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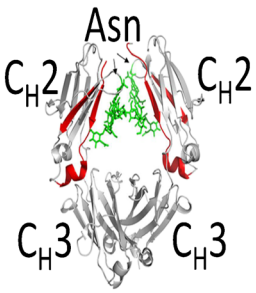
Characterization of Aggregation Propensity of a Human Fc-Fusion Protein Therapeutic by Hydrogen/Deuterium Exchange Mass Spectrometry

Richard Y.-C. Huang,[1] Roxana E. Iacob,[2] Stanley R. Krystek,[3] Mi Jin,[4] Hui Wei,[5] Li Tao,[5] Tapan K. Das,[5] Adrienne A. Tymiak,[1] John R. Engen,[2] Guodong Chen[1]

[1]Bioanalytical and Discovery Analytical Sciences, Research and Development, Bristol-Myers Squibb Company, Route 206 & Province Line Road, Princeton, NJ 08543, USA
[2]Department of Chemistry and Chemical Biology, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA
[3]Molecular Structure and Design, Research and Development, Bristol-Myers Squibb Company, Route 206 & Province Line Road, Princeton, NJ 08543, USA
[4]Biologics Development and Operations, Global Manufacturing & Supply, Bristol-Myers Squibb Company, 6000 Thompson Road, Syracuse, NY 13057, USA
[5]Biologics Development and Operations, Global Manufacturing & Supply, Bristol-Myers Squibb Company, 311 Pennington-Rocky Hill Road, Hopewell, NJ 08534, USA



Abstract. Aggregation of protein therapeutics has long been a concern across different stages of manufacturing processes in the biopharmaceutical industry. It is often indicative of aberrant protein therapeutic higher-order structure. In this study, the aggregation propensity of a human Fc-fusion protein therapeutic was characterized. Hydrogen/deuterium exchange mass spectrometry (HDX-MS) was applied to examine the conformational dynamics of dimers collected from a bioreactor. HDX-MS data combined with spatial aggregation propensity calculations revealed a potential aggregation interface in the Fc domain. This study provides a general strategy for the characterization of the aggregation propensity of Fc-fusion proteins at the molecular level.

Keywords: Aggregation propensity, Hydrogen/Deuterium exchange mass spectrometry, Fc fusion protein

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Introduction

Protein aggregation is a critical issue for the development and manufacturing of protein therapeutics in the biopharmaceutical industry [1]. For example, antibody aggregates could possess biological activity different from that of monomers because of aberrant higher-order structure, resulting in altered therapeutic efficacy and unwanted immune response [2–5]. Protein aggregates can occur through various mechanisms in self-association that are initiated by a number of stress factors, including changes in pH, temperature, and agitation during cell culture expression or downstream purification steps, leading to the formation of soluble/insoluble, covalent/noncovalent, and reversible/irreversible complexes [6, 7]. Among the different types of protein self-association, hydrophobic interactions have been shown to be the predominant interactions in protein aggregation formation, although charge interactions could also play a significant role [7]. Protein–protein interaction surfaces most often are composed of hydrophobic surface residues. Although hydrophobic residues are usually buried within the protein core, when they are exposed on protein surfaces there is often a functional consequence. For example, the changes in protein conformation in solution due to different stress factors could result in the exposure of hydrophobic residues of proteins and alter the charge–charge repulsions between proteins, eventually leading to aggregation [7]. Therefore, there is a need to understand the molecular

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Correspondence to: Guodong Chen; *e-mail:* guodong.chen@bms.com

mechanism of a given protein therapeutic aggregation event that could help design a better therapeutic and mitigate the aberrant therapeutic effects.

One of the most widely used biophysical tools for the characterization of protein aggregation is size-exclusion chromatography (SEC) as it allows the separation of aggregates from monomers for further characterization. Protein aggregates can be quantified by SEC with UV detection, and the mass/identity of aggregates can be characterized by SEC followed by multiangle light scattering and SEC coupled with mass spectrometry (MS) [8]. The SEC method can be validated by analytical ultracentrifugation to determine even very weak association constants and reveal the size distributions of solutions containing multiple species. Higher-order structure characterization of protein aggregates provides detailed information regarding the molecular mechanism of aggregation. However, the large size of protein aggregates, monoclonal antibody (mAb) aggregates in particular, poses a significant challenge for typical high-resolution protein structural characterization tools (i.e., X-ray crystallography and nuclear magnetic resonance). On the other hand, methods such as small-angle X-ray scattering have less restriction on protein size, but poor spatial resolution can lead to a loss of molecular-level information in small-angle X-ray scattering analysis as compared with X-ray crystallography. Recently, protein higher-order structure analysis by MS (i.e., MS-based protein footprinting techniques) has gained increased momentum in the characterization of protein aggregates as it can provide information on protein dynamics and protein–protein interaction interfaces in solution without the restriction of molecular size [9].

