfide isoforms of the two IgG2 mAbs were purified by cation exchange chromatography followed by affinity chromatography utilizing a custom antibody against human IgG2 epitopes specific to IgG2-A isoforms. The purities of the isolated disulfide isoform samples were further evaluated by reversed-phase chromatography.[3] Figure 2 shows the reversed-phase profiles for IgG2#2. Compared to the unprocessed IgG2 sample that comprised several disulfide isoforms (Figure 2a), the purified samples (Figure 2b–d) had much higher homogeneity with one major desired isoform for each. Profiles for IgG2#1 (Figure S1 of the Supporting Information) were similar to those of IgG2#1, except that the A2 form was not purified and studied. These isoforms were stable for up to 6 months at 4 °C, as indicated by the lack of significant changes in their chromatographic profiles (data not shown).

Structural Differences of IgG2 Disulfide Isoforms. The two purified isoforms IgG2-B and IgG2-A1 of IgG2#1 were subjected to H/D exchange in parallel by diluting each sample 10-fold into D_2O buffer at pH 7. After labeling for time periods ranging from 30 s to 8 h, the exchange was quenched and the IgG2 antibody was digested. A total of 335 peptides (from 4 to 62 residues per peptide, with an average of 19 residues) were identified and tracked for H/D exchange by MassAnalyzer. These peptides covered 100% of the IgG2 sequence in both light and heavy chains (see the Supporting Information for a coverage map). Unlike most other HDX data analysis programs, MassAnalyzer implements a comprehensive mathematical model to simulate the deuterium labeling and back-exchange processes and calculates several sets of the most possible protection factors for each backbone amide hydrogen as the final output. As shown in Figure S2 of the Supporting Information, MassAnalyzer utilized the deuterium labeling time courses of all 335 peptides obtained for each isoform as input and calculated 20 sets of possible protection factors of each amino acid residue for each corresponding isoform. Figure 3 compares the average profiles of the 20 sets of possible protection factors between the IgG2-A1 and IgG2-B isoforms. Both the light and heavy chains are shown.

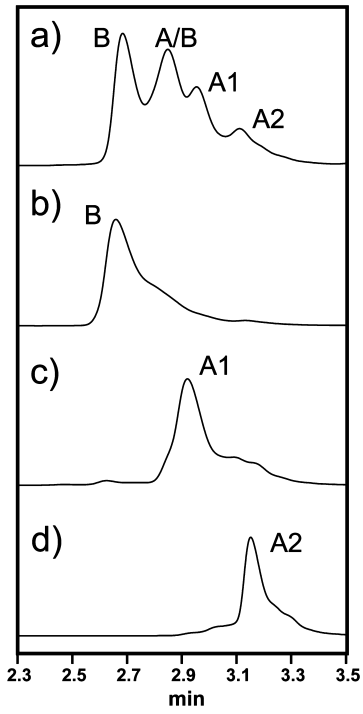

Figure 2. Reversed-phase chromatograms of the purified isoforms from IgG2#2 demonstrating the purity of the isoforms. Panel a is the chromatogram of unpurified IgG2#2; panels b–d are the chromatograms of purified B, A1, and A2 forms, respectively.

It is seen that the overall profiles of the protection factors across the light and heavy chains are similar between IgG2-A1 and IgG2-B, indicating the two isoforms had similar structures. Clear differences, however, are observed in the region near residue 121 (spanning residues 118–129) in the light chain and near residue 200 (spanning residues 185–203) in the heavy chain. In both regions, the IgG2-B isoform displays protection factors higher than those of the IgG2-A1 isoform, suggesting a more solvent-protected local structure in the IgG2-B isoform.

