Elucidation of acid-induced unfolding and aggregation of human immunoglobulin IgG1 and IgG2 Fc

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*Running title: Fc aggregation in acidic conditions is determined by Cᵢᴴ₂ stability

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Keywords: Fc denaturation; Fc aggregation; Fc glycosylation; Quality by Design; NMR; DSC; cation-exchange chromatography

Background: Monoclonal antibodies and Fc-fusion proteins contain an IgG Fc moiety, which is associated with various degradation processes, including aggregation.

Results: Fc unfolding is triggered by the protonation of acidic residues and depends on the IgG subclass and Cᵢᴴ₂ domain glycosylation.

Conclusion: Fc aggregation in acidic conditions is determined by Cᵢᴴ₂ stability.

Significance: Understanding Fc aggregation is important for improving the quality of Fc-based therapeutics.

SUMMARY

Understanding the underlying mechanisms of Fc aggregation is an important prerequisite for developing stable and efficacious antibody-based therapeutics. In our study, high-resolution two-dimensional nuclear magnetic resonance (NMR) was employed to probe structural changes in the IgG1 Fc. A series of ¹H-¹⁵N HSQC NMR spectra were collected between pH 2.5 and 4.7 to assess whether unfolding of Cᵢᴴ₂ domains precedes that of Cᵢᴴ₃ domains. The same pH range was subsequently screened in Fc aggregation experiments that utilized molecules of IgG1 and IgG2 subclasses with varying levels of Cᵢᴴ₂ glycosylation. In addition, differential scanning calorimetry (DSC) data was collected over a pH range of 3-7 to assess changes in Cᵢᴴ₂ and Cᵢᴴ₃ thermostability. As a result, compelling evidence was gathered that emphasizes the importance of Cᵢᴴ₂ stability in determining the rate and extent of Fc aggregation. In particular, we found that Fc domains of the IgG1 subclass have a lower propensity to aggregate compared to those of the IgG2 subclass. Our data for glycosylated, partially deglycosylated, and fully deglycosylated molecules further revealed the criticality of Cᵢᴴ₂ glycans in modulating Fc aggregation. These findings provide important insights into the stability of Fc-based therapeutics and promote better understanding of their acid-induced aggregation process.

ABBREVIATIONS

CEX, cation-exchange HPLC; CHO cells, Chinese Hamster Ovary cells; DSC, differential scanning calorimetry; ESI, electrospray ionization; Fab, fragment antigen-binding; Fc, fragment antigen-binding; GuHCl, guanidine hydrochloride; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; MG, molten globule; MS, mass spectrometry; pI, isoelectric point; QbD, Quality by Design; Q-TOF, quadripole-time-of-flight; rCE-SDS, capillary electrophoresis under reducing/denaturing conditions; RP-HPLC, reversed-phase HPLC; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel
electrophoresis; SEC, size-exclusion HPLC; UV Abs, absorbance in the UV region.

INTRODUCTION

In order to ensure the safety and efficacy of biotherapeutics, it is critical to understand and prevent protein degradation. The presence of aggregates in therapeutic proteins may jeopardize their safety and efficacy by eliciting unwanted immunogenic responses (1, 2). Mitigation of aggregation processes while maximizing biotherapeutic shelf-life remains one of the outstanding challenges in biotechnology.

Monoclonal antibodies (mAbs) continue to represent the leading group of biopharmaceutical products (3–6). All currently approved therapeutic mAbs belong to the IgG class and have a structure schematically depicted in Figure 1. Intact mAbs are composed of two identical light chains (LC) and two identical heavy chains (HC) which are covalently linked via several inter- and intra-chain disulfide bonds. The LCs and HC s form two \( V_L \), \( C_L \) and four structurally homologous domains \( (V_H, \ C_H1, \ C_H2, \ C_H3) \), respectively. The overall IgG structure consists of two identical Fab domains \( (V_L, \ C_L, \ V_H, \ C_H1) \) and one Fc domain \( (C_H2 \) and \( C_H3) \) that are connected by a flexible hinge region. The Fc portion harbors one conserved Asn297 glycosylation site in each of its \( C_H2 \) domains. The Fab and Fc regions of mAbs have different biological functions. The Fab regions are responsible for binding to the antigen whereas the Fc portion plays a role in modulating immune cell activity. In addition to mAbs, there are other classes of biotherapeutics, such as Fc-fusion proteins, that also contain Fc. These molecules are composed of therapeutically active peptide or protein moieties which are attached to either the C-termini or N-termini of an IgG Fc. In such cases, presence of an IgG Fc moiety may result in improved physiological function, ease of production, solubility, etc. However, the Fc region is also associated with a range of degradation processes, including oxidation (7) and aggregation (8). A detailed understanding of how certain structural changes within the Fc domain lead to aggregation represents an important step towards improving the quality of these therapeutic agents.

Fc-based biologics offer significant manufacturing and physiological advantages. Their purification process is greatly simplified by the available selection of affinity resins targeting the Fc portion (9, 10). Presence of a relatively large (~50 kDa) and highly soluble Fc moiety confers increased solubility and half-life (11). In addition, the Fc region engages in specific biologically relevant interactions that may require \( C_H2 \) glycosylation (antibody-dependent, cell-mediated cytotoxicity; complement activation; \textit{in vivo} clearance; etc.) (12–15). Uncovering the various sources of Fc instability that are connected with particular \( C_H2 \) glycoforms will enable production of biologics with enhanced pharmacological properties.

In a typical purification process, mAbs and Fc-fusion proteins are exposed to acidic conditions during viral inactivation and elution from affinity resins (9, 16). It is well known that low pH conditions may result in protein denaturation and aggregation (17, 18). It was shown that acidic pH and high ionic strength can promote formation of nonnative protein structures. Some of the best studied, partially folded, acid-denatured states (A-states or MG-states) are populated at low pH in the presence of salt. For example, an acid molten globule state of cytochrome \( c \) is formed at pH 2.0–2.5 in the presence of 0.5–1.5 M salt (19–21). Apomyoglobin, \( \beta \)-lactamase, and staphylococcal nuclease also exhibit an acid- and salt-induced formation of A-states at low pH (22–25). Monoclonal antibodies and their fragments are no exceptions to this rule. Buchner and co-workers demonstrated that intact mAbs, Fab regions, and even isolated \( C_H3 \) domains form A-states at acidic pH and high ionic strength (26–28). Although stability and structure of these states are highly dependent on the protein and experimental conditions, their common characteristic is a tendency to aggregate (17, 29, 30). Unlike small, single-domain proteins, mAbs are complex glycoproteins composed of several, independently folded domains. Commercial mAb preparations are rather heterogeneous and may contain differentially processed, incompletely glycosylated, and covalently modified forms (31). The understanding of mAb aggregation is challenged by the intrinsic and extrinsic complexity (not to mention the storage history) of antibody preparations.

Despite the aforementioned issues, significant progress has been made in understanding and preventing aggregation in
biopharmaceuticals (for a recent review see 32). It was recognized that IgG aggregation can be induced by various factors and proceed through different mechanisms (33–36). However, the role of individual antibody domains in aggregation remained poorly understood. Recently, we proposed that acid-induced aggregation of mAbs is controlled by the stability of C{sub H}{sub 2} domains located in the Fc region (8). At the time, no structural evidence was generated concerning the extent of C{sub H}{sub 2} unfolding associated with this aggregation process. We are now filling this gap by gathering all of the necessary structural and stability data to implicate the C{sub H}{sub 2} domain. The scope of our study was limited to Fc fragments to allow for the use of high-resolution 2D NMR and to reduce the number of DSC transitions. In addition, various forms of Fc (i.e., with respect to IgG subclass and degree of C{sub H}{sub 2} glycosylation) were analyzed under conditions promoting acid-induced aggregation. As a result, we revealed the aggregation rank order of the most typical IgG Fc domains currently used in biotechnology.

