Correlations between changes in conformational dynamics and physical stability in a mutant IgG1 mAb engineered for extended serum half-life

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Abbreviations: DSC, differential scanning calorimetry; HX-MS, hydrogen/deuterium exchange mass spectrometry; Fab, antigen binding fragment of a monoclonal antibody; Fc, crystallizable fragment of a monoclonal antibody; HC, heavy chain of a monoclonal antibody; LC, light chain of a monoclonal antibody; CH1-CH3, constant domains 1–3, respectively, of the heavy chain of a monoclonal antibody; VH/VL, variable domain of the heavy/light chain of a monoclonal antibody

This study compares the local conformational dynamics and physical stability of an IgG1 mAb (mAb-A) with its corresponding YTE (M255Y/S257T/T259E) mutant (mAb-E), which was engineered for extended half-life in vivo. Structural dynamics was measured using hydrogen/deuterium (H/D) exchange mass spectrometry while protein stability was measured with differential scanning calorimetry (DSC) and size exclusion chromatography (SEC). The YTE mutation induced differences in H/D exchange kinetics at both pH 6.0 and 7.4. Segments covering the YTE mutation sites and the FcRn binding epitopes showed either subtle or no observable differences in local flexibility. Surprisingly, several adjacent segments in the Cε2 and distant segments in the V\text{H}ε, C\text{H}1, and V\text{L} domains had significantly increased flexibility in the YTE mutant. Most notable among the observed differences is increased flexibility of the 244–254 segment of the C\text{H}2 domain, where increased flexibility has been shown previously to correlate with decreased conformational stability and increased aggregation propensity in other IgG1 mAbs (e.g., presence of destabilizing additives as well as upon de-glycosylation or methionine oxidation). DSC analysis showed decreases in both thermal onset (T\text{onset}) and unfolding (T\text{m}1) temperatures of 7°C and 6.7°C, respectively, for the C\text{H}2 domain of the YTE mutant. In addition, mAb-E aggregated faster than mAb-A under accelerated stability conditions as measured by SEC analysis. Hence, the relatively lower physical stability of the YTE mutant correlates with increased local flexibility of the 244–254 segment, providing a site-directed mutant example that this segment of the C\text{H}2 domain is an aggregation hot spot in IgG1 mAbs.

Introduction

The IgG1 framework is the most widely used platform for designing and developing therapeutic monoclonal antibodies (mAbs) for treatment of cancer, autoimmune and infectious diseases.1,2 The Fc region of IgG1 mAbs, whose sequence is primarily dictated by antibody subtype and host, serves a variety of biological functions, the most important of which include binding to specific Fcγ receptors to trigger various immunological events, (e.g., complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC), opsonization),3,5 and determining pharmacokinetic (pK) properties of the mAb through interaction with FcRn receptors.6 Recent work has also implicated the Fc region in reducing mobility of infectious viruses in the mucosa after antibody binding.7 These biological properties are often mediated by the glycosylation profile of the N-linked Asn 297 residue within the C\text{H}2 domain of the IgG1-Fc region.8 Thus, engineering specific sequences to improve the therapeutic utility of IgG1 mAbs is not limited to the amino acid residues of the complementarity-determining regions (CDRs) in the Fab, to enhance antigen binding specificity and affinity.9 The constant regions in the Fc can also be engineered to improve mAb-based therapeutics, including better engagement of the immune system10,11 and extension of the circulation half-life.12,14

Increasing the serum half-life of mAbs is an attractive proposition because it potentially results in changes in their
pharmacokinetic/pharmacodynamic profiles, as well as decreased dosing frequency leading to higher patient compliance.\textsuperscript{15} The serum half-life of IgG1 antibodies is known to be regulated by the neonatal Fc receptor (FcRn) located primarily in the acidic endosomes of endothelial and haematopoietic cells.\textsuperscript{16,17} Free or antigen-bound antibodies enter these cells through pinocytosis or endocytosis, respectively, and are then transported to the endosomes. FcRn interaction then marks the antibodies for either recycling or lysosomal degradation. The recycling mechanism is based on selective binding of IgG to the FcRn receptor in the relatively more acidic (pH <6.5) environment of endosomes, followed by release from the receptor upon exposure to the more basic (pH \textasciitilde 7.4) milieu of the bloodstream.\textsuperscript{18} The binding sites of the Fc region of an antibody to the FcRn receptor, as well as the amino acid residues responsible for the pH-dependent recycling, have been identified and are well documented for human IgG\textsubscript{2,19-21} The pH-dependence of this binding within acidic endosomes is driven by positively-charged histidine residues in human IgG1 antibodies, specifically His 310 and His 436 at pH 6.5 or below, that form salt bridges with negatively-charged Glu 117 and Asp 137 residues of FcRn.\textsuperscript{18,19,21} Under physiological conditions (pH \textasciitilde 7.4) of the bloodstream, however, these histidine residues are no longer charged and hence there is little histidine-mediated binding between the IgG-Fc region and the FcRn receptor.