Hydrogen/deuterium exchange (HDX) MS has been widely used in the biopharmaceutical industry for higher-order structure characterization of protein therapeutics because of a well-developed automatic platform and the commercial availability of data analysis software [10–17]. By monitoring protein amide backbone HDX, which depends on the protein local solvent accessibility and hydrogen bonds, one can characterize the solution conformation and dynamics of proteins, including changes due to the external environment [18] (e.g., chemical modifications [19–22], buffer components [23, 24], and the presence of a binding protein/ligand [25, 26]). Regarding the characterization of protein aggregation, HDX-MS has shown its potential for our understanding of protein self-association interfaces and aggregation-induced protein conformational changes at the molecular level in either the lyophilized state [27] or the solution state [28–30].

In this study we used both HDX-MS and computational analysis, spatial aggregation propensity (SAP) calculations, to characterize the aggregation propensity of a human Fc-fusion protein therapeutic, FcP1.

Fc-fusion proteins are composed of an immunoglobulin (Ig) Fc domain that is directly linked to a proteinaceous molecule. The proteinaceous molecules can be peptides or proteins with significant therapeutic potential resulting from their binding to certain receptors or pathogens. The addition of the Fc domain provides additional beneficial biological and pharmacological properties. For example, the interaction of the Fc domain with neonatal Fc receptor increases the plasma half-life of Fc-fusion proteins, which prolongs the therapeutic activity. Since the first approval of an Fc-fusion protein, Enbrel®, by the US Food and Drug Administration in 1998, Fc-fusion proteins have been intensely investigated for their clinical effectiveness [31, 32]. In the developmental process for FcP1, although a drug with extremely high purity can be obtained, multiple aggregate species were observed for the crude substance from the bioreactor. Understanding the biophysical properties of the aggregate species of FcP1 could help optimize the manufacturing process. FcP1 dimers were purified from the bioreactor, and their conformational dynamics were monitored by HDX-MS. The hydrophobicity of FcP1 was further analyzed by SAP calculations. SAP analysis uses information on both the solvent-accessible area and the hydrophobicity of protein amino acid residues via molecular simulations to identify protein aggregation hot spots [33, 34]. HDX and SAP analyses both pinpointed a potential aggregation interface in the C_H2 region of Fc, which further reveals the importance of the C_H2 region in the aggregation of IgG-type molecules as observed previously [28–30]. To the best of our knowledge, this is the first study that provides molecular-level aggregation information for a human Fc-fusion protein. It also demonstrates the practical utility of the combined approach of HDX-MS and SAP analysis for the investigation of the aggregation propensity of protein therapeutics.

Experimental

Material

The monomers and dimers of FcP1 were obtained from Bristol-Myers Squibb. They were purified from the bioreactor by SEC (Fig. S1) and their concentrations were determined by UV absorbance at 280 nm. Biophysical methods including hydrophobic interaction chromatography and dynamic light scattering were used for the characterization of oligomeric species.

Hydrogen/Deuterium Exchange

For hydrogen exchange labeling, 50 pmol of FcP1 was incubated with D_2O as follows: Labeling was initiated by dilution of each protein (monomer and dimer) with 15-fold 10 mM sodium phosphate buffer made with 99% D_2O (pD 7.0) at room temperature. At each deuterium exchange time point (from 10 s to 4 h) an aliquot from the exchange reaction was removed and labeling was quenched by adjustment of the pH to 2.5 with an equal volume of quench buffer [4 M guanidine hydrochloride, 0.5 M tris(2-carboxyethyl)phosphine hydrochloride, 200 mM sodium phosphate, H_2O]. Quenched samples were immediately frozen on dry ice and stored at -80 °C until analysis.