**EXPERIMENTAL PROCEDURES**

The *E. coli*-derived IgG1 Fc, *CHO*-derived IgG1 Fc, *CHO*-derived IgG2 Fc, and the uniformly {sup 2}H, {sup 15}N-labeled, *E. coli*-derived IgG1 Fc were supplied by the Protein Sciences group at Amgen, Inc. Intact mAbs (IgG1-A, IgG1-B, IgG2-B, and IgG2-C) were supplied by the Process Development group at Amgen, Inc. All purified proteins were verified greater than 95% pure by SDS-PAGE and size-exclusion HPLC. Other reagents and chemicals were of analytical grade or better. All solutions were filtered through a 0.22 µm filter prior to use.

**Protein preparation, identification, and characterization**—The *E. coli*- and *CHO*-derived Fc were supplied in 10 mM sodium acetate with 9% (w/v) sucrose at pH 5.2. The purity and identity of the Fc were verified by RP-HPLC and mass spectrometry (see Figures S1-S3 of Supporting Information for details). The *E. coli*-derived IgG1 Fc material was the most homogeneous. It contained only one minor impurity, a species presumably with an unpaired disulfide. The *CHO*-derived IgG1 Fc contained fully glycosylated species of expected mass and three minor species: 1) a singly oxidized species, 2) a species with an unpaired disulfide, and 3) a partially glycosylated species. The *CHO*-derived IgG2 Fc was more heterogeneous, containing some clips and host cell proteins. Its major fraction was composed of two fully glycosylated species that presumably differed in sulfation.

The IgG1-B-derived Fc fragments with differing levels of C{sub H}{sub 2} glycosylation were prepared as follows. Two milliliters of IgG1-B at 6 mg/mL were incubated with 24 µL of PNGase F (New England Biolabs, Ipswich, MA) in 1X G7 buffer for 45 min at 37 °C. Endoproteinase Lys-C (Roche Diagnostics, Basel, Switzerland) was then spiked into the reaction mixture at a protein/enzyme weight ratio of 200:1. The sample was incubated at 37 °C for an additional 15 min before quenching with 150 mM ammonium acetate at pH 4.7 (37). The sample was then cooled and maintained at 4 °C for immediate purification or frozen at -80 °C to arrest further enzyme activity.

Glycosylated, partially deglycosylated, and fully deglycosylated Fc were purified using the CEX method described below (also see 37). Digested IgG1-B material was loaded onto the column in multiple injections. An Agilent 1200 Series HPLC system with a 12/13 SelValve external valve (Agilent Technologies, Santa Clara, CA) was used to perform the fractionation. Resulting fractions containing the same species were pooled and concentrated, then re-analyzed by CEX. Purity greater than 90% was achieved for all three Fc species based on RP-HPLC and mass spectrometry (see Figure S4 of Supporting Information).

**Aggregation experiments**—Previously, we demonstrated that CEX can be used to measure the aggregation propensity of both intact and fragmented mAbs (8, 35). This same method was applied in the current study where mAb and Fc mixtures were exposed to different solution conditions. In our experiments, each protein was at 0.5 mg/mL final concentration unless noted otherwise. MAb and Fc mixtures were prepared in a native buffer composed of 10 mM sodium acetate at pH 5.2. They were subsequently diluted into various solutions of interest and incubated quiescently at 30 °C for up to several days. Sample aliquots were taken at predetermined intervals and analyzed immediately or stored on ice to reduce further aggregation. The loss of soluble monomer was determined for each individual protein relative...
to an appropriate control stored refrigerated in 10 mM sodium acetate, pH 5.2. Unstable proteins exhibited a faster decrease in the CEX monomer concentration compared to more stable proteins (8, 35).

**Cation-Exchange Chromatography (CEX)—**
Aggregation of mAb and Fc mixtures was investigated by cation-exchange chromatography at pH 5.2 (35, 37). The method was run on an Agilent 1100 Series HPLC system. Chromatography was performed on a ProPac WCX-10 analytical column (weak cation exchange, 4 × 250 mm; Dionex, Sunnyvale, CA) preceded by a ProPac WCX-10G guard column (weak cation exchange, 4 × 50 mm; Dionex) at 25 °C. Protein samples were loaded onto the column and analyzed at a flow rate of 0.7 mL/min. The column was equilibrated with Buffer A (20 mM sodium acetate, pH 5.2), and protein was eluted with a linear gradient of Buffer B (20 mM sodium acetate, 300 mM sodium chloride, pH 5.2) from 0 to 100 % over 35 minutes. Following elution, the column was washed with Buffer C (20 mM sodium acetate, 1 M sodium chloride, pH 5.2) for 5 minutes then re-equilibrated with Buffer A for 16 minutes. Absorbance was measured at 215, 235, and 280 nm. Data were analyzed with Dionex Chromeleon© software and the 280 nm signal was integrated to determine protein peak area.

**Differential Scanning Calorimetry (DSC)—**
DSC measurements were taken using a VP-Capillary DSC system (MicroCal Inc., Northampton, MA) equipped with tantalum 61 cells, each with an active volume of 135 μL. Protein samples, typically at 0.5 mg/mL, were scanned from 20 to 110 °C at a rate of 60 °C/hour following an initial 15-minute equilibration at 20 °C. A filtering period of 16 seconds was used, and the data were analyzed using Origin 7.0 software (OriginLab® Corporation, Northampton, MA). Resulting thermograms were corrected by subtraction of buffer control scans. The corrected thermograms were normalized for protein concentration.

**Nuclear magnetic resonance (NMR)—**
Fc NMR measurements were performed at 25 °C using a Varian INOVA 800 MHz NMR spectrometer (Varian Inc., Palo Alto, CA) equipped with a 5 mm triple resonance probe. The uniformly 1H, 15N-labeled E. coli-derived IgG1 Fc was tested at 5 mg/mL in 10 mM sodium acetate adjusted by HCl to pH 2.5, 3.1, 3.5, and 4.7. 1H,15N HSQC spectra were acquired with 64 experiments run in the 15N dimension (t1) consisting of 16 scans and 1024 data points in the 1H dimension (t2). The total experimental time for each spectral acquisition was 37 minutes. Spectra were processed using NMRPipe (38) and analyzed using NMRView (39). The 1H-15N cross-peak assignments from Liu et al. were used (40).

The weighted average chemical shift difference was calculated as previously described (41).

**Reversed-phase chromatography and mass spectrometry—**
Reversed-phase analysis of the E. coli-derived IgG1 Fc, CHO-derived IgG1 Fc, and CHO-derived IgG2 Fc was carried out on a Waters (Milford, MA) Acquity system, equipped with a Diphenyl 3 μm 1 × 50 mm column (Varian Inc., Palo Alto, CA) as previously described (42). Typically, 5 μg of protein was injected on the column. The column was held at 95 % solvent A (0.1 % TFA in water) and 5 % solvent B (90 % ACN and 0.085 % TFA in water) for 5 min followed by a gradient from 5 % B to 38 % B over 13 min. Fc elution was achieved with a linear gradient from 38 % B to 46 % B in 40 minutes at a flow rate of 0.05 mL/min. The column temperature was maintained at 75 °C throughout the run and detection was at 214 nm.

Reversed-phase analysis of the IgG1-B-derived Fc was carried out on a Waters Acquity UPLC system as previously described (42). Typically, 5 μg of sample was injected onto an Acquity BEH 1.7 μm 1 × 50 mm phenyl column. The column was held at 72 % solvent A (0.1 % TFA in water) and 28 % solvent B (90 % ACN and 0.085 % TFA in water) for 0.7 min. Solvent B was increased to 31.4 % at 0.9 min, to 49.4 % at 3.4 min, and to 90 % at 3.5 min. At 4.10 min, solvent B returned to the starting level (28 %) and remained constant until the end of the assay at 5 min. The column temperature was maintained at 80 °C throughout the run, the flow rate was kept constant at 0.35 mL/min, and the detection was at 214 nm.