The recycling machinery has been widely targeted through random and specific mutations of the IgG primary sequence to increase or decrease the affinity for binding to FcRn and thereby extend or reduce the circulation half-life of IgG-based therapeutics.\textsuperscript{14,22-28} For example, the increased FcRn binding at pH 6.0 by a YTE triple-mutant mAb is mediated by the creation of one additional salt bridge between Glu 256 (E) of Fc-YTE and Gln 2 (Q) of the β2-microglobulin chain of FcRn compared to the original IgG1 Fc structure.\textsuperscript{29} The YTE mutations (M252Y/S254T/T256E) in the CH2 domain of an IgG1 mAb resulted in a nearly four-fold higher in vivo half-life in cynomolgus monkeys, the largest increase in IgG mAb half-life reported to-date for non-human primates.\textsuperscript{23} The YTE triple mutation also resulted in ten-fold higher FcRn binding, four-fold higher tissue bioavailability, and modulated ADCC in non-human primates.\textsuperscript{23} In a recent human clinical trial, the YTE mutant of an IgG1 mAb that targeted respiratory syncytial virus (motavizumab) had two-to four-fold longer in vivo half-life compared to the original mAb.\textsuperscript{15} The X-ray crystal structures of the original human IgG1 Fc (PDB ID 3AVE) and the Fc-YTE (PDB ID 3FJT) mutant reveal no apparent structural differences between the 2 molecules.\textsuperscript{14,30} A recent study, however, suggested that the YTE mutant has relatively lower physical stability in solution than the same mAb without the mutations.\textsuperscript{31} One possibility is that these stability differences are mediated by changes in structural dynamics of specific sequences in the mAbs due to the YTE mutations. Although the interrelationships between dynamics and stability of proteins are complex,\textsuperscript{32} growing evidence suggests that physical destabilization of mAbs can, in at least some cases, be mediated by changes in local dynamics of specific sequences.\textsuperscript{33,34}

Hydrogen/deuterium exchange coupled to mass spectrometry (H/D-MS) is now a well-established technique to explore the local dynamics of the amide backbone of mAbs with peptide-level resolution.\textsuperscript{35,36} H/D-MS has been used to study changes in mAb conformational dynamics imparted by stabilizing and destabilizing excipients\textsuperscript{34} and salts,\textsuperscript{33} mutation in the IgG1 CH3 domain,\textsuperscript{37} deglycosylation,\textsuperscript{35} chemical and post-translational modifications,\textsuperscript{38,39} thermal and freeze-thaw stress,\textsuperscript{40} and cytotoxic drug conjugation\textsuperscript{41} in IgG mAbs.

The work reported here investigated the nature of the relative differences in physical stability between an IgG1 mAb (mAb-A) and its corresponding YTE-mutant (mAb-E) by examining effects of the YTE-mutations on amide backbone local dynamics measured by amide H/D exchange, conformational stability measured by differential scanning calorimetry (DSC), and aggregation propensity measured by size exclusion chromatography (SEC). Since solution pH plays a very important role in the interaction of antibodies with FcRn receptors (i.e., binding at pH 6.0 and release at pH 7.4), we explored differences in flexibility and stability between the 2 mAbs at both pH values. We then correlated the observed changes in physical stability to changes observed in the amide backbone dynamics due to the YTE mutations.