Chromatography and MS

Each frozen sample was thawed rapidly and injected into a custom Waters nanoACQUITY UPLC HDX Manager™ [35]

and analyzed with a Xevo G2 mass spectrometer (Waters, Milford, MA, USA) as previously described [29]. Protein samples were digested online with use of a Poroszyme immobilized pepsin cartridge (2.1 mm × 30 mm, Applied Biosystems). The digestion temperature was set to 15 °C and the digestion was performed for 30 s. The cooling chamber of the UPLC system, which housed all the chromatographic elements, was 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 6 min by a 5–40% acetonitrile–water gradient at 40 µL/min. The separation column was a 1.0 mm × 100.0 mm ACQUITY UPLC BEH C_{18} column (Waters) containing 1.7-µm particles and the back pressure averaged 8800 psi at 0.1 °C. The average amount of back-exchange with this experimental setup was 18–25%, based on analysis of highly deuterated peptide standards. Mass spectra were obtained with a Waters Xevo G2 time-of-flight mass spectrometer equipped with a standard electrospray ionization source (Waters). The instrument configuration was as follows: capillary voltage 3.2 kV, trap collision energy 6 V, sampling cone voltage 35 V, source temperature 80 °C and desolvation temperature 175 °C. Mass spectra were acquired over an *m*/*z* range of 100 to 1900. Mass accuracy was ensured by calibration with 500 nM [Glu^1]-fibrinopeptide B, and was less than 10 ppm throughout all experiments. All comparison experiments were performed under identical experimental conditions such that deuterium levels were not corrected for back-exchange and are therefore reported as relative [36]. All experiments were performed in duplicate. The error of measuring the mass of each peptide was ± 0.20 Da in this experimental setup, consistent with previously obtained values [37–39]. Deuterium uptake was calculated by subtraction of the centroid of the isotopic distribution for peptide ions from undeuterated protein from the centroid of the isotopic distribution for peptide ions from the deuterium-labeled sample. The resulting relative deuterium levels were plotted versus the exchange time with use of the software program DynamX 3.0™ (Waters). Identification of the peptic peptides was accomplished through a combination of exact mass analysis and MS^E [40] with use of ProteinLynx Global SERVER 2.5 (Waters), as previously described [41, 42]. All assignments, deuterated spectra, and data processing were manually checked and verified. No bimodal distribution was observed in the acquired mass spectra.

SAP Calculations

SAP calculations were performed on the FcP1 homology model with the BIOVIA Discovery Studio modeling environment, version 4.1 (Dassault Systèmes BIOVIA, San Diego, CA, USA). The homology model of FcP1 was generated with use of the Fc and hinge regions of human IgG1 template (Protein Data Bank code 1HZH) combined with the crystal structure of antigen-binding protein (ABP). Following sequence alignment of FcP1 with the IgG1 template, the conserved framework backbone and side chain atoms were used along with the ABP coordinates to generate an initial model followed by side