The mass spectrometric analysis was carried out in positive ion mode on a Waters Q-TOF premier or LCT premier equipped with an electrospray ionization (ESI) source. The capillary and cone voltages were set at 3200 V and 60 V,
RESULTS

Effect of acidic conditions on IgG1 Fc structure via NMR analysis—Although Fc is a relatively large protein (~50 kDa), recent studies demonstrated that it is amenable to high-resolution 2D NMR analysis (7, 43). Furthermore, resonance assignments from Liu et al. made investigation of pH effects on IgG1 Fc structure straightforward (40). In the current study, the Fc conformation was assessed between pH 2.5 and 4.7 by acquiring a series of $^1$H-$^{15}$N HSQC spectra of the uniformly $^2$H, $^{15}$N-labeled E. coli-derived IgG1 Fc. Due to the low ionic strength of the protein solutions (see Experimental Procedures), no evidence of aggregation was seen in any of the Fc samples throughout the experiment. At pH 4.7, the amide peaks of Fc were highly dispersed, which was consistent with a folded conformation (see Figure 2A). A similar degree of dispersion was seen at pH 3.5, although a number of peaks were reduced in intensity (Figure 2B). In addition, some new, low intensity peaks emerged that were not present at higher pH. At pH 3.1, a subset of native resonances disappeared while a different set of peaks appeared (Figure 2C). Spectral properties of these new peaks were characteristic of a disordered, largely unfolded protein conformation. They resembled the minor resonances that were barely visible at pH 3.5. The remaining native resonances disappeared at pH 2.5 where NMR showed limited peak dispersion consistent with an unfolded state (Figure 2D).

At pH 3.5 and 4.7, the number of assigned resonances available for analysis was 116 and 117, respectively. This represented 51% of the 227 Fc amino acid residues. Many of the missing peaks originated from the vicinity of the hinge region or were due to peak overlap between the different pH spectra. The number of native resonances dropped to 46 at pH 3.1, and none were present at pH 2.5.

The weighted average, residue-specific, chemical shift changes between pH 3.5-4.7 and 3.1-3.5 are shown in Figure 3. The most prominent changes between pH 3.5-4.7 were clustered around residue positions 250-255 and 310-315 (Figure 3A). These regions overlap with two short C$_{12}$ α-helices that interface with the C$_{13}$3 domains (see Discussion). In addition, notable chemical shift changes ($\geq 0.05$ ppm) were associated with positions corresponding to D280, Q295, L306, and T335 of C$_{12}$ and G385 and K447 of C$_{13}$. Similar regions produced peaks with reduced intensity at pH 3.5, which likely reflected changes in the C$_{12}$ conformational dynamics. Moreover, some of the native resonances that were present at pH 4.7 completely disappeared at pH 3.5, among them resonances from K290 and possibly W277 and V412 (see Table 1).

Peaks indicating the presence of a folded C$_{12}$ domain were virtually non-existent at pH 3.1, suggesting that major unfolding had occurred (Figure 2C). Therefore, estimates for the chemical shift changes between pH 3.1-3.5 were only available for C$_{13}$3 domains (Figure 3B). At least three residue positions showed significant chemical shift changes at pH 3.1: R344, W381, and K447. Of interest is the R344 residue, the residue near the loop connecting the C$_{12}$ and C$_{13}$3 domains. Changes at this position are likely the result of structural changes in the adjacent C$_{12}$ domain. This is supported by the simultaneous disappearance of many of the C$_{13}$3 resonances originating from positions 366-380 and 428-438 (Table 1). Both of these segments contain residues forming the C$_{12}$-C$_{13}$3 domain interface which likely gets disrupted because of C$_{12}$ unfolding (see Discussion).

A further reduction in pH from 3.1 to 2.5 resulted in the disappearance of all remaining folded resonances (Figure 2D). The NMR spectrum of Fc at pH 2.5 was now consistent with an unfolded protein conformation devoid of stable tertiary or secondary structure. The high affinity C$_{12}$3-C$_{13}$3 interaction was likely disrupted also, as demonstrated by the lack of native resonances originating from the domain contact area. Specifically, this was reflected by the absence of native amide peaks from the following residues: L351, E357, S364, L368, K370, T394, D399,
F405, and K409. Since all of these positions were in a native-like environment at higher pH, the data were consistent with a scenario where dissociation and unfolding of the C\textsubscript{H}3-C\textsubscript{H}3 inter-chain complex occurred simultaneously (see Discussion).

**CEX analysis of Fc aggregation**—Previously, we demonstrated the utility of CEX in measuring the aggregation propensity of both intact and fragmented mAbs (8, 35). Similar to SEC, CEX is a non-denaturing chromatographic technique that can effectively separate aggregates from monomers. However, in contrast to SEC, CEX can resolve complex mixtures composed of similarly sized proteins. Working with protein mixtures allows us to monitor the aggregation of different molecules simultaneously and under identical conditions. Hence, CEX was selected to establish the rank order of Fc aggregation as a function of C\textsubscript{H}2 glycosylation and subclass (IgG1 vs. IgG2).

In addition to separating aggregates from monomers, CEX is useful in detecting degraded or chemically modified proteins (31, 37). This was an added benefit as our goal was to measure Fc aggregation with minimal interference from chemical degradations. Our initial studies were focused on finding conditions to induce Fc aggregation within a short period of time at moderately elevated temperatures. First, we performed a pH screening experiment using protein solutions buffered with 10 mM sodium acetate to mimic the conditions that were used for NMR. The samples contained a mixture of four different molecules: three full-length mAbs (IgG1-A, IgG2-B, and IgG2-C) and E. coli-derived IgG1 Fc. The choice to use the three mAbs was dictated by our previous experience with these molecules (8, 35). They served as internal controls to optimize solvent composition and incubation time to assess Fc aggregation. In agreement with the NMR results, aggregation was not observed in these low (10 mM) acetic acid solutions at pH 3-5 even after 2 days of storage at 30 °C (data not shown). This was consistent with the important role of the ionic strength and acid concentration in low pH mAb aggregation (8). Subsequently, the pH screening was repeated in the presence of high (100 mM) sodium acetate with and without 50 mM NaCl (the corresponding solutions are abbreviated as 100A\textsubscript{x} and 100A\textsubscript{N}, where A stands for sodium acetate, \textit{x} is the pH, and \textit{N} is NaCl). All four molecules were premixed in 10 mM sodium acetate at pH 5.2 prior to being exposed to the low pH conditions. Figures 4A and 4B show CEX chromatographic traces for samples incubated in 100A37N and 100A34, respectively. Figure 5 summarizes results from various conditions in terms of percent monomer recovery based on CEX. It is evident that IgG1-A aggregated only at pH 3.4 (Figures 5A and 5E), whereas aggregation of Fc and the two IgG2s occurred at pH 3.7-4.1 (Figures 5B and 5F, 5C and 5G, and 5D and 5H, respectively). Previously, we observed that low pH aggregation of mAbs was dependent on C\textsubscript{H}2 glycosylation and the IgG subclass (8). In particular, glycosylated IgG1 mAbs were more resistant to aggregation compared to their glycosylated IgG2 counterparts, whereas an aglyco-IgG1 (an IgG1 mAb devoid of C\textsubscript{H}2 glycosylation) was the least stable molecule tested (8). Consistent with these findings, Figure 5 reveals the following aggregation rank order of the four molecules (listed from the highest aggregation propensity to the lowest): \textit{E. coli}-derived IgG1 Fc (i.e., aglyco IgG1 Fc) > IgG2-C > IgG2-B > IgG1-A. Aggregation propensity of Fc in 100A34 and 100A37N was particularly high and resulted in the loss of 30-40 % of monomer at \textit{t} = 0 (Figures 5D and 5H, respectively). Thus, the CEX data demonstrated an increased instability of aglyco-IgG1 Fc compared to glycosylated mAbs. Furthermore, the aggregation rank order for these molecules was the same in either the 100A\textsubscript{x} or 100A\textsubscript{N} conditions, which indicated the following: 1) the underlying aggregation mechanism was largely unaffected by NaCl; 2) the rate and extent of Fc aggregation could be appropriately modulated by varying the ionic strength. Since covalent modification and fragmentation was not evident in these experiments (see Figure 4), protein aggregation was the major degradation process. In summary, sufficient evidence was gathered to support the low pH approach for generating Fc aggregation data.