To examine whether the structural difference observed between IgG2-A1 and IgG2-B was affected by solution conditions, the HDX comparison of the two disulfide isoforms was also performed under two additional conditions: pH 5.0 as well as pH 7.0 in the presence of 2 M guanidine. The protection factor profiles of the two disulfide isoforms, as shown in Figure S3 of the Supporting Information, exhibited patterns similar to that measured at pH 7.0. To better visualize the difference between the IgG2-A1 and IgG2-B isoforms, differential protection factors were calculated by subtracting log(protection factor) of IgG2-A1 from that of IgG2-B. The differential protection factor plots of the light and heavy chains under the three H/D exchange conditions are shown in Figure 4. It is clearly shown that at pH 7.0, the IgG2-B isoform was

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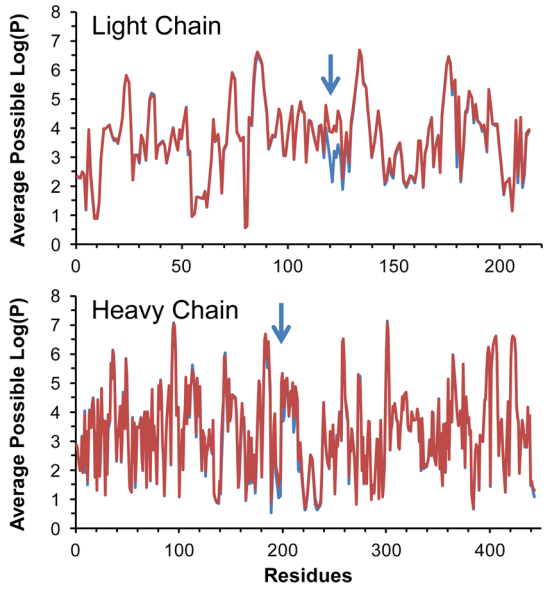

Figure 3. Protection factor plots of A1 (blue) and B (red) isoforms of IgG2#1 (pH 7). The vertical axis is the average logarithm (10-based) value of possible protection factors.

more solvent-protected within two discrete local regions in the light chain and three discrete regions in the heavy chain. In the light chain, these more protected regions in the IgG2-B isoform were comprised of residues 118−129 (sequence FPPSDEQLKSGT) and 179−181 (sequence LTL). In the heavy chain, these more protected regions consisted of residues 185−203 (sequence SVVTVPSSNFGTQTYTCNV), 210−219 (sequence TKVDKTVERK), and 140−142 (sequence TAA). The differential protection profiles measured under the other two solution conditions shown in Figure 4 supported a similar conclusion. Figure 5 shows the deuterium incorporation of two representative peptides of light-chain residues 117−125 and heavy-chain residues 187−201 from these regions over the labeling time course at pH 7.0.

To understand whether the localized structural differences observed between different disulfide isoforms are molecule specific, we conducted a similar HDX analysis for the isoforms of another IgG2 mAb (IgG2#2). In this case, three disulfide isoforms, IgG2-A1, IgG2-A2, and IgG2-B, were isolated and subjected to H/D exchange at pH 7.0. The protection factor profiles of the three isoforms are shown in Figure S4 of the Supporting Information, while the differential protection factor plots of IgG2-B and IgG2-A2 as compared to IgG2-A1 are shown in Figure 6. The data demonstrated that, similar to the case of IgG2#1, the B isoform of IgG2#2 had increased solvent protection compared to that of its A1 isoform in certain local regions of both the light and heavy chains. These regions included residues 118−135 (sequence FIFPPSDEQLKSGTASVV) in the light chain and residues 183−200 (sequence