**Results**

**YTE mutation decreased the conformational stability of only the CH2 domain**

DSC was used to examine the conformational stability of mAb-A and mAb-E (YTE-mutant of mAb-A). Representative DSC thermograms comparing the 2 mAbs are shown in Figure 1. Both the mAbs display 3 thermal melting (T\textsubscript{m}) transitions at both pH 6.0 and 7.4. The T\textsubscript{m1} value (Fig. 1) has been shown previously to represent the thermal unfolding of the CH12 domain.\textsuperscript{42} The T\textsubscript{m2} and T\textsubscript{m3} values (Fig. 1) have been shown to represent the unfolding of either the Fab or the CH13 domain depending on the antibody and solution conditions.\textsuperscript{32,43} In addition, the T\textsubscript{onset} values, marking the temperature at which the first structural transition initiates (an important value for formulation stability considerations),\textsuperscript{44} were also determined. A summary of the 3 T\textsubscript{m} and T\textsubscript{onset} values are listed in Table 1. The YTE mutation decreased T\textsubscript{m1} values by 6.5°C and 6.7°C, at pH 6.0 and 7.4, respectively, and decreased T\textsubscript{onset} values by \textasciitilde 7°C at both pH values. The T\textsubscript{m2} and T\textsubscript{m3} values were not affected by the YTE mutation. Comparing the effect of pH 6.0 to 7.4 on protein conformational stability as measured by DSC, both mAbs had similar values for T\textsubscript{m1} and T\textsubscript{m2} at both pH values. However, both mAb-A and mAb-E had \textasciitilde 4.7°C higher values for T\textsubscript{m3} and \textasciitilde 1.4°C lower T\textsubscript{onset} values at pH 6.0 compared to 7.4. In summary, the YTE mutation decreased conformational stability in the CH12 domain while the other domains in the 2 mAbs were similar in terms of their thermal stability.

**YTE mutation increased the aggregation propensity of mAb-E under accelerated storage conditions**

The aggregation propensities of the 2 mAbs at pH 6.0 and 7.4 under accelerated storage conditions were measured by SEC at
50°C over 28 d. **Figure 2A** shows the loss of IgG monomer and formation of fragments, soluble, and insoluble aggregates for the mAb samples. Peaks in the SEC chromatograms were assigned based on molecular weight as described previously. Both mAbs had greater than 99% monomer content at time zero and small amounts of fragments and soluble aggregates. Quantitation of the various mAb species after storage was determined relative to the respective peak areas at time 0. Loss in total integrated area of the chromatogram was attributed to formation of insoluble aggregates. The YTE mutation caused faster monomer loss at 50°C at both pH 6.0 and 7.4, but had no effect on the rate of fragment formation. In addition, the YTE mutant produced higher amounts of soluble aggregates at either pH value, which became evident after 14 d at 50°C. Although minimal amounts were observed until 14 d, the YTE mutant (mAb-E) also produced significantly higher amounts of insoluble aggregates (≈9–11%) compared to mAb-A (≈4–5%) after storage for 28 d at 50°C. To better display these differences in soluble, insoluble, and total aggregate content after incubation for 28 d, the data are shown as bar charts in **Figure 2B** and 2C, respectively. Both mAbs produced higher amounts of insoluble aggregates than soluble aggregates after 28 d storage. At both pH 6.0 and 7.4, the YTE mutant produced higher amounts of soluble and insoluble aggregates. The total aggregate content for the YTE mutant after 28 d of storage is roughly 2-fold higher than the original mAb at both pH values.

**Identifying the effects of YTE mutation on the backbone flexibility**

Amide H/D exchange with mass spectrometric detection was used to measure the effects of the YTE mutation on protein amide backbone flexibility. As an initial step in the analysis of both mAbs by H/D-exchange mass spectrometry, a peptic peptide map was developed. A total of 167 common peptides were reproducibly obtained by pepsin digestion of both mAb-A and mAb-E, resulting in 92% and 96% sequence coverage in the light chain and heavy chain, respectively, as shown in Figure S1. The shapes of the different deuterium uptake profiles illustrate the different degrees of flexibility in different regions of the mAb. Similar deuterium uptake profiles for all 167 common peptides found in both mAb-A and mAb-E at pH 6.0 and 7.4 are presented in Figures S2 and S3. Differences in deuterium uptake between mAb-A and mAb-E at various exchange times were found in 4 of
the 6 peptides shown in Fig. 3 (HC 244–254, HC 255–264, HC 320–332, and LC 179–194) at both pH 6.0 and 7.4. However, not all of these differences exceed the 99% confidence interval for significance (see below). The segment locations in the primary sequence of mAb-A or mAb-E corresponding to peptide numbers are tabulated in Table S1.

