**REPORT**

**L^{445}P mutation on heavy chain stabilizes IgG\(_4\) under acidic conditions**

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**ABSTRACT**

IgG\(_4\), a common type of therapeutic antibody, is less stable during manufacturing processes compared with IgG\(_1\). Aggregation and fragmentation are the two main challenges. Here, we report instability of the heavy chain (HC) C-terminal region under acidic conditions, which leads to cleavage and aggregation. Leu\(^{445}\), at the C-terminal region of the HC in IgG\(_4\), plays a critical role in its acid-induced fragmentation and subsequent aggregation. We found that mutating HC C-terminal Leu\(^{445}\) to Pro (the corresponding residue in IgG\(_1\)) in IgG\(_4\)_CDR-X significantly reduces fragmentation and aggregation, while mutating Pro\(^{445}\) to Leu in IgG\(_4\)_CDR-X promotes fragmentation and aggregation. HC C-terminal Gly\(^{446}\) cleavage was observed in low pH citrate buffer and resulted in further fragmentation and aggregation, whereas, glycine buffer can completely inhibit the cleavage and aggregation. It is proposed that cleavages occur through acid-induced hydrolysis under acidic conditions and glycine stabilizes IgG\(_4\) via two main mechanisms: 1) product feedback inhibition of the hydrolysis reaction, and 2) stabilization of protein conformation by direct interaction with the peptide backbone and charged side chains. Experiments using IgG\(_4\) molecules IgG\(_4\)_CDR-Y and IgG\(_4\)_CDR-Z with the same CH domains as IgG\(_4\)_CDR-X, but different complementarity-determining regions (CDRs), indicate that the stability of the HC C-terminal region is also closely related to the sequence of the CDRs. The stability of IgG\(_4\)_CDR-X is significantly improved when binding to its target. Both observations suggest that there are potential interactions between Fab and CH2-CH3 domains, which could be the key factor affecting the stability of IgG antibodies.

**Introduction**

Immunoglobulin (Ig) G is the most abundant in human serum among the five Ig classes: IgA, IgD, IgE, IgM, and IgG. IgGs have been extensively used as therapeutic antibodies. The IgG family is further divided into four subclasses, IgG\(_1\), IgG\(_2\), IgG\(_3\), and IgG\(_4\) as determined by minor differences found in the constant region of their heavy chain amino acid sequences. IgG\(_1\), the predominant Ig serum subclass, is commonly used in therapeutics owing to its potent effector function and excellent stability. IgG\(_4\), however, has emerged as an important subclass for cancer immunotherapeutic antibodies, despite being less stable than IgG\(_1\). Two IgG\(_4\) antibodies, nivolumab and pembrolizumab, have been successfully developed as immune checkpoint modulators broadly used in cancer treatment. The IgG\(_4\) backbone is widely selected in agonist and antagonist therapeutic antibodies in immunotherapy due to its abated effector function. However, IgG\(_4\) is natively unstable and prone to forming aggregates, a major manufacturing difficulty.\(^{1,2}\) Many protein engineering efforts have been made to stabilize IgG\(_4\) molecules, such as S\(^{228}\)P mutation to stabilize the hinge region.\(^{3}\)

Both IgG\(_1\) and IgG\(_4\) are composed of two identical heavy chains (HC \(\gamma\)) and light chains (LC \(\alpha\) or \(\kappa\)). They have similar overall structures and a high degree of sequence homology in both heavy chain and light chain constant regions. Although few in number, the amino acid differences are found across all of the CH domains (Figure 1). Most of the differences are in the hinge region, but there are differences also in the CH2-CH3 domains that modulate effector function.\(^{4,5}\) Antigen-binding fragment (Fab)-arm exchange in IgG\(_4\) has been observed in \(vivo\), and is promoted by the Ser\(^{228}\) residue in the hinge region\(^{6}\) and the Arg\(^{409}\) residue at the noncovalent CH3-CH3 interface.\(^{7}\) To prohibit the Fab-arm exchange observed in natural IgG\(_4\), a mutation of Ser\(^{228}\) to Pro (the corresponding residue found in IgG\(_1\)) in the hinge region is adopted in recombinant therapeutic IgG\(_4\).\(^{8}\) Another major difference between IgG\(_1\) and IgG\(_4\) is the altered FG loop structure in the CH2 of IgG\(_4\), which is mainly induced by Ser replacing Pro\(^{331}\) in IgG\(_1\). In IgG\(_4\) this leads to reduced effector function because the FG loop cannot interact with the Fc receptors. The third major difference between IgG\(_1\) and IgG\(_4\) is the location of the HC and LC attachment. In IgG\(_1\), the LC is attached to the fifth Cys of HC, while in IgG\(_4\) attachment occurs at the third Cys. Although these structural and sequence differences are subtle,\(^{9}\) studies have shown that IgG\(_4\)S more likely undergo aggregation\(^{5,9}\) and are more susceptible to acid-induced aggregation\(^{10}\) and thermal denaturation\(^{11}\) than IgG\(_1\). IgG\(_4\) is ranked as the least stable...
antibody by intrinsic propensity of acid-induced aggregation of IgG subclasses. IgG1 < IgG2 < IgG4.\textsuperscript{11}