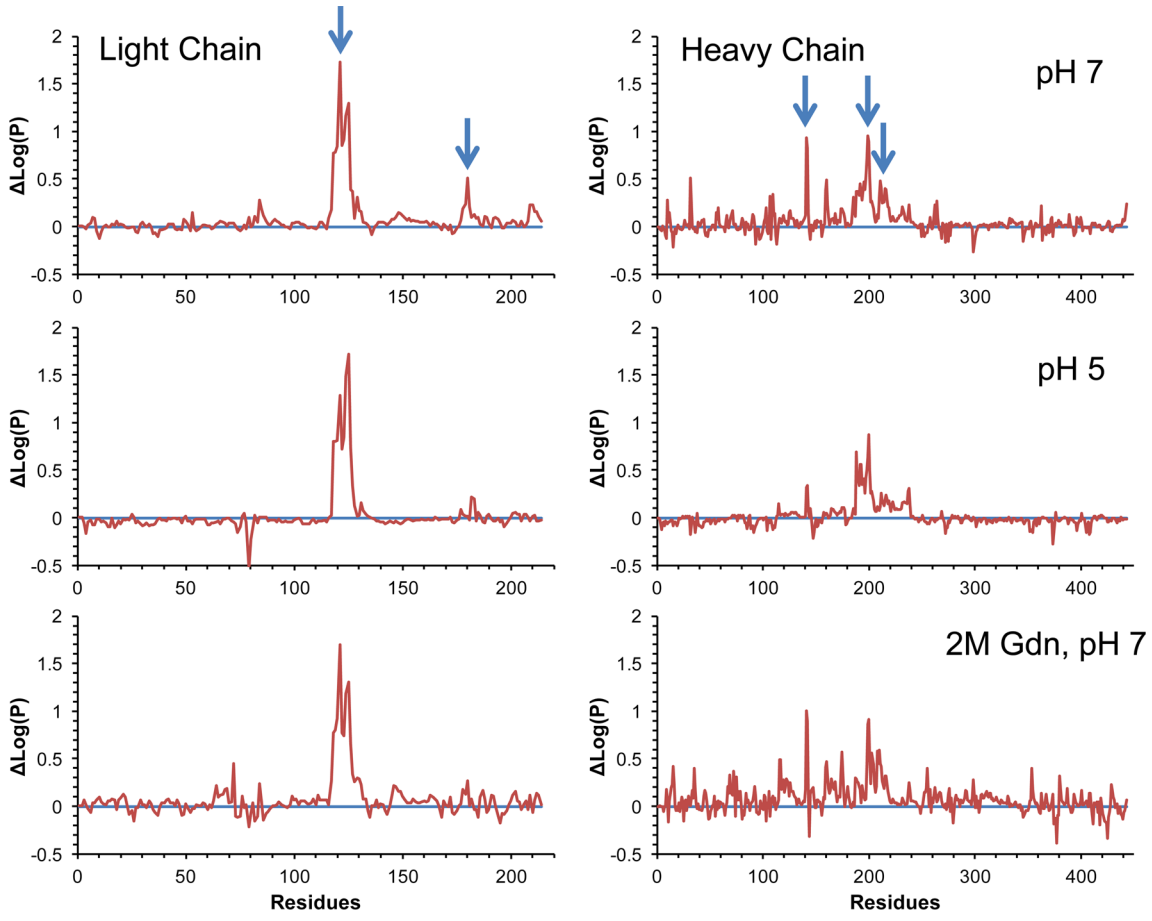

Figure 4. Differential protection factor plots of IgG2#1 light-chain (left) and heavy-chain (right) residues between IgG2-B (red) and IgG2-A1 (blue) isoforms under different H/D exchange conditions, using IgG2-A1 as the reference. More protected regions in the B isoform are denoted with arrows.

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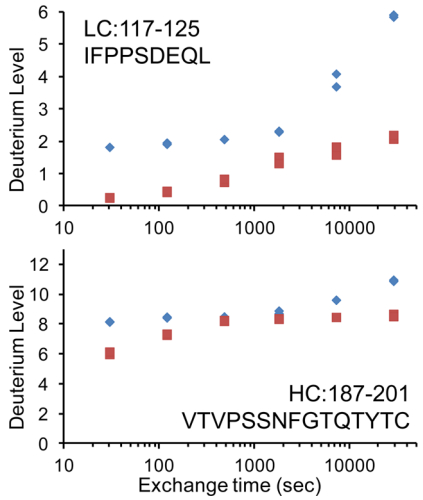

Figure 5. Time courses of two representative peptides from IgG2#1 with more protected residues in the IgG2-B form (red) vs the IgG2-A1 form (blue). Duplicate runs were conducted for each time point. All data points were corrected for back-exchange using fully deuterated controls as well as back-exchange internal standards.

VVTVPSSNFGTQTYTCNV) as well as residues 137−141 (sequence TAALG) in the heavy chain, which were in agreement with the observations in the case of IgG2#1. By contrast, the protection profile of the A2 isoform was much closer to that of the A1 isoform, with only minor differences seen in the regions of residues 154−207 in the heavy chain. Interestingly, the A2 isoform showed less solvent protection than the A1 isoform in these identified regions in the heavy chain, while no significant difference was observed in the light chain between A2 and A1.