To more clearly display the effects of the YTE mutation on deuterium uptake for all peptide segments, the data are presented as differential deuterium uptake as described previously. The differential deuterium uptake, i.e., $\Delta \Delta m(t) = \Delta m_{\text{mAb-E}}(t) - \Delta m_{\text{mAb-A}}(t)$ where $\Delta m$ represents the measured mass increase upon deuteration for each peptide, is plotted as a vertical bar (Fig. 4 for pH 6.0 and Fig. 5 for pH 7.4) and $t$ denotes the labeling time. A positive value for $\Delta \Delta m$ indicates that the YTE mutation caused faster exchange, corresponding to increased flexibility, while a negative value indicates slower exchange, corresponding to decreased flexibility. The dotted lines denote the 99% confidence interval ($\pm 0.59$ Da) for a statistically significant difference in exchange. The 99% confidence interval was determined by pooling all standard deviation values for triplicate measurements in our earlier studies with another IgG1 mAb33,34 and this study. Differential exchange that exceeds the 99% confidence interval is mapped onto a homology model for the mAb in Figure 6A, and in more detail for the Fc portion only in Figure 6B. The YTE mutation-induced increases in local flexibility are colored in yellow, decreases in blue, and no changes in gray. The absence of H/D exchange data is shown in white. The H/D exchange results are discussed in detail in the next section.

The YTE mutation caused localized increases in backbone flexibility at pH 6.0

At pH 6.0, nearly all of the significant differences in exchange were positive and localized to narrow regions, indicating that the YTE mutation increases flexibility in only a limited number of segments (see Fig. 4). The highest positive deuterium uptake differences were observed in the HC 244–255 region of the C_{H2} domain at $10^3$ s followed by 180 s, a region covered by the overlapping peptides HC 238–254, HC 244–254, HC 244–255, HC 245–254, HC 247–254 and HC 247–255 (corresponding to peptide numbers 52, 54–58 in Fig. 4). The mutation also

Figure 2. Accelerated storage stability of mAb-A and mAb-E after incubation at 50 °C for up to 28 d as measured by SEC. The mAbs were formulated in 50 mM sodium phosphate and 150 mM sodium chloride at pH 6.0 and 7.4. (A) The percentage of monomer, fragments, soluble aggregates, and insoluble aggregates of mAb-A and mAb-E analyzed by SEC after incubation at 50 °C for 0, 2, 4, 7, 14, and 28 d relative to day zero. Error bars represent one standard deviation for duplicate analyses of duplicate vials. Bar charts representing the percentage of soluble, insoluble, and total aggregate content in mAb-A and mAb-E after incubation for 28 d at 50 °C are shown at (B) pH 6.0 and at (C) pH 7.4.
caused 2 other segments in the C11-1 domain (HC 174–188 corresponding to peptide 42 in Fig. 4) and the C12-2/C13 interface (HC 320–332 corresponding to peptide 81 in Fig. 4) to exchange significantly faster. In addition, the YTE mutation caused several other segments (HC 28–46, HC 144–168, HC 159–177, and LC 147–174 corresponding to peptides 12, 36, 40, and 159 in Fig. 4) to exchange faster than mAb-A. Only one segment, LC 179–194 (corresponding to peptide 165 in Fig. 4), exchanged more slowly in the YTE-mutant.

Mutation-induced differences in local flexibility at pH 7.4 were similar to differences at pH 6.0

Deuterium uptake by the representative peptides was faster at pH 7.4 than at pH 6.0 (see Figure 3A vs. 3B), as expected from the known pH dependence of intrinsic deuterium exchange.45 Looking across all of the peptides in Figure 5, it can be seen that most of the differences in deuterium uptake were at early exchange times (30s and 180s) at pH 7.4 rather than later times (180s and 10^3 s) at pH 6.0. The shift arises from faster intrinsic exchange at pH 7.4.45 Essentially all of the peptide segments that had significant differences in deuterium uptake at pH 6.0 also had significant differences at pH 7.4 (Figs. 4 and 5), with only 2 exceptions noted. One segment in the C1 domain (LC 147–174 corresponding to peptide 159 in Figs. 4 and 5) had increased deuteration at 10^3 s in the mutant mAb-E at pH 6.0, but not at pH 7.4. Another segment in the C12-2 domain (HC 269–280 corresponding to peptide 68 in Figs. 4 and 5) had increased deuteration at 10^3 s at pH 7.4, but not at pH 6.0.