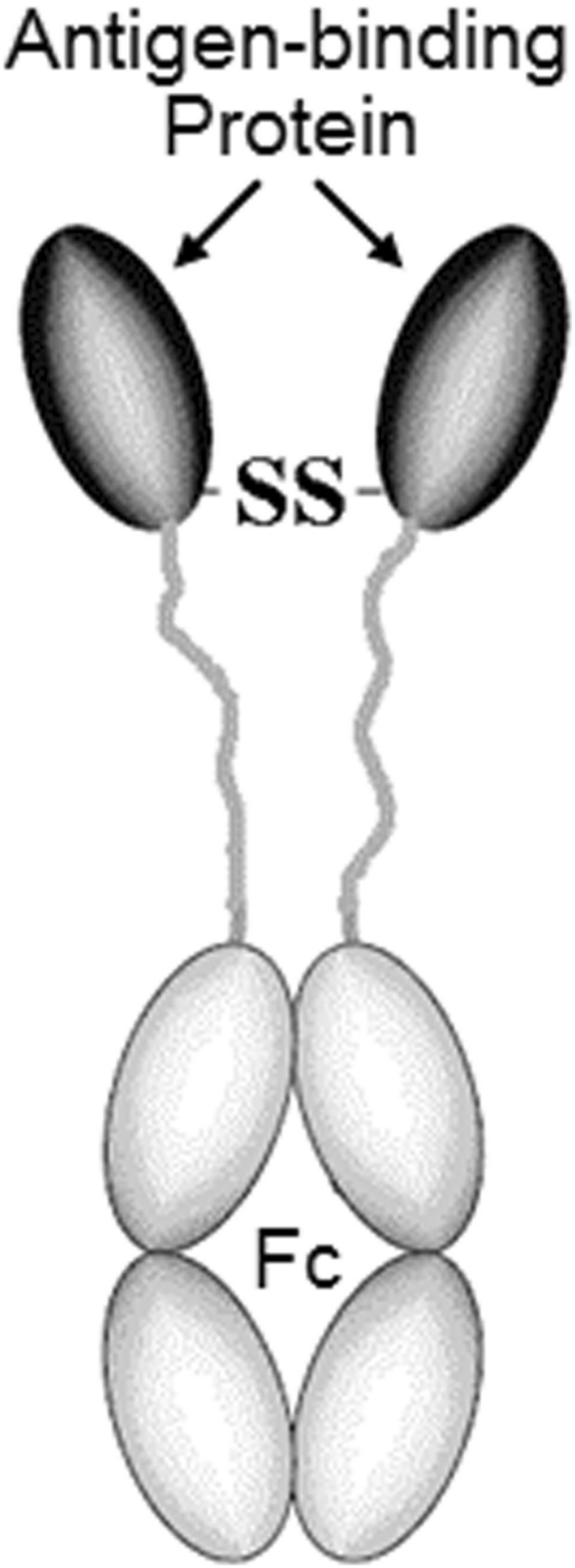

Fig. 1. FcP1 structure. FcP1 is composed of antigen-binding protein, a linker region, and the human immunoglobulin G (IgG) Fc domain

chain generation and optimization for any missing atoms by standard methods within Schrödinger software (Protein Preparation Wizard and Prime) [43, 44]. With use of Prime loop modeling protocols [43, 44], the modified hinge region was built connecting the Fc region to the ABP molecule. The overall model was then optimized for hydrogen bonding and protonation state, followed by restrained minimization (Schrödinger release 2015-2: Maestro, version 10.2, Biologics Suite 2015-2: BioLuminate, version 1.9 (Schrödinger, New York, NY, USA).

Results and Discussion

Structural Dynamics of FcP1

FcP1 is a human Fc-fusion protein with ABP linked to the modified, glycosylated, Fc domain of human IgG1. As shown in Fig. 1, the double chains of ABP–Fc are formed through the bridging of a single chain by an interchain disulfide bond located at the C-terminal region in ABP. ABP forms a rigid structure that is composed of an eight β-sheet–loops bundle. ABP is linked to the human IgG1 Fc domain through a flexible, unstructured linker region. Although there is currently no crystal or nuclear magnetic resonance structure available for the full-length FcP1, one can extract some structural information from HDX kinetics by monitoring the protein dynamics in solution. For example, Fig. 2 shows the mapping of HDX data onto the available crystal structures of ABP and the human IgG1 Fc domain (Protein Data Bank code 3AVE). In ABP, the outer loop regions and C-terminal unstructured region, because of greater solvent exposure and faster conformational dynamics, showed faster HDX kinetics than β-sheet–loop–β-sheet structural regions. The inner β-sheet regions showed low and steady deuterium uptake, demonstrating their relatively low solvent accessibility and/or hydrogen bond formation. The linker region is predicted to be unstructured, and as expected from HDX kinetics (Fig. S2), region 113–144 of the linker region had a significant amount of deuterium uptake at 10 s and the level of deuterium uptake stayed steady for later exchange time points, suggesting its unstructured nature. The IgG1 Fc domain, which is composed of multiple β-sheet–loop structures, showed relatively slower HDX as compared with ABP. The overall HDX of the C_H2 domain in Fc was faster than that of the C_H3 domain, indicating faster dynamics for the C_H2 domain in solution, which is in accordance with the observation that C_H2 has less conformational stability and unfolds faster than other domains in IgG under stress conditions [45]. In general, the HDX data agree well with the known structural information on ABP and IgG Fc.