To correlate these regions with their spatial locations in the three-dimensional (3D) structure, homology models of the IgG2-A1 forms of the two IgG2 mAbs were generated by using the crystal structure of human IgG1 as a template (PDB entry 1HZH).[16] In Figure 7, the regions displaying a conformational difference between IgG2 isoforms are shown in the 3D structure models of IgG2-A1 isoforms. Regions colored blue are the residues that showed increased solvent protection (pH 7) in the IgG2-B isoform relative to the IgG2-A1 isoform, while those colored red are the residues that showed decreased solvent protection in the IgG2-A2 isoform.

DISCUSSION

IgG2 antibodies have κ and λ subtypes, with the former molecules forming a relatively large population of all disulfide isoforms. The IgG2 λ molecules form only a small percentage

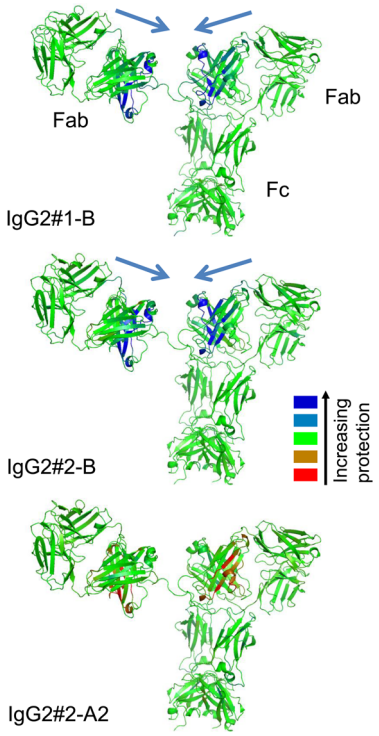

Figure 7. Differential protection factors of IgG2-B and IgG2-A2 (compared to IgG2-A1 at pH 7) mapped onto the homology models of IgG2-A1 of IgG2#1 and IgG2#2. More protected regions are colored in blue and less protected regions red.

of the IgG2-B form,[3] probably because of the presence of an additional serine reside at the C-terminus of the light chain, causing spatial restriction in the hinge to prohibit IgG2-B formation.[17] The two IgG2 antibodies selected in this study were both of the κ subtype. So far, no crystal structure of the intact human IgG2 antibody has been reported.

The covalent structures of these disulfide isoforms and their chromatographic elution behaviors have been extensively studied.[2,5] Previous studies have also shown that the disulfide isoforms do exist in human native IgG2.[2] Under the slightly reducing conditions at neutral pH in the presence of cysteine/cystine pairs in human blood, it was observed that the IgG2-A1 form converts slowly to the B form.[6,17] The A2 isoform, on the other hand, remains stable.[7] At neutral pH in the absence of redox conditions, however, all disulfide isoforms remain stable.[3] It is known that disulfide redox reactions further slow under mildly acidic conditions. The samples used in this study were formulated in a mildly acidic formulation in the absence of a cysteine/cystine pair, and the disulfide isoforms were therefore

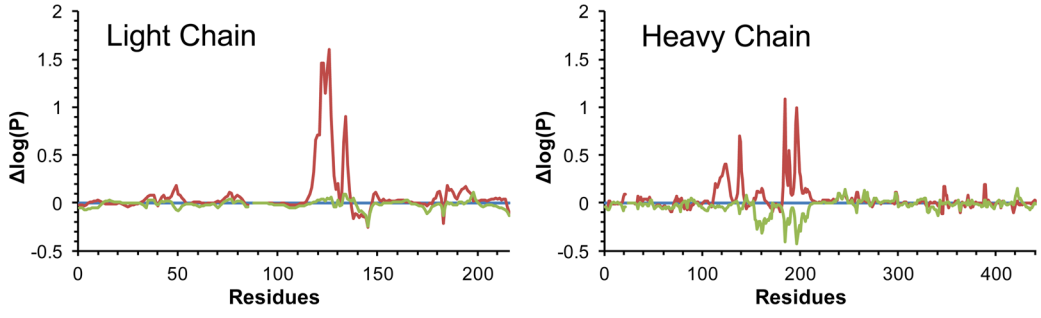

Figure 6. Differential protection factor plots for IgG2-B (red) and IgG2-A2 (green) of IgG2#2 at pH 7, as compared to that of IgG2-A1 (blue).