Backbone flexibility of the peptide segments containing the YTE mutation

Three overlapping peptide segments covering the YTE mutation sites were detected in the peptic peptide maps of both mAb-A and mAb-E. The H/D exchange kinetics of one of the 3 segments (HC 255–264) is shown in Figure 3, while data for the 2 others are shown in Supplemental Figures S2 and S3. The point mutations resulted in trends showing slightly faster deuterium exchange in these 3 segments in mAb-E, but observed differences in deuterium uptake at every exchange time were less than the 99% confidence interval for a significant difference. Under typical exchange conditions (i.e., the EX2 kinetic limit), the kinetics of exchange depend on both backbone dynamics and on intrinsic exchange.47,48 When comparing homologous backbone segments with different amino acid sequences, the rate of intrinsic exchange must be considered. We used tabulated values for intrinsic exchange45,46 to calculate the intrinsic exchange by these segments at pD 6, as shown in Figure 7. All 3 segments in mAb-A have slightly faster intrinsic exchange compared to mAb-E. The differences in the calculated intrinsic exchange rates oppose the trend observed experimentally in the 2 mAbs. Therefore, the differences in backbone flexibility in the mutation region are likely somewhat larger than they appear in Figure 3. Given the complex, multi-exponential nature of H/D exchange kinetics, no suitable method has yet been developed to directly correct the experimental results for these differences. It remains possible that the small differences in backbone flexibility induced by the YTE mutation at the mutation sites would be statistically significant if they could be corrected for intrinsic exchange effects.

**Discussion**

Previous work has shown that the YTE mutations in the C12-2 domain of IgG1 mAbs resulted in ten-fold higher FcRn binding and four-fold longer serum half-life in animal models.23 In-house
formulation work (data not shown) and characterization stud-
ies,31 however, have shown a relative decrease in physical stability
of various mAbs containing the YTE mutations. The purpose of
this study was to probe the potential mechanisms of this
destabilization by examining the YTE mutation-induced changes
in backbone flexibility of an IgG1 mAb and to correlate results to
DSC and SEC analysis of physical stability. To accomplish this
purpose, 2 IgG1 mAbs were generated with identical primary
sequences in the Fab domains, one molecule containing the YTE
triple mutations in the Fc domain (mAb-E), while the other mol-
ecule had the native IgG1 sequence for the Fc domain (mAb-A).
The N-linked glycosylation patterns in the Fc region of mAb-A
and mAb-E were overall similar, containing primarily G0F and
G1F oligosaccharides (see Figure S4 and Table S2).

DSC is a robust and widely utilized technique to assess the
thermal stability of mAbs.49 The endothermic unfolding transi-
tions have been found to be independent of each other and gener-
ally irreversible for mAbs.50 In the case of 3 distinct transitions,
the peaks can be attributed to unfolding of the C_{H2} domain fol-
lowed by the Fab, and then the C_{H3} domain in order of increasing
melting temperatures.42 Although both mAbs displayed high
thermal stability as measured by DSC, typical for IgG molecules,
the mutant mAb-E had relatively lower T_{m1} values at both pH
6.0 and 7.4 (Fig. 1 and Table 1). The YTE mutant had ~7°C
lower T_{onset} value and ~6.7°C lower T_{m1} value compared to
mAb-A. There were no differences in the other thermal melting
transition temperatures (T_{m2} and T_{m3}) between mAb-A and
mAb-E. In addition, accelerated stability studies monitoring
mAb dimer and multimer formation by SEC analysis showed
that the YTE mutant has a higher aggregation propensity
(Fig. 2). Despite these observed relative differences in physical
stability, the YTE molecules can be formulated as biopharma-
ceuticals to be sufficiently stable during manufacturing and long-
term storage by appropriate design of formulation conditions,
including solution pH, excipients, and dosage form selection.

An examination of the X-ray crystal structures of Fc-YTE of
mAb-E (PDB ID 3FJT) and the Fc of mAb-A (PDB ID 3AVE)
indicated that there is little structural difference between the
2.14,30 However several adjacent as well as distant segments from
the mutation site in the C_{H2} domain were shown in the current
work to have increased flexibility due to the YTE mutations.
Earlier reports indicated that single as well as concerted mutations
far from the Fc binding epitopes in the Fc region enhanced the

Figure 4. Differential deuterium uptake at 5 exposure times for 167 com-
mon peptide segments of mAb-E relative to mAb-A at pH 6.0. Average
mass corresponding to each exposure time for each segment is calcu-
lated based on 3 independent measurements. The horizontal axes
denote the ordinal peptide numbers assigned based on peptide mid-
points spread in ascending order from the N-to-C termini of the heavy
chain followed by the light chain of mAb-A or mAb-E. Positive and nega-
tive vertical bars imply increased or decreased deuterium uptake, respec-
tively, for a segment in mAb-E compared to mAb-A. The dashed lines at
±0.59 Da indicate the 99% confidence limits for significant differences.
Domain locations shown on the top of the figure are approximate as
some segments span 2 neighboring domains. Alternate shades of white
and gray in the background demarcate domain boundaries. Shades in
pink demarcate segments with residues of Fc epitopes of Fc-FcRn bind-
ing interfaces. The red circles denote segments that contain the YTE
mutations. Segment locations in the mAb-A/mAb-E sequence corre-
sponding to peptide numbers can be found in Table S1 in the support-
ing information.
Figure 5. Differential deuterium uptake at 5 exposure times for 167 common peptide segments of mAb-E relative to mAb-A at pH 7.4. Refer to the legend for Figure 4 for description of the figure.