Our next experiment was performed on a mixture composed of three different Fc moieties: \textit{E. coli}-derived IgG1 Fc, CHO-derived IgG1 Fc, and CHO-derived IgG2 Fc. This mixture was subjected to aggregation in the 100A31N and 100A35N conditions as outlined above. The CEX overlays corresponding to aggregation in 100A35N are shown in Figure 6A. Quantitative aggregation
results in 100A31N and 100A35N are summarized in Figures 6B and 6C, respectively. Despite the heterogeneous nature of the Fc samples, the CHO-derived IgG1 Fc (including all minor forms) was evidently more resistant to aggregation compared to its aglycosylated (E. coli) variant or the CHO-derived IgG2 Fc. Aggregation of the latter two molecules appeared similar in 100A35N but differed in 100A31N. In particular, aglyco-IgG1 Fc lost ~55% of monomer at $t = 0$ but aggregated more slowly afterwards (gray symbols in Figure 6C). The initial monomer loss of the CHO-derived IgG2 Fc was less than 40%, but the remaining monomer disappeared rapidly (open symbols in Figure 6C). Since all three molecules were premixed at pH 5.2 prior to the low pH exposure, this result indicated a lack of stability of the aglyco-IgG1 and glyco-IgG2 Fc. Consequently, the rank order of Fc aggregation was found to be: aglyco-IgG1 Fc $\geq$ IgG2 Fc (CHO) $>$ IgG1 Fc (CHO). This was consistent with our earlier findings (8), as well as the aggregation rank order that was drawn from Figure 5. Therefore, a conclusion was made that the 100A31N and 100A35N conditions primarily promoted a C$_H2$-dependent aggregation mechanism.

Our last aggregation experiment utilized differentially glycosylated Fc fractions generated from another IgG1 mAb, IgG1-B. The success of this experiment depended on: 1) the ability of CEX to resolve Fc fragments with different levels of C$_H2$ glycosylation; 2) the optimization of PNGase F treatment to achieve an optimal ratio of glycosylated, partially deglycosylated, and fully deglycosylated Fc for purification. The ability of CEX to separate differentially glycosylated Fc was verified by analyzing PNGase F treated and untreated IgG1-B following Lys-C limited proteolysis (data not shown). Subsequently, IgG1-B and PNGase F concentrations were varied along with incubation temperature and duration to achieve an optimal rate of digestion and desired ratio of glycosylated, partially deglycosylated, and fully deglycosylated Fc. Storage temperature and duration after digestion were also assessed to ensure that this ratio was sufficiently maintained over the course of purification (see Experimental Procedures). The resulting Fc fractions were verified by RP-HPLC and mass spectrometry (see Figure S4), mixed together, and subjected to an aggregation process in 100A31N. The corresponding CEX results are shown in Figures 7A and 7B. In agreement with the data in Figure 6C, glycosylated Fc was more resistant to aggregation compared to its fully deglycosylated variant. The partially deglycosylated Fc exhibited an intermediate stability. The initial monomer loss of the glycosylated, partially deglycosylated, and fully deglycosylated Fc was 8%, 31%, and 63%, respectively. Thus, the rank order of Fc aggregation was found to be: fully deglycosylated Fc $>$ partially deglycosylated Fc $>$ glycosylated Fc. Such results provided compelling evidence for the importance of C$_H2$ glycosylation in determining the rate and extent of Fc aggregation.

**Effect of acidic conditions on Fc stability via DSC analysis**—Thermostability of E. coli-derived IgG1 Fc, CHO-derived IgG1 Fc, and CHO-derived IgG2 Fc was assessed under conditions mimicking the CEX aggregation experiments (see above). DSC samples were made by diluting protein stock solutions into different 100AX buffers, except for pH 7 controls that were prepared in 100 mM sodium phosphate (100NaPi70). Figure 8 shows DSC traces for the three Fc variants. A summary with all of the DSC data as a function of pH and apparent $T_m$ is given in Figure 9 and Table 2. Although IgG Fc’s are known to consist of two different, independently folded domains, C$_H2$ and C$_H3$, some of the DSC profiles contained an additional high temperature peak. We will refer to this peak as an “A-state” in accordance with Buchner and co-workers (26).

At pH 7.0, the DSC profile of the E. coli-derived IgG1 Fc was characterized only by the presence of C$_H2$ and C$_H3$ transitions (Figure 8A). Between pH 3.5-5.2, an additional A-state transition was present, giving rise to a characteristic three-peak profile (Figures 8B-E). Below pH 3.5, the aglycosylated C$_H2$ domain was unfolded and no longer produced a peak. As a consequence, the corresponding DSC profile contained only C$_H3$ and A-state transitions (Figure 8F).

Results for CHO-derived IgG1 Fc were similar to E. coli-derived IgG1 Fc with respect to the C$_H3$ and A-state transitions (Figure 8). The latter transition was now seen across the entire pH range, including pH 7.0 (Figure 8A). As expected, glycosylated C$_H2$ had a higher $T_m$ compared to its aglycosylated counterpart, confirming the important stabilizing role of C$_H2$ glycans (see
Figure 9A and Table 2). The magnitude of this difference increased as pH decreased: the initial $T_m$ difference of $-6^\circ C$ reached $-13^\circ C$ when the pH dropped from 7.0 to less than 4.0. C$_{H3}$ of the CHO-derived Fc also appeared to have a higher $T_m$ but only at a pH below 3.5 (Figure 9A). Such a result indicated that C$_{H3}$ domains in the aglyco-Fc were affected by the C$_{H2}$ instability (unfolding) at pH < 3.5, in agreement with the NMR data above.

The DSC profiles of the CHO-derived IgG2 Fc (Figures 8 and 9) generally resembled those of the CHO-derived IgG1 Fc. The IgG2 Fc differed notably from IgG1 Fc in the following ways: 1) the A-state transition was less frequently observed; 2) the C$_{H3}$ transition had a lower $T_m$ across the entire pH range; 3) the $T_m$ of the C$_{H2}$ transition was comparable at pH 5.2-7.0, but a further reduction in pH revealed a stability difference. Specifically, the $T_m$ of the IgG2 C$_{H2}$ was much more dependent on pH and decreased significantly. The maximal difference seen in this study ($-7^\circ C$ at pH 3.5) agreed with our previously reported value obtained at pH 3.5 in the presence of 500 mM NaCl (8).

The following domain properties were the same across all three Fc types (see Figure 9): 1) the $T_m$ of C$_{H2}$ was higher at pH 7.0 compared to 5.2, and it decreased in a non-linear fashion upon solution acidification; 2) the $T_m$ of C$_{H3}$ was nearly identical between pH 7.0 and 5.2 but decreased in a non-linear fashion upon further acidification; 3) the $T_m$ of the A-state seemed to be lower at pH 7.0 than at 5.2. It proceeded through a maximum at pH $-5.2$ before undergoing a non-linear decrease upon further pH reduction.

In order to understand the nature of the A-state transition, the concentration dependence of the thermograms for CHO-derived IgG1 Fc was investigated. For this purpose, 0.1, 0.2, 0.3, and 0.4 mg/mL protein samples were prepared in 100A35 and subsequently analyzed by DSC. Although the signal at low protein concentrations was weak, triplicate runs reproducibly revealed the concentration-dependent nature of the A-state. The characteristic three-peak profile was seen in all cases, but the enthalpy of the A-state peak visibly decreased at 0.1 mg/mL protein (see Figure S5 of Supporting Information).