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stable. Under these conditions, the interconversion between sulfhydryl and disulfide is also minimal.

In this study, we utilized the high-resolution HDX-MS technique to map the local structural differences among the three IgG2 disulfide isoforms. HDX analysis of the disulfide isoforms for two IgG2 antibodies led to the identification of a similar set of discrete local regions with differential solvent protection resulting from the alternative disulfide bond linkages.

The 3D structural models shown in Figure 7 indicate that all the regions with more solvent protection in the IgG2-B isoform were located at the lower end of the two Fab arms close to the hinge region, centered on the sides that face each other. In the IgG2-B isoform, two of the four disulfide bonds in the hinge region are replaced by alternative linkages that bridge the hinge region to the CH1 and CL domains (Figure 1). Therefore, our results suggest that the two Fab arms in the IgG2-B isoform were pulled together by the nonclassical disulfide bonds to form a close contact (Figure 8). As a consequence, the IgG2-B isoform had a global structure more compact than that of the IgG2-A1 isoform. The change in global packing affects the size of the molecule, which is easily detected by many biophysical and chromatographic techniques. A more dramatic change was observed in the light chain (Figure 4), suggesting that the light chain may play a stronger role in the interaction of the two Fab domains. The folding of each individual domain, on the other hand, remains unaffected by the alterative disulfide linkages, as suggested by the very similar protection factor profiles between the IgG2-A1 and IgG2-B isoforms in other regions (Figure 3). Interestingly, while the heavy-chain residues immediately before the hinge disulfide are more protected in the IgG2-B form, the light-chain residues at the proximity of the C-terminal disulfide are not affected.

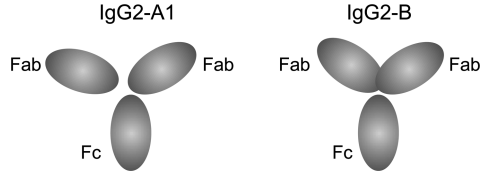

Figure 8. Cartoon models of human IgG2 disulfide isoforms A1 and B suggested from the differential protection factors between the isoforms.

The increased solvent protection in IgG2-B seen in this study agrees with earlier observations about its more compact and less flexible nature. Local structural information obtained in this study also provides a more detailed understanding on the interaction that brings the two Fab arms "together", and not "down", as suggested earlier for the IgG2-B isoform.[3] More specifically, the HDX analysis performed here identified a set of discrete regions in the IgG2-B isoform to form intramolecular contacts between two Fab arms, which may not be present in the IgG2-A isoforms. This additional intramolecular interaction may contribute to the additional thermal transition of IgG2-B at a high temperature of approximately 85 °C, as observed by differential scanning calorimetry.[3] This transition suggests the presence of an additional unfolding event in IgG2-B compared to IgG2-A in a structural domain unique to the IgG2-B isoform. We speculate that the additional transition involves the two Fab arms brought into contact with each other by the nonclassical disulfide bonds described in this paper.

In a previous study, Dillon et al. reported that different disulfide isoforms of an IgG2 antibody can covert from one to another under reduction−oxidation conditions created by addition of a cysteine/cystine mixture at neutral pH. In the presence of 1 M guanidine, the IgG2 disulfide isoforms converted to IgG2-A1, while in the absence of guanidine, they converted to IgG2-B.[3] This observation indicates that the nonclassical interchain disulfide bonds in the IgG2-B isoform are energetically more stable under native conditions, probably because of the presence of the Fab−Fab interactions near the hinge region as identified by HDX analysis. Under a mild denaturing and redox condition, the chaotropic reagent may disrupt the Fab−Fab interaction and drive the conversion to the IgG2-A1 isoform when the interchain disulfide bonds are transiently reduced in the IgG2-B isoform. It is worth noting that under nonredox condition where the disulfide bonds remain intact, the presence of guanidine seemed not to be able to disrupt intramolecular Fab−Fab interactions as suggested by the similar solvent protection difference between IgG2-A and IgG2-B isoforms with or without 2 M guanidine (Figure 4). This is probably due to the restriction of the interchain disulfide bonds that brought two Fab arms into the proximity of each other.