Figure 6. Effect of the YTE mutation (mAb-E) on the local flexibility of the native mAb (mAb-A) at pH 6.0 as measured by H/D-MS plotted on the homology models of (A) intact mAb-A and (B) Fc domain of mAb-A. Changes in flexibility of particular peptide segments are colored as shown in the legend and are derived from the differential exchange data shown in Figure 4.
IgG1/FcRn binding.\textsuperscript{25,27} Using the residue numbering scheme of mAb-A or mAb-E, these mutations are P233S/T, F244L, T253Q, V267A/E, N318D, A333V, N364D, A381V/T, M431L.\textsuperscript{25,27} Several of these previously reported mutations (e.g., F244L, T253Q, V267A/E, N318D, and A333V) are in the peptide segments (HC 244–255, HC 269–280, HC 320–332) that showed increased local flexibility due to the YTE-mutations in our work. Hence, it can be inferred that residues in the segments with increased flexibility in the C\textsubscript{H2} domain and the C\textsubscript{H2}-C\textsubscript{H3} interface may have indirect interactions with the conformation of the 255–259 segment, which is the location for YTE-mutations as well as one of the FcRn binding epitopes of Fc.

Additional segments in the Fab region (HC 28–46, HC 144–168, HC 159–177, HC 174–188, and LC 147–174 corresponding to peptide 9, 36, 40, 42 and 158 in Figs. 4 and 5) had increased flexibility in the YTE mutant and the LC 179–194 had decreased flexibility. It is surprising that these segments with increased flexibility in different domains. Molecular dynamics simulations have shown that the 2 arms of the Fab can have polar and non-specific interactions with regions of the Fc not far from the FcRn binding sites.\textsuperscript{51} Furthermore, recent studies have shown that mAbs with identical Fc but differing Fab sequences,\textsuperscript{52} as well as mAbs and Fc fusion proteins with the same Fc sequences,\textsuperscript{33} can bind FcRn differentially with varying pK properties. The H/D exchange data in our study further support the notion that certain segments of the F\textsubscript{ab} sequence may have some yet-to-be-defined indirect interactions with the 255–259 region in the C\textsubscript{H2} domain of the Fc, one of the FcRn binding epitopes. At the same time, the YTE mutation did not cause any changes in the thermal unfolding temperatures of the F\textsubscript{ab} region measured by DSC (Fig. 1 and Table 1). These results further highlight the complex interrelationships between protein flexibility and conformational stability.\textsuperscript{32}

The YTE mutation increased the flexibility of the 244–255 region of the C\textsubscript{H2} domain at both pH 6.0 and 7.4. This region, covered by 6 overlapping segments (peptide numbers 52, 54–58 in Figs. 4 and 5), had the largest magnitude of increase among all differences detected by H/D-MS. This segment is adjacent to the 255–259 segment containing the YTE mutations. A visual comparison of the deuterium uptake kinetic profiles of segments covering the 244–255 region at pH 6.0 show very similar overall shapes across 3 different IgG1 mAbs (mAbs A and E in this study and mAb-B in previous studies) examined in our laboratories, with the midpoint of deuterium uptake at \(\sim 10^3\) s.\textsuperscript{33,34} Moreover, similar kinetic profiles are evident in H/D-MS studies at pH \(\sim 6\) with other IgG1 mAbs in other laboratories.\textsuperscript{35,38,54} Hence, it can be concluded that this region has similar dynamic behavior across all of these distinct IgG1 mAbs.