**DISCUSSION**

*High-resolution structural analysis of Fc unfolding—Analysis of chemical shift perturbations via $^1$H-$^{15}$N HSQC NMR is a powerful approach that allows a detailed understanding of protein structural changes. Changes in chemical shifts may originate from changes in hydrogen bonding as there is a strong correlation between hydrogen bond energies and amide proton or amide nitrogen chemical shifts (45). Protein unfolding is expected to result in chemical shift and peak intensity changes, but some regions of a molecule may exhibit greater changes in conformational dynamics or in the electronic/electrostatic environment than others. This may especially be the case with proteins composed of structurally distinct domains, such as mAbs and their Fab and Fc fragments.*

An IgG Fc molecule is composed of two types of independently folded domains, C$_{H2}$ and C$_{H3}$. Thus, denaturation of Fc is expected to be a multi-stage process where changes in one domain are not fully coupled with changes in another. Until now this view was supported by evidence from differential scanning calorimetry and optical spectroscopy studies (46, 47). It has also been known that C$_{H2}$ domains tend to be less stable than C$_{H3}$ under a large variety of conditions. Our residue-specific 2D NMR analysis provides direct evidence for both of these notions. We demonstrate that structural changes in C$_{H2}$ and C$_{H3}$ are largely uncoupled and that unfolding of C$_{H2}$ precedes that of C$_{H3}$. This mainly follows from: 1) the chemical shift and peak intensity changes at pH 3.5, indicating increased susceptibility of C$_{H2}$ to structural perturbations (Figure 3A); 2) the absence of native peaks from C$_{H2}$ at pH 3.1, in contrast to the presence of at least 46 assigned resonances from C$_{H3}$ (Figures 2C and 3B).

Equilibrium GuHCl unfolding of isolated C$_{H2}$ was previously shown to be consistent with a two-state process (48). Results from our study suggest a more complex unfolding scenario that invokes partially denatured conformations. Feige and co-authors derived their conclusion based on evidence provided by intrinsic fluorescence and circular dichroism. Because of limited sensitivity and structural resolution, optical spectroscopy may not always reveal near-native protein conformations accessible by NMR. The observed discrepancy may also be explained by: 1)
differences in the experimental pH (the GuHCl unfolding studies were performed at pH 7.5); 2) differences in the mode of protein denaturation (chaotrope- vs. acid-induced); 3) differences in the unfolding mechanism of isolated C\textsubscript{H}2 domains vs. C\textsubscript{H}2 domains within the context of an IgG Fc. All of these aspects could possibly be addressed by performing NMR-based unfolding experiments on isolated C\textsubscript{H}2. However, such studies were beyond the scope of our investigation.

Structural characteristics of C\textsubscript{H}2 at pH 3.5 are indicative of partial denaturation caused by protonation of acidic side chains. Particularly susceptible are the E380 and K338 residues that form salt bridges with K248 and K338, respectively. Both glutamic acid residues reside in C\textsubscript{H}3 and their side chains are expected to be largely protonated at pH 3.5 \((pK_a \text{ of free glutamic acid is } 4.07)\). Their C\textsubscript{H}2 partners, K248 and K338, lay in close proximity to the two C\textsubscript{H}2 \(\alpha\)-helices that undergo the most significant perturbation at pH 3.5 (Figure 3A). The crystal structure of IgG1 Fc (PDB entry 1HZH (49)) provides evidence that the K248-E380 and K338-E430 ion pairs may be required for stabilization of the C\textsubscript{H}2-C\textsubscript{H}3 interaction area. Therefore, it is plausible that the loss of such interactions due to the E380 and E430 protonation could modulate the structure of the C\textsubscript{H}2-C\textsubscript{H}3 interface. It is important to note that the same region represents the Protein A-binding site and its impairment causes acid elution of mAbs from Protein A resins. Some C\textsubscript{H}2 destabilization may also be brought about by the protonation of D312, a residue that resides in one of the two C\textsubscript{H}2 helices and interacts with K317. Indeed, the amide peak of D312 shows a significant chemical shift perturbation and intensity loss at pH 3.5 (Figure 3A). Another interesting observation is related to the L306 and W277 side chains that are packed against each other in the hydrophobic core of the C\textsubscript{H}2 domain. At pH 3.5, the L306 resonance undergoes a notable chemical shift change, whereas the peak for W277 apparently disappears (Table 1). Moreover, chemical shift changes are evident for the amide peak of the Q295 residue that is located near the hinge region and is far removed from the aforementioned helices. This indicates that changes in the C\textsubscript{H}2 domain structure extend well beyond the C\textsubscript{H}2-C\textsubscript{H}3 interface (see Figures 10A and 10B). The potential outcome of these changes is the exposure of buried hydrophobic surfaces that can prime Fc for aggregation. The scenario and regions involved appear to be different from the computationally predicted aggregation-prone regions in C\textsubscript{H}2 (50). However, because of the differences in the experimental pH (SAP analysis was performed at pH 6.5), a direct comparison of the data may not be appropriate.

In contrast to C\textsubscript{H}2, structural changes in C\textsubscript{H}3 are limited at pH 3.5. We only note the chemical shift changes for G385 and K447 and the potential absence of the V412 resonance (Table 1). At pH 3.5, native resonances from C\textsubscript{H}3 remain relatively unperturbed, indicating that it maintains an overall folded structure (Figures 2B and 3A).

The reduction in pH from 4.7 to 3.5 is associated with only a partial denaturation of C\textsubscript{H}2, whereas pH reduction from 3.5 to 3.1 results in a major loss of tertiary and secondary structure. In contrast, unfolding of C\textsubscript{H}3 only takes place below pH 3.1, as evidenced by the dispersion of its amide resonances. Although many of the native C\textsubscript{H}3 resonances are missing at pH 3.1, this is likely related to the denaturation of C\textsubscript{H}2 rather than C\textsubscript{H}3. This is supported by the location of the majority of the missing peaks at the C\textsubscript{H}2-C\textsubscript{H}3 interface (positions 366−380 and 428−438, see Figures 10C/D and Table 1). However, the absence of some of the native C\textsubscript{H}3 peaks may simply be caused by an overlap with resonances originating from denatured C\textsubscript{H}2. Furthermore, there is indirect DSC evidence suggesting only a slight difference in C\textsubscript{H}3 \(T_m\) between the \(E.\ coli\) - and \(CHO\)-derived IgG1 Fc at pH below 3.5 (Figure 9A). In the case of these two Fc moieties, the difference in C\textsubscript{H}3 stability is likely determined by the presence or absence of C\textsubscript{H}2 glycans. Since the magnitude of the C\textsubscript{H}3 \(T_m\) difference is rather small, we can deduce that C\textsubscript{H}2 denaturation has only a minor impact on the stability of the adjacent C\textsubscript{H}3. The C\textsubscript{H}3-C\textsubscript{H}3 contact area also remains relatively unperturbed, but some changes within these domains do take place, as evidenced by the W381 and Y391 resonances. The side chains of these two residues form a tertiary contact by packing against each other in the hydrophobic core. At pH 3.1, the W381 resonance exhibits notable changes in its chemical shift and intensity, whereas the peak for Y391 disappears entirely (Figure 3B and Table 1). Furthermore, V369 is another deeply buried C\textsubscript{H}3 residue that is missing its native resonance at pH
3.1 (Table 1). Therefore, it can be concluded that although the C_{i3} domains are folded at this pH, their tertiary structure is not fully native.

A further reduction in pH from 3.1 to 2.5 brings about major unfolding of C_{i3}, as evidenced by the disappearance of resonances from its folded state (Figure 2D). An important feature of C_{i3} domain is the formation of a tightly bound C_{i3}-C_{i3} dimer. Upon inspection of the Fc crystal structure, a number of C_{i3}-C_{i3} inter-domain interactions can be found. Among them, the ion pairs E357-K439 and D399-K409 clearly stand out. Although no direct NMR evidence is currently available, one can hypothesize that protonation of the E357 and D399 residues might contribute to C_{i3} unfolding at pH 2.5. Protonation of these side chains would be expected to result in destabilization of the C_{i3}-C_{i3} dimer due to the loss of 4 inter-domain salt bridges. Another interaction that may be influenced by the same mechanism is the ion pair formed between D356 and K439. A more detailed analysis of the specific role of acidic side chains in Fc stability will be presented elsewhere.