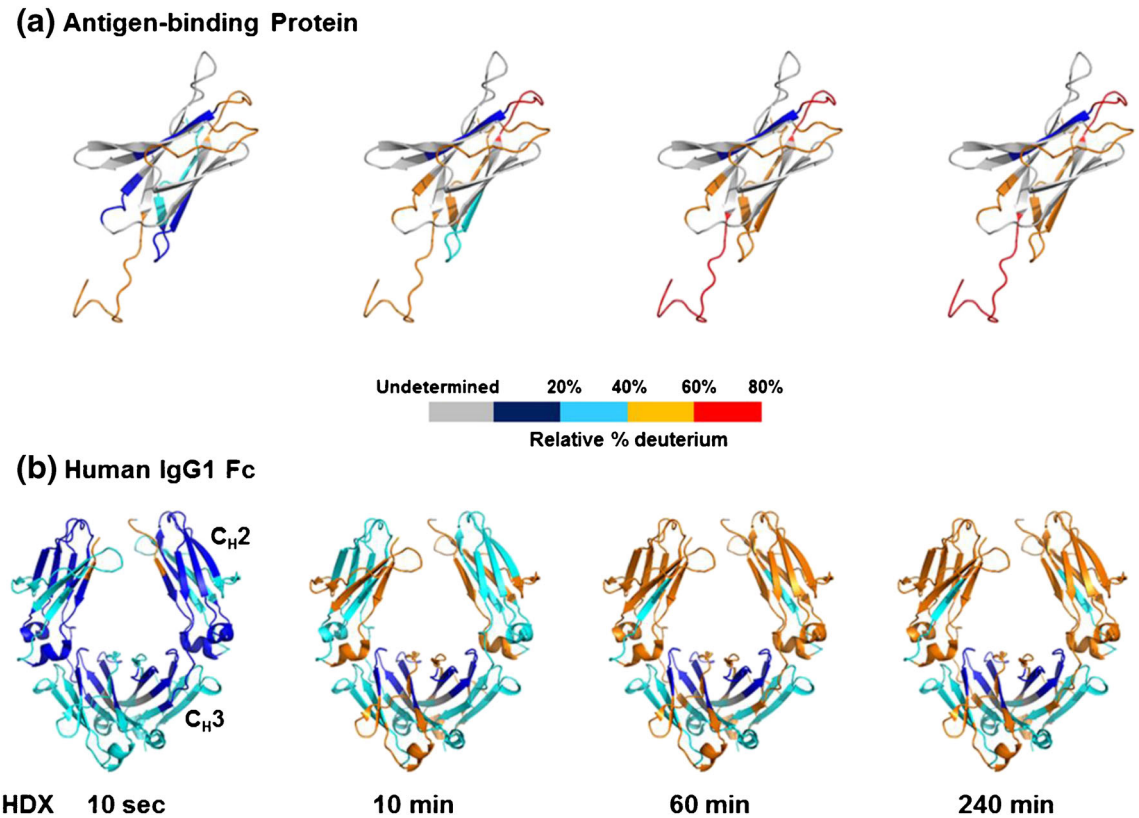

Fig. 2. Hydrogen/deuterium exchange (*HDX*) kinetics mapped onto **a** antigen-binding protein and **b** the human IgG Fc domain (Protein Data Bank code 3AVE). Regions were colored from *blue* to *red* on the basis of the extent of deuterium uptake (%)

HDX-MS Revealed Potential Aggregation Interfaces in the Fc Domain

In the discovery and developmental process for protein therapeutics, in-depth understanding of the protein aggregation propensity and the protein aggregation mechanism is important to mitigate the extent of aggregation by redesigning the molecule or optimizing formulation conditions. However, the size of protein aggregates often poses significant challenges to the analytical strategies that aim to obtain molecular-level data on protein aggregation. Recent advances in HDX-MS have shown its potential in the characterization of protein aggregates. By monitoring the HDX profiles of purified monomers versus oligomers, one could pinpoint the potential aggregation interfaces with a spatial resolution of 10–20 amino acids on proteolytic digestion. For example, the reduction of HDX in a given peptide region of an oligomer as compared with its monomer can indicate regional solvent protection, which could be due to protein self-association in that region. On the other hand, a reversed trend of HDX data in oligomers would suggest aggregation-induced protein conformational changes.

In this study, HDX-MS was applied to interrogate the FcP1 monomer and FcP1 dimer. HDX-MS coupled with proteolytic digestion provided approximately 82% sequence coverage of full-length FcP1. Some regions of the protein could not be identified because of the presence of complex glycans [46]. Pairwise comparison of the HDX profiles of 62 peptic peptides showed comparable deuterium uptake for most of the peptides (Fig. S2). However, six peptides in the region covering ^{145}LGGSSVFLFPPKPKDTLM^{162} together with region ^{211}RVVSVL^{216} in the Fc C_H2 domain showed HDX reduction in the dimer as compared with the monomer, suggesting some amide hydrogens in these areas contribute to aggregation interface for FcP1 (Fig. 3). The reduced deuteration in region 145–162 mostly happened at the earlier exchange time points, within 10 min, suggesting that the self-association in this