Increased flexibility of the 244–255 sequence caused by the YTE mutation correlates with decreased thermal stability of the C\textsubscript{H2} domain and increased aggregation propensity. Significant increases in flexibility of this same segment have been correlated previously in our labs in another IgG1 mAb with arginine and sodium thiocyanate-induced decreases in conformational stability, and concomitant increases in the aggregation propensity.\textsuperscript{33,34} Furthermore, several reports by others have correlated increased flexibility of this same primary sequence segment in the C\textsubscript{H2} domain with methionine oxidation,\textsuperscript{38,39,54} destabilization by deglycosylation,\textsuperscript{35} changes in glycosylation\textsuperscript{38} and even decreased stability due to drug conjugation to free cysteine residues.\textsuperscript{41} The 244–255 segment in the C\textsubscript{H2} domain is relatively apolar in nature, with 2 valine residues and 2 phenylalanine residues, and its phenyl rings pack closely with the glycans in crystal structures.\textsuperscript{30} It is possible that the increased flexibility of the peptide backbone disrupts the packing interactions between the peptide backbone and the glycan chains, leading to unfolding of this hydrophobic segment. This unfolded apolar segment, in turn, may act as a hotspot for subsequent structural alterations that could lead to irreversible aggregation in IgG1 mAbs,\textsuperscript{55–57} for example, as observed by SEC analysis in this study.
Materials and Methods

Materials

Two highly purified IgG1 mAbs, mAb-A and the corresponding YTE (M255Y/S257T/T259E) mutant of the same IgG1, designated mAb-E, were supplied by MedImmune, Gaithersburg, MD. The residue number is slightly different from numbering reported elsewhere14,23 due to the presence of 3 extra amino acids in the sequence of mAb-A/mAb-E. Residue numbers of other IgG1 mAbs mentioned in the Discussion section of this report are adjusted according to the sequence numbering scheme for mAb-A/mAb-E by adding +3 to the residue numbers. Both mAbs were produced in the same cell line and had overall similar N-linked glycosylation patterns in the C_{142} domain containing primarily G0F and G1F oligosaccharides (see Fig. S4 and Table S2). In addition, mAb-E had ~50% C-terminal lysine heterogeneity while mAb-A had little to no C-terminal lysine. The relatively elevated levels of C-terminal lysine heterogeneity of mAb-E was not considered while interpreting our results, as it has been shown previously to have no effect on the pK\_a, stability or conformational dynamics of mAbs.60,61

Both mAb-A and mAb-E showed ~99% purity as measured by SDS-PAGE analysis (data not shown), and greater than 99% monomer content as measured by SEC (see Results). The mAbs were provided at 100 mg/mL and were buffer exchanged into 50 mM sodium phosphate, 150 mM sodium chloride at pH 6.0 or 7.4. The protein concentration was adjusted to 10 mg/mL based on absorbance at 280 nm measured with an Agilent 8453 UV-visible spectrophotometer. Extinction coefficient values of 1.47 (mg/mL)^{-1} cm^{-1} and 1.42 (mg/mL)^{-1} cm^{-1} were used for calculation of concentrations of mAb-A and mAb-E, respectively. Anhydrous sodium phosphate dibasic (code number 42437), and monobasic (code number 389870010) were purchased from Acros Organics. Sodium chloride (product number M-11628), LC-MS grade water, acetonitrile, and isopropanol were purchased from Fisher Scientific. TCEP (Catalog # C4706), guanidine hydrochloride (Catalog # 50933), porcine pepsin, acetic acid, deuterium oxide (99+\%D; catalog number 151882) were purchased from Fluka/Sigma Aldrich. All chemicals used were of the highest purity grade commercially available from these vendors.

Differential scanning calorimetry

DSC experiments and corresponding data analysis were performed as described previously.34 The mAb-A and mAb-E samples were diluted to 0.5 mg/mL in buffers containing 50 mM sodium phosphate and 150 mM sodium chloride, either at pH 6.0 or 7.4, using the appropriate dilution buffer and filtered by 0.22 μm filter before analysis. All analyses were done in triplicate.

Accelerated storage stability study and analysis by size exclusion chromatography

Both mAb-A and mAb-E were prepared at 0.5 mg/mL in 50 mM sodium phosphate, 150 mM sodium chloride at both pH 6.0 and 7.4. The stock mAb formulations were filtered by passing through 0.22 μm filters (Millipore, Billerica, Massachusetts), aliquoted, 0.5 mL each, into separate 3 mL type 1 glass vials (West Pharmaceutical Services, Exton, Pennsylvania), stoppered with rubber stoppers (West Pharmaceutical Services, Exton, Pennsylvania), crimped, and then stored in a 50°C incubator. Sample filtration, aliquoting, and vial sealing were performed in a pre-sanitized laminar flow hood. Samples were taken out of the incubator and analyzed by SEC at days 0, 2, 4, 7, 14, and 28. SEC was performed and data were analyzed in the same manner as described in our earlier work34 with the following exception. Duplicate vials were analyzed 2 times each by SEC for each incubation time and condition. Hence, the error values for the amounts of mAb monomer, soluble aggregates and fragments measured by SEC were derived from 4 different measurements. The error values for insoluble aggregate content were calculated by propagating the variances in the measurements of monomers, soluble aggregates and fragments.