**C_{i2}/C_{i3} stability and Fc aggregation in acidic conditions**—Direct investigation of Fc aggregation by $^1$H-$^15$N HSQC NMR is precluded by the large size of products of this reaction. To forestall unwanted aggregation, our NMR measurements were performed under low ionic strength conditions. These experiments provided information on the location and extent of structural changes in C_{i2} and C_{i3}, which is necessary for improved understanding of Fc aggregation. Our DSC experiments allowed us to extend this analysis to the CHO-derived IgG1 and IgG2 Fc for which isotopically enriched material was not available. The intrinsic susceptibility of C_{i2} to pH-induced changes is reflected by its $T_m$ vs. pH profile. For example, the pH profile of C_{i2} from the *E. coli*-derived IgG1 Fc is less linear compared to its CHO-derived counterpart (Figure 9A). Similarly, the CHO-derived IgG2 Fc shows less linear dependency on pH compared to the CHO-derived IgG1 Fc (Figure 9B). The increased susceptibility of aglycosylated C_{i2} is likely caused by the absence of stabilizing interactions from carbohydrates; the reasons for the higher susceptibility of IgG2 C_{i2} are less obvious. Previously, we speculated on the role of the C_{i2} sequence variations between the IgG1 and IgG2 subclasses (8). In particular, we hypothesized that the region surrounding the $Y_{IgG1300F_{IgG2}}$ substitution (Table 3) could be associated with IgG2 C_{i2} instability at low pH. Our NMR data at pH 3.5 for *E. coli* IgG1 Fc lend support to this idea. As revealed by the chemical shift analysis, the structural environment of Q295 undergoes a significant change at this pH (Figure 3A). Q295 is located near a tertiary contact formed by the side chains of His268, Glu294, and $Y_{IgG1-300F_{IgG2}}$. It is reasonable to presume that protonation of Glu294 at pH 3.5 would destabilize the His268-Glu294 charge-charge interaction. The specific residue located at position 300 may further influence the energy of this contact. Indeed, the $T_m$ vs. pH profiles of C_{i2} shown in Figure 9 are generally consistent with protonation of a glutamic acid residue(s). It would then follow, that the mechanism of destabilization of the region including Q295 is similar to the one proposed for the two C_{i2} helices at the C_{i2}-C_{i3} interface (see above). Such scenarios tie together the widespread structural perturbations in C_{i2} at pH 3.5 with the location of key acidic residues in the Fc structure. Other mechanisms, including anion binding, may contribute to this destabilization, especially when the ionic strength is increased.

The above considerations are consistent with our previous studies on low pH mAb aggregation. Ten different IgG2s that were studied earlier showed an increased aggregation propensity compared to five different IgG1s (8). This subclass dependency correlated with reduced thermostability of IgG2 C_{i2} relative to IgG1 C_{i2}. In addition, regardless of the subclass, C_{i2} glycosylation was found to be an important attribute that determined stability and aggregation of Fc and intact mAbs. Some of these conclusions have already been confirmed outside of our group (50).

It is interesting to note that the $T_m$ vs. pH profiles for the C_{i3} domain of CHO-derived IgG1 and IgG2 Fc are very similar, but offset by 6-7 °C (Figure 9B). There are only three sequence positions that differ in these domains between the IgG1 and IgG2 subclasses (see Table 3): D_{IgG1-356E_{IgG2}}, L_{IgG1-358M_{IgG2}} and V_{IgG1-397M_{IgG2}}. Therefore, some or all of these residues must be responsible for the reduced stability of IgG2 C_{i3}. Commercial IgG1s tend to contain either D356/L358 or E356/M358 substitutions (8, 51).
Thus, an opportunity exists to assess the impact of these residues without the need for new mutant construction. Our preliminary experiments on an IgG1 containing D356/L358 vs. an IgG1 with E356/M358 revealed no difference in their C_{H3} domain stability (data not shown). Given that this finding is confirmed in the case of other IgG1s, this result leaves the $V_{\text{IGG1:397M}}$ mutation as the culprit for the reduced stability of IgG2 C_{H3}.

The CHO-derived IgG1 Fc provided the most complete pH profile for the A-state transition (Figure 9). For reasons not fully understood, the E. coli-derived IgG1 Fc and CHO-derived IgG2 Fc failed to produce this transition at some of the pH values. One possible explanation for this is the narrow temperature range (20-110 °C) that was probed in our experiments. Since the A-state transition tends to occur at $\geq 100$ °C, it may have been necessary to extend the DSC measurements up to at least 120 °C. Another possibility is to use higher protein concentrations for improved detection of this low enthalpy transition. Nevertheless, three important conclusions can be made based on the available data. First, the A-state transition does not seem to be affected by C_{H2} glycosylation (or C_{H2} (de)stabilization as a result thereof), suggesting that it forms independent of C_{H2} (Figure 9A). This dovetails nicely with earlier findings which show that the A-state is formed by isolated C_{H3} domains (28). Such an agreement serves to confirm that the A-state formation might be C_{H3}-dependent even in the case of a full-length Fc. Second, the $T_m$ vs. pH profiles of the A-state for CHO-derived IgG1 and IgG2 Fc appear similar but are offset by several degrees °C (Figure 9B). The reason for this is currently unknown and further studies in this area are warranted. Third, the protein concentration dependence of the A-state suggests that its formation requires the association of several Fc molecules (Figure S5). This is again consistent with the previous report from Thies and co-workers (28). In particular, they showed that C_{H3} domains formed a defined oligomer consisting of 12-14 subunits at pH 2 in the presence of salt. Although an assessment of the Fc oligomeric structure was beyond the scope of our investigation, the appearance of a well-defined DSC transition is consistent with the formation of a distinct protein complex or aggregate.

There are still many questions regarding the A-state that need to be addressed in order to fully understand Fc aggregation. Semantically, it is unclear whether it is appropriate to call it an acid-denatured state if it is present even at neutral pH (Figure 9). Our findings to date favor the idea that Fc aggregation in acidic conditions is primarily determined by its C_{H2} domains. Nevertheless, questions on the possible contributing role of the A-state (or C_{H3} domains) in Fc aggregation remain unanswered. There is also a necessity to discuss the value of calorimetrically determined stability in predicting Fc aggregation. For instance, is there a correlation between DSC and increased aggregation of aglyco-IgG1 Fc or glyco-IgG2 Fc compared to glyco-IgG1 Fc? Figure 9 shows that thermostability of the A-state does not correlate with aggregation. For example, the $T_m$ vs. pH profiles of the A-states for aglyco- and glyco-IgG1 Fc overlay almost perfectly (Figure 9A). Yet, aggregation propensity of these two Fc moieties is dramatically different (Figure 6). The A-state transition of the glyco-IgG2 Fc occurs at higher temperatures than that of the glyco-IgG1 Fc (Figure 9B). Yet, it is the CHO-derived IgG2 Fc that aggregates more readily (Figure 6). The same reasoning applies to C_{H3} domains. Their stability in the aglyco- and glyco-IgG1 Fc is similar until pH < 3.5 (Figure 9A). Yet, aggregation of the aglyco-Fc is already underway at pH 3.7 (Figures 5D and 5H). As far as the IgG1 and IgG2 Fc, their C_{H3} profiles are substantially shifted along the x-axis (Figure 9B). Assuming that C_{H3} denaturation was driving aggregation, aggregation of IgG2 Fc would always be higher compared to both glyco- and aglyco-IgG1 Fc. Is this really the case? Figure 9C shows an overlay of the $T_m$ vs. pH profiles for E. coli-derived IgG1 Fc and CHO-derived IgG2 Fc. Analysis of these profiles along with the results from Figure 6 helps to identify domains that correlate with aggregation. The aglyco-IgG1 Fc experiences a much larger loss of monomer at $t = 0$ in 100A31N (Figure 6C), which is consistent with the lower stability of its C_{H2} domains (Figure 9C). If Fc aggregation was primarily dependent on C_{H3}, one would have predicted the opposite result. Thus, we can conclude that C_{H2} stability differences from calorimetry are generally reflective of Fc aggregation propensity. However, a prediction based solely on $T_m$ is not recommended because aggregation may also be influenced by structural perturbations or kinetic aspects that escape DSC detection.
On the relationship of C_{H2} glycosylation and product quality—The overall goal of biopharmaceutical production is the achievement of the highest possible yield and purity with reduced aggregate levels. The recently introduced concept of Quality by Design (QbD) provides a description of the desired state for manufacturing (52). Understanding the relationship between product quality and manufacturing is one of the main expectations of QbD. In this section we will discuss how knowledge of Fc aggregation mechanisms may help improve quality of Fc-based therapeutics.