Alignment of the constant regions of IgG1 and IgG4 shows (Figure 1, EU numbering) there are a total of 24 residue differences distributed throughout the CH1 (seven residues), hinge region (six residues), and the CH2 and CH3 domains (11 residues total). The impact of these Fc region residues on IgG4 stability has been studied extensively by point mutating the IgG4 residues to that of a corresponding residue in the IgG1 backbone.\textsuperscript{3} In addition to the hinge region, the instability of IgG4 is mainly considered to be caused by hydrophobic motifs located in the CH2 domain.\textsuperscript{4} Phe\textsuperscript{396} in IgG4, equivalent to Tyr\textsuperscript{296} in IgG1, is believed to contribute to the high tendency of aggregation in IgG4. The R\textsuperscript{109}K mutation in the CH3 domain of IgG4 reduced its sensitivity to low pH and inhibited aggregate formation.\textsuperscript{5} However, other mutations in the Fc (Q\textsuperscript{235}R, E\textsuperscript{350}D, M\textsuperscript{234}L, E\textsuperscript{419}Q and L\textsuperscript{445}P) had little or no impact on IgG4 stability. Along with the impact of individual amino acids on IgG4 stability, the interactions among domains are also important.\textsuperscript{6,12} Fc stability is significantly reduced when altering the interaction of Fab with CH2.\textsuperscript{8} Liu et al.\textsuperscript{10} and Yageta et al.\textsuperscript{13} revealed that change in the interaction of CH3 domains on two HCs resulted in more aggregation in IgG4 compared with IgG1.

Both IgG1 and IgG4 share the same last two C-terminal residues, Gly\textsuperscript{446} and Lys\textsuperscript{447}. Lys\textsuperscript{447} cleavage is well studied and can reach greater than 95% in both natural and manufactured recombinant antibodies.\textsuperscript{14} The generally observed heterogeneity from Lys\textsuperscript{447} cleavage is acceptable in manufactured antibodies as it appears to have little impact on an antibody’s stability and function.\textsuperscript{15} No Gly\textsuperscript{446} cleavage was reported, except due to amidation of Pro\textsuperscript{445} in IgG1 or Leu\textsuperscript{445} in IgG4. Amidation of Pro\textsuperscript{445} in IgG1 or Leu\textsuperscript{445} in IgG4 is another common C-terminal modification that has been observed in manufactured antibodies, but not in endogenous human IgG antibodies.\textsuperscript{14} This C-terminal amidation is believed to be catalyzed by peptidylglycine \(\alpha\)-amidating monooxygenase, which is constitutively expressed in Chinese hamster ovary (CHO) cells. The level of amidation has been reported to increase with increasing copper concentrations in cell culture medium, but the level of observed amidation is typically less than 5%.\textsuperscript{14} Despite not being reported in endogenous IgGs, amidation is commonly observed in biologically active peptides. C-terminal amidation typically showed no impact on antibody stability and function, and as such, is not considered to be an unnatural modification in recombinant IgGs. To date, there are no reports demonstrating that the heavy chain C-terminal residues (-LGK in IgG4 and -PGK in IgG1) have any effect on the stability of IgG1 or IgG4 molecules. Here, we are the first to report that Leu\textsuperscript{445}, the third to last residue in the IgG4 heavy chain, plays a significant role in acid-induced (pH < 4.0) cleavages and aggregation. When present in the sequence, cleavages could occur at multiple sites of the heavy chain C-terminal region in our in-house IgG4 antibody, IgG4 CDR-X. Surprisingly, fragmentation and aggregation are significantly reduced by a single mutation: L\textsuperscript{445}P (P is the corresponding residue at this position in IgG1). Different acidic buffers that are commonly used in antibody manufacturing processes were tested for their impact on IgG4 stability. We found that glycine buffer completely abolished the heavy chain fragmentation and aggregation. In contrast, citrate and acetate promoted both to different degrees, and acetate buffer was found to mainly induce Gly\textsuperscript{446} cleavage. Our studies also show that the potential interaction between Fab and CH2-CH3 domains could be critical to the stability of IgG4 molecules.