Preparation of deuterated labeling buffers

Calculated weights of anhydrous sodium phosphate monobasic, dibasic and sodium chloride were added to 90 atom % D\_2O to prepare 50 mM sodium phosphate, 150 mM sodium chloride at pH 6.0 and 7.4. (90 atom % D was used for consistency with our previous work33,34). Addition of the chemicals did not result in any appreciable change in volume and hence concentrations of buffer components are accurate to 1% of their reported molarities. The pH values reported are directly from pH meter readings without correction for the deuterium isotope effect on pH measurement.62

Hydrogen/deuterium-exchange mass spectrometry

Sample preparation for hydrogen/deuterium exchange experiments followed by LC-MS analyses were performed as described in detail elsewhere.33 H/D exchange sample preparations were performed using the H/DX PAL robot (LEAP Technologies, Carrboro, NC) equipped with temperature-controlled sample drawers. The mAb samples at 10 mg/mL in 50 mM sodium...
phosphate and 150 mM sodium chloride at either pH 6.0 or 7.4 were diluted 20-fold in 90% deuterated buffer at the same pH at 25°C to initiate deuterium exchange. Exchange reactions were quenched after 30, 180, 103, 104 and 105 s using quench buffer (0.5 M TCEP, 4 M guanidine hydrochloride, and 0.2 M phosphate, pH 2.4) held at 1°C resulting in a final pH of ~2.5. Immediately following quench, samples were digested by passing through an immobilized pepsin column prepared in-house,53,64 desalted and concentrated on a reversed phase trap (Peptide Concentration and Desalting Microtrap; Bruker-Michrom, Auburn, CA, USA), separated on a C-18 reversed phase column and mass analyzed (Agilent 6220 time-of-flight LC-MS system). All the columns and tubing were housed inside a refrigerated compartment maintained at 0°C to minimize back-exchange. Deuterium recovery measured separately using fully deuterated peptides ranged from 64–85%.

Mass spectrometry data analysis
A peptic peptide map shown in Figure S1 covering 96% of the heavy chain and 92% of the light chain was constructed from common peptic peptides generated by digestion of both mAb-A and mAb-E. Peptide identities were confirmed using a combination of accurate mass measurement (±10 ppm) and tandem mass spectrometry data from a time-of-flight mass spectrometer and a linear ion trap mass spectrometer, respectively.65 Three replicate sets of H/D-exchange data were processed using HDExaminer (Sierra Analytics, California). Each set of data was manually curated independently to minimize bias by the analyst. Deuterium uptake data averaging and plotting were achieved using an R script. The 99% confidence interval was calculated by combining all of the standard deviations from earlier studies in our laboratories with another IgG1 mAb33,34 and from this study with mAb-A and mAb-E. The newly calculated value for the 99% confidence levels based on approximately 9600 triplicate measurements, with error propagation for the differential measurement, is the same as our previously-established value of ±0.59 Da. All significant differences in deuterium content between the 2 mAbs were mapped onto the homology model of mAb-A.

Construction of the homology model
The homology model for the F\textsubscript{ab} and hinge regions of mAb-A were produced with the in silico KOL-Padlan structure66 as a template, using Modeller version 9.12.67 The F\textsubscript{c} domain was derived from PDBID: 3AVE,30 which has the same amino acid sequence as mAb-A. Mutations were introduced into this model to represent the YTE residues. Very few changes were observed in the structure after the mutations. Hence, the same homology model was used to represent both mAb-A and mAb-E. Of note, the F\textsubscript{c} domain of YTE has been crystallized (3FJT), and the secondary and tertiary structure folds of 3FJT are highly homologous with 3AVE.14 However, the 3FJT structure contains 2 extra residues not present in mAb-A or mAb-E. Therefore, it was not used as a starting structure. The glycan structures in 3AVE were maintained, containing homodimeric G0F sequences, which represent a high population of carbohydrate species found in mAb-A and mAb-E by intact mass spectrometry measurements (Fig. S4).

Disclosure of Potential Conflicts of Interest
RE, SMB, and HSS are employees of MedImmune. RM is a graduate student and CRM, DDW and DBV are professors at The University of Kansas.

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Supplemental Material
Supplemental data for this article can be accessed on the publisher’s website.

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