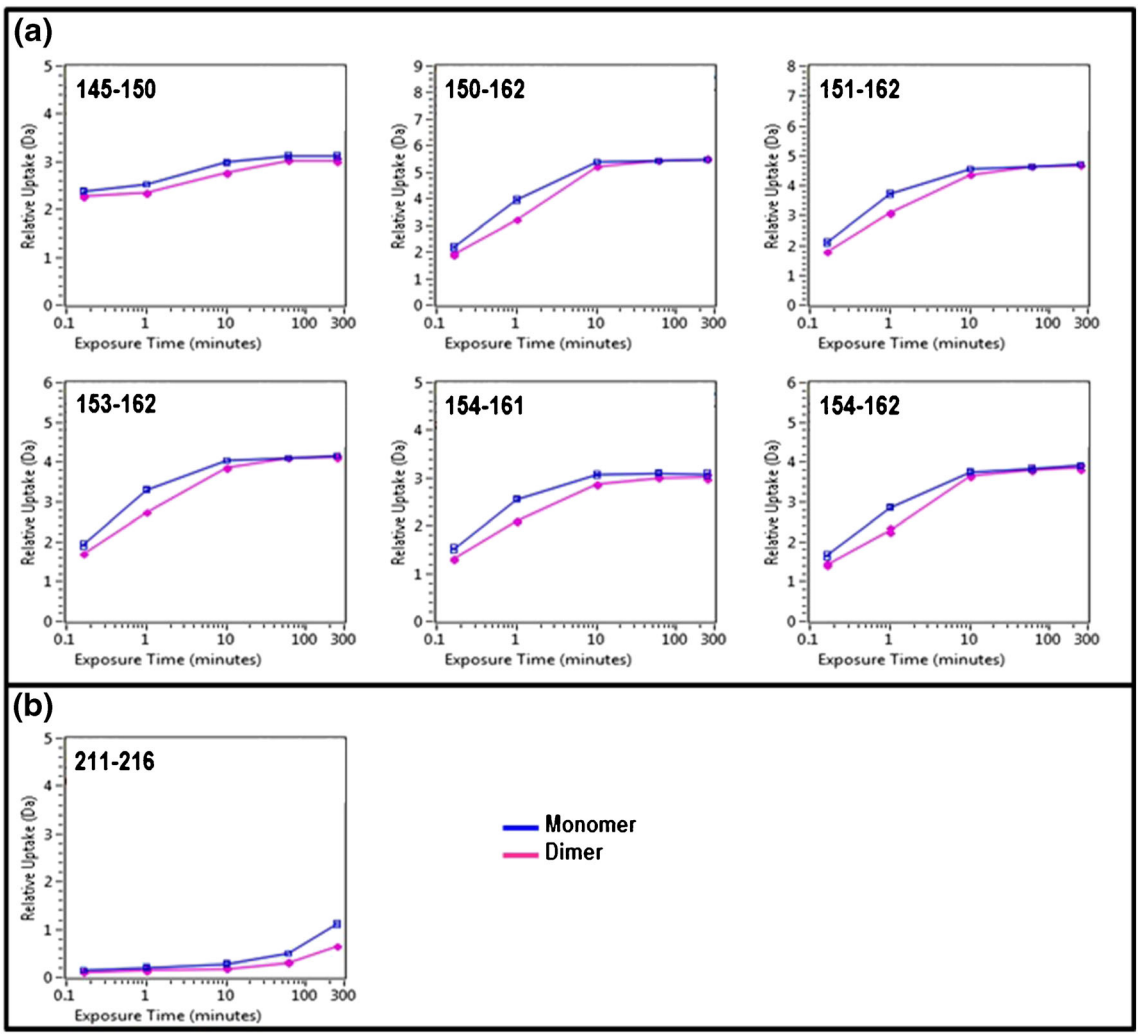

Fig. 3. HDX kinetics (measurements in duplicate) indicates six peptide regions covering region ^{145}LGGSSVFLFPPKPKDTLM^{162} and region ^{211}RVVSVL^{216} in C_H2 of FcP1 as aggregation hot spots. The FcP1 dimer (*pink*) compared with the monomer (*blue*) showed HDX reduction at early exchange time points in region 145–162, suggesting the protein self-association in this region was dynamic

region was dynamic. On the other hand, the HDX reduction in region 211–216 happened at longer exchange times (more than 10 min), which is indicative of slower interaction dynamics. On the basis of the crystal structure of the human IgG Fc domain (Protein Data Bank code 3AVE), both regions are located in the C_H2 domain and are at the same interface (Fig. 4). Region 145–162 is composed of β-sheet–loop–α-helix and is directly linked to the flexible linker/hinge region. The flexible structural nature of this region could potentially lead to fast association dynamics as observed by HDX. Region 211–216 is composed of β-sheet and is partially covered by the glycan, which led to slow HDX kinetics (Fig. 3). The reduced deuteration in peptide 211–216 compared with region 145–162 may explain its slower association dynamics.