**Results**

**Acid-induced fragmentation and aggregation of IgG4 CDR-X**

Lys\textsuperscript{447} cleavage and amidation at position 445 in the C-terminal region of IgG heavy chains (-PGK in IgG1 and -
LGK in IgG₄ are well studied. Gly⁴₄₆ cleavage, without amidation of Leu⁴⁴⁵, was first observed in IgG₄₋CDR-X, an IgG₄ prepared in-house, after it was exposed to acidic stress during the low pH hold step. IgG₄₋CDR-X was expressed in stable CHO pool and purified using ProA affinity chromatography using either a 50 mM sodium acetate or 100 mM sodium citrate elution buffer (both at pH 3.5). For the sodium acetate elution condition, the eluted protein was titrated down to pH 3.5 with 1 M acetic acid and held for 1 hour at room temperature. After the 1-hr hold, pH was neutralized using 1 M Tris/zma base. For the sodium citrate eluent condition, the elution was neutralized with 1 M Tris/zma base immediately without a hold step at low pH. Insoluble and soluble protein aggregates were observed consistently in different lots during the low pH buffer hold (data not shown). The masses of the IgG’s HC, with and without deglycosylation, were determined via mass spectrometry (MS). Furthermore, the deglycosylatedFc/2 region, produced with IdeS digestion, was also analyzed using MS (Table 1). The mass determined for the light chain exactly matched the theoretical mass. However, the measured HC mass of the IgG purified with the sodium acetate elution buffer and held at low pH for 1 hr (Table 1, sample_1) was ~57 Da less than the theoretical mass (taking into account for C-terminal Lys cleavage (~128.2 Da) and N-terminal Pyro-Q formation (~17 Da)). This mass difference was observed in all HC glycosylation forms G0F, G1F and G2F. The deglycosylated HC and Fc/2 also have a ~ 57 Da loss. This same HC mass loss was observed in various production lots of IgG₄₋CDR-X samples purified with the same procedure. In contrast, the mass of samples purified by ProA with immediate pH neutralization do not show this mass loss (Table 1, sample_2). This 57 Da loss is consistent with C-terminal Gly⁴₄₆ truncation.

Antigen-binding affinity, determined by surface plasmon resonance (SPR), was used to evaluate the effect of C-terminal modification on antibody function. Affinity loss was not found in samples with C-terminal cleavages (data not shown), suggesting that C-terminal residues have very limited or no effect on antigen binding. This is consistent with other reports that C-terminal modifications do not affect antibody function.

**L-glycine inhibits and citrate promotes acid-induced C-terminal fragmentation and subsequent aggregation of IgG₄₋CDR-X**

The aforementioned experiments illustrated that IgG₄₋CDR-X purified by ProA column using a sodium citrate elution and immediately neutralized had the expected HC and LC sequences (Table 1). This confirms that the cell culture was not the cause of the C-terminal Gly⁴₄₆ cleavage in IgG₄₋CDR-X. The Gly⁴₄₆ cleavage of IgG₄₋CDR-X appeared to occur only under acidic conditions (low pH hold step) during its production. Factors, including buffer type, buffer concentration, salt concentration, temperature, pH and low pH hold time could potentially affect Gly⁴₄₆ cleavage on the C-terminus of the IgG₄ HC. In this study, we tested the key factors impacting Gly⁴₄₆ cleavage: buffer type, pH and low pH hold time. Sodium acetate, sodium citrate, and L-glycine buffers were selected as ProA elution buffers based on their common use in therapeutic antibody manufacturing processes. Samples were purified via ProA column using the aforementioned elution buffers and then immediately neutralized after elution. Mass analysis was performed to confirm that the heavy chain sequence was intact, except for C-terminal lysine cleavage. These samples were then buffer exchanged into their respective buffers: 20 mM sodium acetate, citrate, or L-glycine (all at pH 3.5). These samples were then incubated at room temperature for up to 6 hrs. Samples before and after low pH treatment were analyzed by appearance for precipitation, MS for potential cleavages, size exclusion-high performance liquid chromatography (SE-HPLC) for aggregation, and SPR (MASS-2) for affinity, as necessary. The data on precipitation, aggregation, and C-terminal truncation of these samples are shown in Figure 2.