In the same study,[3] Dillon et al. also demonstrated that the IgG2-B isoform lowers antigen binding potency relative to that of the IgG2-A1 isoform. Our HDX data provided some rationale for their observation from the structural perspective. The lower antigen binding affinity of the IgG2-B isoform was likely attributed to restricted orientations of the two Fab arms relative to each other, resulting from the alternative disulfide bond linkages from the hinge region to the Fab arms as well as the Fab−Fab interactions. Loss of the Fab domain flexibility in IgG2-B could limit its ability to access epitopes and form multivalent interactions (avidity) with the targeted cell-surface receptors, leading to reduced antigen binding potency in the cell-based assay. As for the Fc effector functions, the HDX comparison of two isoforms did not suggest any conformational difference in the Fc domain; we therefore did not expect that the two disulfide isoforms would have a significant difference in the Fc effector functions, including both FcγR and complement factor C1q binding, which was supported in a Cys-Ser mutation study.[9]

Similar to the IgG2-B isoform, IgG2-A2 was observed by its distinct retention time from the classical IgG2-A1 in both reversed-phase and cation exchange chromatography.[2,3] However, disulfide mapping of the IgG2-A2 isoform suggested it has very similar disulfide bond linkages, including the interchain ones as IgG2-A1. Therefore, the leading cause for the different chromatographic behaviors of IgG2-A2 is not clear.[7] Our HDX data showed that IgG2-A2 had less solvent protection in some Fab regions close to the hinge than the IgG2-A1 isoform did (Figure 7 bottom), suggesting that the A2 isoform had more flexible Fab domains. The less protected Fab domains of the A2 isoform may provide extra hydrophobic interactions and charge−charge interactions with RP-HPLC and CEX columns, respectively, providing an explanation for the observation that A2 elutes later than other isoforms in both assays.[2,3] Furthermore, the lower level of solvent protection in the IgG2-A2 Fab domains may also explain the lower thermal stability and higher aggregation propensity of IgG2-A2 compared to the other disulfide isoforms (manuscript in preparation). The order of compactness from IgG2-B to IgG2-A1 to IgG2-A2 discovered in this study is also consistent with

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the conclusion derived from the charge state distribution during LC−MS analysis of these isoforms.[3] It is interesting to note, however, that although the A2 isoform has more flexible Fab domains, its disulfide bonds near the hinge region are more resistant to reduction, leading to a hypothesis that the upper hinge of A2 behaves like the interior of a globular protein.[7]

■ ASSOCIATED CONTENT

Supporting Information

Sequence coverage map of IgG2#1, reversed-phase chromatograms of the purified isoforms from IgG2#1 demonstrating the purity of the isoforms, plots showing 20 sets of possible protection factors for residues in IgG2#1 isoform A1, average protection factor plots of IgG2#1 comparing A1 and B isoforms under different H/D exchange conditions, and average protection factor plots of IgG#2 comparing A1, A2, and B isoforms. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

HDX, hydrogen/deuterium exchange; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

■ REFERENCES

(1) Milstein, C., and Frangione, B. (1971) Disulphide bridges of the heavy chain of human immunoglobulin G2. *Biochem. J. 121*, 217−225.

(2) Wypych, J., Li, M., Guo, A., Zhang, Z., Martinez, T., Allen, M. J., Fodor, S., Kelner, D. N., Flynn, G. C., Liu, Y. D., Bondarenko, P. V., Ricci, M. S., Dillon, T., and Balland, A. (2008) Human IgG2 antibodies display disulfide-mediated structural isoforms. *J. Biol. Chem. 283*, 16194−16205.