Correlation Between HDX-MS and SAP Results

The utility of complementary approaches in protein higher-order structure analysis is important to verify observations and rationalize differences in experimental measurements. For the characterization of protein aggregation propensity, SAP analysis can provide information at the molecular level complementary to the results obtained from HDX-MS. SAP is an algorithm that computationally estimates the effective exposed hydrophobicity for specific patches of atoms on a protein surface. SAP identifies the location and size of these aggregation-prone regions. Chennamsetty et al. [33, 34, 47] demonstrated that SAP calculations can be used to determine critical regions of aggregation in therapeutic antibodies. The aggregation-prone motifs for the Fc region of antibodies have been identified with use of SAP and other methods [47]. In the FcP1 aggregation study, although no structure is currently available for the full-length protein, the crystal structures of ABP and the human IgG1 Fc domain are available. We generated the homology model of FcP1 using the Fc and hinge regions of human IgG1 template combined with the ABP structure and performed SAP analysis as described in "Experimental". Figure 5 shows the FcP1 model with SAP mapping. Some residues in the N-terminal region of ABP and the Fc domain showed high SAP values, indicating their higher tendency to self-associate. The most significant site of potential aggregation was identified for ten residues in region 138–239 with SAP values greater than 0.4 (Fig. 5b, Table S1). Not surprisingly, Pro142, Glu143, Leu144, Leu145, Ser149, Phe151, and Phe153 with SAP values greater than 0.4 are nearby or within region 145–162, which showed HDX reduction on FcP1 dimerization. No residues with high SAP values were observed in region 211–216. Combined HDX and SAP results suggest that region 145–162 could be the key aggregation interface in FcP1 considering its hydrophobicity and conformational dynamics. Given that region 145–162 is located in the dynamic C_H2 domain and is linked to the unstructured linker region, the stress-induced conformational changes in the C_H2 domain could potentially expose the hydrophobic residues in region 145–162 and initiate the aggregation. The FcP1 self-association in region 145–162 could further reduce the local solvent accessibility or alter the hydrogen bond network in region 211–216, resulting in the observed HDX reduc-

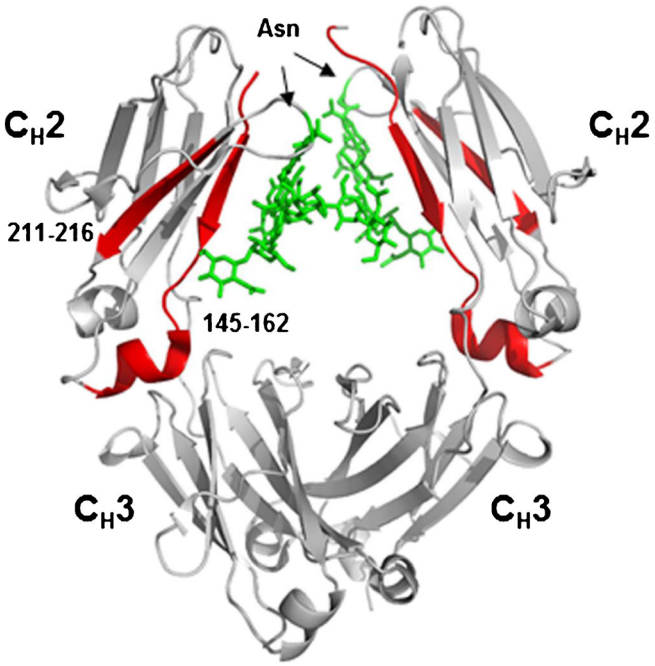

Fig. 4. Structural location of C_H2 regions ^{145}LGGSSVFLFPPKPKDTLM^{162} and ^{211}RVVSVL^{216} (*red*) in a human IgG Fc domain crystal structure (Protein Data Bank code 3AVE). The N-linked glycans are in *green*

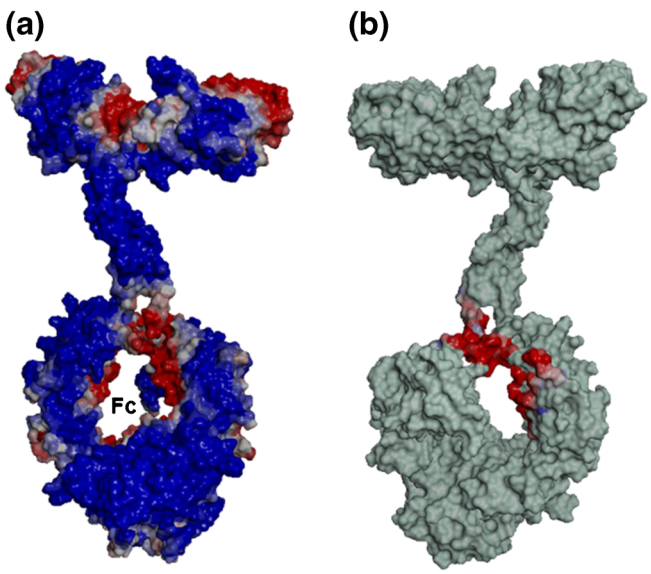

Fig. 5. Spatial aggregation propensity (SAP) for the FcP1 homology model. **a** The SAP values at $R = 10$ Å are mapped onto the molecular surface of the FcP1 homology model, where *red regions* represent hydrophobic, aggregation-prone regions and *blue regions* are hydrophilic exposed regions predicted to be less likely sites for aggregation. **b** The most significant site of potential aggregation was identified by SAP calculations and is shown in *red*. The site consists of residues (highest SAP score to lowest) Glu143, Leu144, Leu145, Phe151, Ser139, Ser149, Pro239, Pro142, Pro138, and Phe153