As seen in Figure 2(d), mass analysis determined that Gly⁴₄₆ cleavage was present in IgG₄₋CDR-X held in sodium acetate buffer. The level of Gly⁴₄₆ cleavage increased with incubation time. After 6 hrs of incubation, 100% of the molecules had Gly⁴₄₆ cleaved (Figure 2). Additionally, soluble aggregates increased from 8.6% to 26% (as determined by SE-HPLC) for aggregation, and SPR (MASS-2) for affinity, as necessary. The data on precipitation, aggregation, and C-terminal truncation of these samples are shown in Figure 2.

**Table 1. MS analysis of heavy chain of IgG₄₋CDR-X purified by different procedures**

| Samples | Theoretical mass (Da) (PTM) | Determined mass (Da) | Mass difference (Da) |
|---------|-----------------------------|---------------------|---------------------|
| Sample 1⁴ | 50245.1 (pyro Q-Lys + G0F) | 50188.0 | –57.1 |
| Sample 1 | 50407.2 (pyro Q-Lys + G1F) | 50350.0 | –57.2 |
| Sample 1 | 50569.3 (pyro Q-Lys + G2F) | 50512.5 | –56.8 |
| Sample 1 | 48800.9 (pyro Q-Lys + 1 Da) | 48743.5 | –57.0 |
| Sample 1 | 23775.7 (Fc/2 by IdeS)⁵ | 23718.5 | –57.2 |
| Sample 2⁶ | 50245.1 (pyro Q-Lys + G0F) | 50245.0 | –0.1 |
| Sample 2 | 50407.2 (pyro Q-Lys + G1F) | 50407.0 | –0.2 |
| Sample 2 | 50569.3 (pyro Q-Lys + G2F) | N/D | N/A |
| Sample 2 | 48800.9 (pyro Q-Lys + 1 Da) | 48801.0 | 0.1 |
| Sample 2 | 23775.7 (Fc/2 by IdeS)⁵ | 23775.5 | 0.2 |

⁴Eluted from ProA column with 50 mM acetate buffer and held in the same buffer for 1 hr before neutralization.
⁵Eluted from ProA column with 100 mM acetate buffer and immediately neutralized.
⁶De-N-glycosylation was done with PNGase F treatment, which results in conversion of asparagine to aspartic acid, a 1 Da mass increase.
⁷Fc/2 was generated by IdeS digestion.

Figure 2(d) shows the mass analysis results for IgG₄₋CDR-X. The level of Gly⁴₄₆ cleavage increased with incubation time. After 6 hrs of incubation, 100% of the molecules had Gly⁴₄₆ cleaved (Figure 2). Additionally, soluble aggregates increased from 8.6% to 26% (as determined by SE-HPLC) for aggregation, and SPR (MASS-2) for affinity, as necessary. The data on precipitation, aggregation, and C-terminal truncation of these samples are shown in Figure 2.
Asn$^{434}$ (Figure 2(d)). After 6 hrs of incubation, about 90% of protein was precipitated and soluble aggregates increased to 45.6% (Figure 2(d)). Antibodies in the precipitation had all of their HC cleaved, with the majority cleaved at Gly$^{446}$. In contrast, the L-glycine buffer produced no C-terminal truncation, aggregation, or precipitation over the course of the 6-hr incubation (Figure 2(c,d)). These data indicate that IgG$_{4}$-CDR-X experienced C-terminal cleavage as a result of acid induced hydrolysis, and that the loss of those residues resulted in much higher levels of aggregation, which suggests HC C-terminal residuals are important for stabilizing antibodies. Cleavage also appears to be buffer dependent, where citrate buffer appears to result in the greatest level of cleavage, acetate buffer only exhibits Gly$^{446}$ cleavage, and L-glycine buffer prevents cleavage. L-glycine buffer was thus selected for production of IgG$_{4}$-CDR-X.