(3) Dillon, T. M., Ricci, M. S., Vezina, C., Flynn, G. C., Liu, Y. D., Rehder, D. S., Plant, M., Henkle, B., Li, Y., Deechongkit, S., Varnum, B., Wypych, J., Balland, A., and Bondarenko, P. V. (2008) Structural and functional characterization of disulfide isoforms of the human IgG2 subclass. *J. Biol. Chem. 283*, 16206−16215.

(4) Allen, M. J., Guo, A., Martinez, T., Han, M., Flynn, G. C., Wypych, J., Liu, Y. D., Shen, W. D., Dillon, T. M., Vezina, C., and Balland, A. (2009) Interchain disulfide bonding in human IgG2 antibodies probed by site-directed mutagenesis. *Biochemistry 48*, 3755−3766.

(5) Zhang, B., Harder, A. G., Connelly, H. M., Maheu, L. L., and Cockrill, S. L. (2010) Determination of Fab−hinge disulfide connectivity in structural isoforms of a recombinant human immunoglobulin G2 antibody. *Anal. Chem. 82*, 1090−1099.

(6) Liu, Y. D., Chen, X., Zhang-van Enk, J., Plant, M., Dillon, T. M., and Flynn, G. C. (2008) Human IgG2 antibody disulfide rearrangement in vivo. *J. Biol. Chem. 283*, 29266−29272.

(7) Liu, Y. D., Chou, R. Y. T., Dillon, T. M., Poppe, L., Spahr, C., Shi, S. D., and Flynn, G. C. (2014) Protected hinge in the immunoglobulin G2-A2 disulfide isoform. *Protein Sci. 23*, 1753−1764.

(8) Wang, X., Kumar, S., and Singh, S. K. (2011) Disulfide scrambling in IgG2 monoclonal antibodies: Insights from molecular dynamics simulations. *Pharm. Res. 28*, 3128−3144.

(9) Lightle, S., Aykent, S., Lacher, N., Mitaksov, V., Wells, K., Zobel, J., and Oliphant, T. (2010) Mutations within a human IgG2 antibody form distinct and homogeneous disulfide isomers but do not affect Fc gamma receptor or C1q binding. *Protein Sci. 19*, 753−762.

(10) Bagal, D., Valliere-Douglass, J. F., Balland, A., and Schnier, P. D. (2010) Resolving disulfide structural isoforms of IgG2 monoclonal antibodies by ion mobility mass spectrometry. *Anal. Chem. 82*, 6751−6755.

(11) Zhang, Z., and Smith, D. L. (1993) Determination of amide hydrogen exchange by mass spectrometry: A new tool for protein structure elucidation. *Protein Sci. 2*, 522−531.

(12) Wales, T. E., and Engen, J. R. (2006) Hydrogen exchange mass spectrometry for the analysis of protein dynamics. *Mass Spectrom. Rev. 25*, 158−170.

(13) Zhang, Z., Zhang, A., and Xiao, G. (2012) Improved protein hydrogen/deuterium exchange mass spectrometry platform with fully automated data processing. *Anal. Chem. 84*, 4942−4949.

(14) Dillon, T. M., Chou, R. Y.-T., Chen, Y., Bondarenko, P. V., Wiltzius, J. J., and Woehle, D. (2013) IgG2 disulfide isoform separation. PCT Int. Appl. 2012:WO2013086448A2.

(15) Zhang, Z. (2009) Large-scale identification and quantification of covalent modifications in therapeutic proteins. *Anal. Chem. 81*, 8354−8364.

(16) Saphire, E. O., Parren, P. W., Pantophlet, R., Zwick, M. B., Morris, G. M., Rudd, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R., and Wilson, I. A. (2001) Crystal structure of a neutralizing human IGG against HIV-1: A template for vaccine design. *Science 293*, 1155−1159.

(17) Liu, Y. D., Wang, T., Chou, R., Chen, L., Kannan, G., Stevenson, R., Goetze, A. M., Jiang, X. G., Huang, G., Dillon, T. M., and Flynn, G. C. (2013) IgG2 disulfide isoform conversion kinetics. *Mol. Immunol. 54*, 217−226.

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