tion. Overall, good correlations were obtained between HDX data and SAP results, indicating region 145–162 as a potential aggregation interface in FcP1. In addition, the combined results from HDX and SAP analysis provide useful insights into the potential aggregation mechanism of FcP1.

Involvement of the C_H2 Region in the Aggregation of IgG-Type Molecules

The sequence and structure of the C_H2 region in the Fc domain are highly conserved in IgG molecules and result in some common features observed for IgG aggregation in the literature. For example, it has been proposed that the C_H2 domain can trigger IgG aggregation at low pH [48], which is likely caused by reduced conformational stability of the Fc C_H2 region as compared with other domains in IgG1 molecules under stress conditions [45]. Recently, Arora et al. [28] used HDX-MS to characterize the protein interfaces involved in the reversible self-association of an IgG1 mAb. In their studies, HDX was applied to mAb in different concentrations prepared from lyophilized material. Regions in complementarity-determining regions were observed with HDX reduction at high concentration, suggesting Fab–Fab self-interaction. Regions in the C_H1/C_H2 domain exhibited more deuterium uptake under aggregation conditions, indicating aggregation-induced conformational changes in C_H1/C_H2 domains. Conformation changes were also observed in an HDX-MS study by Iacob et al. [29] on the conformational comparison between a mAb monomer and dimer, where the Fc C_H2 domain was more rapidly deuterated in the dimer, suggesting an increase in structural flexibility in this region on dimer formation. In addition to the aggregation study, it has been shown that the conformation of the Fc C_H2 region is also sensitive to the removal of glycan in the Fc domain [21, 49]. These data point to the overall sensitivity of the Fc C_H2 region to its environment and storage conditions. In the present work, we observed that regions in the C_H2 domain are relatively more dynamic than regions in the C_H3 domain, confirming that the C_H2 domain has less conformational stability than other domains in IgG molecules. The same Fc C_H2 region reported previously (i.e., region 145–162), instead of becoming more solvent exposed on FcP1 aggregation, was deuterated less on dimer formation, which is indicative of the potential aggregation interface of FcP1. The HDX reduction occurred mostly at the early HDX time points, suggesting the dynamic nature of this region. This is in agreement with the common knowledge that the Fc C_H2 domain unfolds faster under stress conditions and therefore it is less stable than other domains. The different behavior of this region in the aggregation of FcP1 versus mAbs is probably because FcP1 lacks the Fab domain, which is composed of highly reactive complementarity-determining regions. Although FcP1 has a different structure and different biophysical properties for the N-terminal domain, the biophysical properties of the common Fc C_H2 domain are still sensitive to the changes of the external environment. The conformational changes in the C_H2 domain of FcP1 could be induced under stress conditions, resulting in the exposure of hydrophobic residues and initiation of the aggregation.

Conclusions

In-depth understanding of aggregation propensity of protein therapeutics is an important task for optimization of protein therapeutics in the biopharmaceutical industry to prevent aberrant therapeutic effects. As presented in this study, we have demonstrated, for the first time, the utility of both HDX-MS and SAP analysis in the molecular-level characterization of the aggregation propensity of a human Fc-fusion protein therapeutic. Unlike mAbs, which form aggregation mostly through Fab–Fab interactions, HDX-MS data indicated amide hydrogens in regions ^{145}LGGSSVFLFPPKPKDTLM^{162} and ^{211}RVVSVL^{216} in C_H2 of FcP1 as aggregation hot spots. SAP analysis of FcP1 based on the model structure composed of ABP and the human IgG Fc domain indicated that several residues near or within C_H2 region 145–162 are solvent exposed and hydrophobic, resulting in high aggregation propensity in this region. The aggregation of FcP1 in C_H2 region 145–162 was likely caused by its neighboring unstructured linker region, which facilitated the exposure of the hydrophobic residues in region 145–162 under stress conditions and initiated the self-association. In this study, HDX-MS and SAP analysis not only provided complementary information with good correlation, but also shed light on the potential mechanism of FcP1 aggregation. This strategy combining both HDX-MS and SAP analysis could be applied to the analysis of aggregates for protein therapeutics.

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