In the past few years, understanding of the importance of C_{H2} glycosylation has improved dramatically (14, 15). Apart from its biological function, C_{H2} glycosylation may now be viewed as one of the attributes determining mAb aggregation during production and storage (8, 50). The majority of therapeutic mAbs are produced in a glycosylated form via the CHO cell expression system. Subsequent purification steps are streamlined and tightly controlled to ensure consistency and reproducibility of product quality. However, existing purification platforms rarely result in bulk material that is devoid of incompletely glycosylated species. The presence of such molecules is routinely assayed via capillary electrophoresis under reducing and denaturing conditions (rCE-SDS) (14). Disulfide reduction and denaturation of IgGs forces them to dissociate into constituent light and heavy chains that can be separated based on size. Migration of the latter is influenced by the presence or absence of C_{H2} glycans, resulting in the detection of two distinct heavy chain peaks. Typical rCE-SDS estimates for non-glycosylated heavy chain (NGHC) are on the order of 1% for different mAb preparations (personal communication by Nancy Nightlinger and Alison Wallace of Amgen Inc.). Assuming that there is only one NGHC per antibody molecule, this would translate into 2% of partially glycosylated species in the purified bulk. Is there a particular role for these species in mAb and Fc aggregation? Is their presence a quality attribute that reflects risks of aggregation in biopharmaceutical preparations?

Recent studies showed that aglycosylated mAbs and Fc are unstable in acidic conditions or at higher pH and elevated temperature (8, 50). However, no information was available on the aggregation propensity of a fully glycosylated molecule relative to its aglycosylated and partially glycosylated forms. To the best of our knowledge, Figure 7 provides the first illustration of the effect of all three levels of C_{H2} glycosylation in the case of Fc. The data shown therein were generated from a protein mixture to ensure identical conditions for establishing aggregation propensity. It is evident that Fc aggregation correlates strongly with the degree of C_{H2} glycosylation. Since IgG aggregation in acidic conditions proceeds predominantly via a C_{H2}-dependent pathway (see above), we can argue that such results are relevant also for intact mAbs and Fc-fusion proteins.

The fate of partially glycosylated species is determined by their exposure to stress conditions triggering nonnative aggregation. A typical manufacturing process is a matrix of conditions, some of which are known to be denaturing (acidic pH during viral inactivation, freezing and thawing, stirring, etc.). Therefore, it is possible that biopharmaceutical preparations containing increased levels of such species are at a higher risk of forming aggregates. Even a fraction of denatured, partially glycosylated species that could form immunogenic aggregates may negatively impact product quality. Moreover, the probability of aglycosylated species (species that lack both heavy chain carbohydrates) may be low, but it is far from negligible. Such species are of even greater concern because of their increased aggregation propensity (Figure 7) and the inability of most current techniques to detect them. In particular, their direct assessment is complicated by the prevalence of partially glycosylated mAbs.

The CEX traces in Figure 6A attest to the fact that C_{H2} glycosylation and IgG subclass must be the main determinants of Fc aggregation. This follows from their overriding effects on stability, even when considering all of the major and minor species that are associated with Fc heterogeneity. Therefore, considering all of the evidence generated in this and preceding work (8), there is sufficient knowledge to be applied in a practical test. It is likely that opportunities exist to improve the quality of biotherapeutics by increasing the level of C_{H2} glycosylation, assuming that this does not interfere with functional requirements (Fc effector functions, in vivo clearance, etc.). This may be achieved by improving cell culture and/or purification processes. Alternatively, C_{H2}
aggregation may be mitigated by finding appropriate formulation and/or protein engineering strategies. If tested, some of these ideas may prove beneficial in reducing aggregation issues associated with mAb- and Fc-based therapeutics.

The discussion above should not be viewed as an argument against the production of aglycosylated biologics. To the best of our knowledge, they can be successfully developed given that sufficient efforts are spent to optimize manufacturing, storage, and delivery conditions. Our goal was to highlight that the aggregation propensity of fully glycosylated, partially glycosylated, and aglycosylated mAbs can vary significantly. And, that the cause of protein instability may not necessarily be associated with the main product, but with some of its unstable forms that escape detection.

CONCLUSIONS

A combined use of 2D NMR, DSC, and CEX proved useful for gaining a detailed understanding of the mechanisms of unfolding and aggregation of IgG Fc. Fc aggregation under acidic conditions was found to be primarily determined by the CH2 domain stability. This process appeared to be triggered by CH2 unfolding associated with the protonation of specific acidic residues. The rate and extent of Fc aggregation were shown to be highly dependent on the subclass (IgG2 Fc was less stable than IgG1 Fc) and the degree of CH2 glycosylation. The ionic strength of the solution played an important role in Fc aggregation under acidic conditions.

Acknowledgements—The authors thank Randall Bass, Feng He, Gerald W. Becker, Linda O. Narhi, Michael J. Treuheit, and David N. Brems for general support, technical assistance, and helpful discussions. They also thank Gerd R. Kleemann for help with the IgG schematic and Prof. Melanie Cocco (University of California, Irvine, CA) for access to the UCI NMR facility.

REFERENCES

TABLE 1

Fc residues that lost their native amide resonances upon pH reduction

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<td>V412&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>all&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup> Relative to the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum acquired at pH 4.7 (Figure 2A).

<sup>b</sup> Disappearance of native C_H3 resonances at pH 3.5 and 3.1 may not necessarily reflect C_H3 unfolding. It may also result from peak overlap with resonances originating from denatured C_H2.

<sup>c</sup> Disappearance of the native resonance from this residue is uncertain because of peak overlap.

<sup>d</sup> This residue resides between C_H2 and C_H3 domains.

<sup>e</sup> Since native resonance assignments for the hinge residues were incomplete (see text), implications of C_H2/C_H3 denaturation on the hinge conformation are not fully understood.
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100 mM sodium acetate solutions were prepared from sodium acetate salt as opposed to acetic acid titrated with NaOH.

b Measured pH.
c pH 7 samples were prepared in 100 mM sodium phosphate (100NaPi70).
d Melting transition was not detected or well defined.
**TABLE 3**
Sequences of the analyzed Fc fragments

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<tr>
<td>IgG2 Fc</td>
<td>375 SDAVEWSNGQPENNYKTTPVLDSDGSSFLYSLKTVDKSRWQQGNVFS</td>
</tr>
<tr>
<td>IgG1 Fc</td>
<td>425 CSVMHEALHNHYTQKSLSLPGK</td>
</tr>
<tr>
<td>IgG1-B Fc</td>
<td>425 CSVMHEALHNHYTQKSLSLPGK</td>
</tr>
<tr>
<td>IgG2 Fc</td>
<td>425 CSVMHEALHNHYTQKSLSLPGK</td>
</tr>
</tbody>
</table>

\(^a\) Residue numbering is listed in Eu format (44).  
\(^b\) The N-terminus of this recombinant Fc contained an additional Met residue.  
\(^c\) Since this Fc was derived from IgG1-B via Lys-C proteolysis (see Experimental Procedures), its N-termini were determined by the location of the Lys-C cleavage site (37).
FIGURE LEGENDS

Figure 1. Schematic diagram of an IgG molecule. As indicated by the dashed line, an IgG structure consists of one Fc and two identical Fab regions. Thin blue lines represent intra- and inter-chain disulfide bonds. Structure of a carbohydrate unit attached to Asn297 of the C2 domain is shown separately. Abbreviations used: LC, Light Chain; HC, Heavy Chain; G, galactose; GN, N-acetylglucosamine; F, fucose; M, mannose.

Figure 2. $^1$H-$^{15}$N HSQC spectra of the uniformly $^2$H, $^{15}$N-labeled E. coli-derived IgG1 Fc at pH 4.7 (A), 3.5 (B), 3.1 (C), and 2.5 (D). The spectra were recorded at 25 °C.

Figure 3. The weighted average chemical shift changes of the uniformly $^2$H, $^{15}$N-labeled E. coli-derived IgG1 Fc. The graphs show residue-specific perturbations induced by a pH reduction from 4.7 to 3.5 (A) and from 3.5 to 3.1 (B). The location of the hinge region, as well as the C$_{13}$/C$_{13}$ domains is indicated in panel B. Residue numbering is listed in Eu format (44).

Figure 4. Real-time aggregation of a protein mixture composed of three full-length mAbs (IgG1-A, IgG2-B, and IgG2-C) and the E. coli-derived IgG1 Fc at 30 °C. Shown are CEX overlays for samples incubated in 100 A37N (A) or 100 A34 (B). The green, blue, and red traces correspond to 0, 4, and 21 h time points, respectively. The black traces in (A) and (B) are the 100 A52N and 100 A52 controls, respectively. Protein monomers eluted between 17-39 min, whereas the majority of aggregates eluted at 41-42 min, corresponding to the column washing step with 1 M salt (see Experimental Procedures). The Fc concentration in these experiments was 1.5 mg/mL. All other proteins were at 0.5 mg/mL.

Figure 5. Aggregation kinetics of a protein mixture composed of three full-length mAbs (IgG1-A, IgG2-B, and IgG2-C) and the E. coli-derived IgG1 Fc at 30 °C. Shown are CEX overlays for samples incubated in 100 A37-N (A) or 100 A34 (B). The green, blue, and red traces correspond to 0, 4, and 21 h time points, respectively. The black traces in (A) and (B) are the 100 A52N and 100 A52 controls, respectively. Protein monomers eluted between 17-39 min, whereas the majority of aggregates eluted at 41-42 min, corresponding to the column washing step with 1 M salt (see Experimental Procedures). The Fc concentration in these experiments was 1.5 mg/mL. All other proteins were at 0.5 mg/mL.

Figure 6. Real-time aggregation of the E. coli-derived IgG1 Fc, CHO-derived IgG1 Fc, and CHO-derived IgG2 Fc at 30 °C. (A) CEX overlays where the pink, green, blue, and red traces correspond to 0, 2, 7.5, and 21.5 h time points in 100 A35N. The black trace is pH 5.2 control. The Fc sample heterogeneity resolved by CEX resulted in appearance of many minor species. The CHO-derived IgG2 Fc species eluted between 16-23 min, whereas the CHO- and E. coli-derived IgG1 Fc species eluted between 23-29 min and 29-34 min, respectively (see Experimental Procedures and Supplemental Figures S1-S3 for details). Aggregation kinetics in 100 A35N (B) and 100 A31N (C) are expressed in % recovery of Fc monomer relative to pH 5.2 control. Closed, gray, and open circles correspond to the CHO-derived IgG1 Fc, E. coli-derived IgG1 Fc, and CHO-derived IgG2 Fc, respectively. The error bars were calculated from the standard deviation of replicate injections over several runs. The CHO-derived IgG1 Fc represented the most stable Fc in these experiments.

Figure 7. Real-time aggregation of glycosylated (Peak 1), partially deglycosylated (Peak 2), and fully deglycosylated (Peak 3) IgG1-B Fc in 100 A31N at 30 °C. (A) CEX overlays where the green, blue, and red traces correspond to 0, 2.5, and 4.5 h time points, respectively. The black trace is pH 5.2 control. (B) Aggregation kinetics expressed in % recovery of Fc monomer relative to pH 5.2 control. The error bars were calculated from the standard deviation of replicate injections over several runs.

Figure 8. DSC thermograms of the E. coli-derived IgG1 Fc, CHO-derived IgG1 Fc, and CHO-derived IgG2 Fc in 100 NaPi70 (A), 100 A52 (B), 100 A45 (C), 100 A40 (D), 100 A35 (E), and 100 A30 (F).
Experimentally measured pH of these samples is listed in Table 2. Note that in panel F the *E. coli*-derived IgG1 Fc was at pH 3.3, whereas both of the *CHO*-derived Fc were at pH 3.0.

Figure 9. **Apparent T_m versus pH profiles for the *E. coli*-derived IgG1 Fc, *CHO*-derived IgG1 Fc, and *CHO*-derived IgG2 Fc.** (A) An overlay of the *E. coli*-derived IgG1 Fc (black symbols and solid lines) and *CHO*-derived IgG1 Fc (open symbols and dashed lines). (B) An overlay of the *CHO*-derived IgG2 Fc (gray symbols and solid lines) and *CHO*-derived IgG1 Fc (open symbols and dashed lines). (C) An overlay of the *E. coli*-derived IgG1 Fc (black symbols and solid lines) and *CHO*-derived IgG2 Fc (gray symbols and solid lines). Circles, triangles, and squares correspond to the C_{H2}, C_{H3}, and A-state transitions, respectively.

Figure 10. **Ribbon diagrams of IgG1 Fc (PDB entry 1HZH (49)) illustrating the location of structural perturbations at pH 3.5 (A, B) and 3.1 (C, D).** In all cases the hinge region is on the top, and the C_{H3} domains are at the bottom. In B and D the molecule is rotated by 90° along the vertical axis parallel to the plane of the page. Unperturbed residue positions for which resonance data is available are colored blue. Residues colored red undergo weighted average chemical shift changes equal or exceeding 0.05 ppm (see the text and Figure 3). Positions colored magenta correspond to residues that lost their native resonances (see the text and Table 1). Residue positions for which resonance data is not available are shown in gray. The figures were generated using PyMOL (DeLano Scientific LLC, South San Francisco, CA).
Figure 1
Figure 3

(A) pH 3.5-4.7

(B) pH 3.1-3.5

Weighted Average Chemical Shift Change (ppm)

Residue Number
Figure 4
Figure 5
Figure 6

A

B

C

TIME, h

0 20 40 60 80 100 120

% soluble monomer

CHO IgG1 Fc
E. coli IgG1 Fc
CHO IgG2 Fc

Elution time, min

16 20 24 28 32 42

UV Abs (280 nm)

0 10 20 30 40 50 60 70 100 200

Aggregates

B

% soluble monomer

0 20 40 60 80 100

CHO IgG1 Fc
E. coli IgG1 Fc
CHO IgG2 Fc

Time, h

0 20 40 60 80 100 120

C

% soluble monomer

0 20 40 60 80 100

CHO IgG1 Fc
E. coli IgG1 Fc
CHO IgG2 Fc

Time, h

0 20 40 60 80 100 120
Figure 7

A

Elution time, min

UV Abs (280 nm)

28 29 30 31 32 33 34 41 42 43 44 45

0 10 20 30 40 50

Peak 1

Peak 2

Peak 3

Aggregates

B

Time, h

% soluble monomer

0 10 20 30 40 50 60 80 100

Peak 1

Peak 2

Peak 3
Figure 8

A, B, C: Graphs showing the change in heat capacity ($C_p$) with temperature for different IgG Fc forms in E. coli and CHO. The graphs illustrate the stability and conformational changes at various temperatures.

D, E, F: Graphs displaying the $C_p$ for E. coli IgG1 Fc, CHO IgG1 Fc, and CHO IgG2 Fc at different temperatures, highlighting the thermal stability of each form.

E. coli IgG1 Fc
CHO IgG1 Fc
CHO IgG2 Fc
Figure 9

A

B

C

pH

T_{m}, ^{\circ}C

3 4 5 6 7

10

30

50

70

90

110

10

30

50

70

90

110

3 4 5 6 7

10

30

50

70

90

110

3 4 5 6 7

10

30

50

70

90

110

3 4 5 6 7

10

30

50

70

90

110

A-state (CHO IgG1 Fc)

CH3 (CHO IgG1 Fc)

CH2 (CHO IgG1 Fc)

CH3 (E. coli IgG1 Fc)

CH2 (E. coli IgG1 Fc)

A-state (E. coli IgG1 Fc)

A-state (CHO IgG2 Fc)

A-state (E. coli IgG2 Fc)

A-state (CHO IgG2 Fc)

A-state (E. coli IgG2 Fc)

A-state (CHO IgG2 Fc)
Elucidation of acid-induced unfolding and aggregation of human immunoglobulin IgG1 and IgG2 Fc
Ramil F. Latypov, Sabine Hogan, Hollis Lau, Himanshu Gadgil and Dingjiang Liu
J. Biol. Chem. published online November 14, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.297697

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