Due to the pronounced effect of sodium citrate we selected it to study the pH dependency of HC C-terminal cleavage. pH dependency was studied with 100 mM citrate at pH 7.0, 4.0, and 3.5. The experiments were carried out by eluting CHO-expressed IgG$_{4}$-CDR-X from a ProA column using citrate buffers at pH 4.0 and 3.5, incubating at room temperature for 1 hr, and then neutralizing with 1 M Tris, while for the pH 7.0 condition the eluent was immediately neutralized and then incubated. The samples were analyzed with MS, data are shown in Figure 3. At pH 7.0, no Gly$^{446}$ cleavage or protein aggregation increase were detected. At pH 4.0, Gly$^{446}$ cleavage was observed on 25% of the antibodies and no other types of cleavage were detected. However, at pH 3.5, many more C-terminal amino acids were truncated and no intact IgG$_{4}$ antibody was found after 1 hr incubation. Also, significant precipitation was observed. These pH dependent cleavages reveal that truncation of the C-terminus is most likely due to acid-induced hydrolysis, and that citrate buffer with a pH below 3.5 greatly elevates C-terminal cleavages.

**Point mutation L$^{445}$P on IgG$_{4}$-CDR-X heavy chain significantly reduces heavy chain C-terminal truncation and antibody aggregation**

Researchers have reported that the last few C-terminal residues in the HC of IgG$_{4}$ had limited or no impact on its stability.$^{7}$ Leu at position 445 in IgG$_{4}$ is the only residue different from IgG$_{1}$ in the HC C-terminal region. To evaluate the effect of Leu$^{445}$ on the stability of IgG$_{4}$-CDR-X, the L$^{445}$P mutation was incorporated in IgG$_{4}$-CDR-X and the P$^{445}$L mutation was incorporated into IgG$_{1}$ _CDR-X. Both antibodies have the same complementarity-determining region (CDR) and LC. The proteins were transiently expressed in CHO cells. Affinity by SPR and $T_{m}$ by differential scanning calorimetry (DSC) were determined in order to assess potential structural changes caused by the mutations (Table 2). No affinity or $T_{m}$ changes were observed in either mutated IgG forms compared to the non-mutated partner. The $T_{m}$ of the two IgG$_{4}$s were about 12°C higher than those of the IgG$_{1}$s, which is consistent with the differences reported in the literature.$^{16}$ Comparable $T_{m}$ and affinity between wild types and mutants indicated no significant structural change after mutation.

Both wild type and mutant IgG$_{1}$ and IgG$_{4}$ CDR-X IgGs were subjected to low pH stress in 100 mM sodium citrate buffer at pH 3.5. The samples taken at 1 hr and 24 hrs were analyzed via MS and SE-HPLC. The data on aggregation and protein loss are summarized in Figure 4 and cleavages of the antibody in both pellet and supernatant after 24 hrs incubation in Table 3. The wild type IgG$_{4}$ experienced complete C-terminal truncation within 1 hr with the following cleavage species being detected: HC missing-NHYTQKSLSLGK, -HYTQKSLSLGK, -TQKSLSLGK or -
KSLSLSLGK (Figure 3). In contrast, the IgG4 L445P CDR-X mutant had two cleavage species after 24 hrs: Gly446 (found mostly in the precipitate) and -QKSLSLSLGK (found in both supernatant and precipitate). Approximately 40% of the IgG4 L445P CDR-X molecules experienced no cleavage (except for C-terminal Lys), suggesting that Pro445 can play a significant role in stabilizing IgG4 at low pH. In the experiments with the IgG1 constructs, no cleavage was observed in the wild type IgG1 CDR-X, while complete cleavage was observed in the IgG1 P445L CDR-X mutant with only one cleaved HC species (missing -LSLSLGK) detected after 24 hrs of incubation.

Protein loss via precipitation was observed in some of the samples after 24 hrs of incubation (Figure 4(c)). The two IgG4 CDR-X constructs experienced significant mass loss (wild type 42% and IgG4 L445P 22%), while there were no obvious mass losses in IgG1 and IgG1 P445L CDR-X mutant samples. Soluble aggregation in supernatant assessed by SEC showed increases in both IgG wild types and IgG4 L445P CDR-X mutant during the 24 hrs of stress (Figure 4(a)), but the percentage of aggregates varied (Figure 4(b), main peak area changes). Fragmentation was found in the IgG1 P445L CDR-X mutant. The IgG4 L445P CDR-X mutant experienced ~50% less aggregate formation compared to wild type IgG4 CDR-X. The IgG1 P445L CDR-X mutant experienced an ~55% reduction in monomers compared to wild type IgG1 CDR-X. These data illustrate clear differences in the stability of wild type and mutated IgGs, indicating that Leu445 is the key factor leading

Figure 3. Deconvoluted heavy chain mass spectra showing pH-dependent C-terminal cleavages of IgG4 CDR-X. (a) Neutral pH, protein was eluted from ProA column with 100 mM citrate buffer, pH 3.5, and immediately neutralized; (b) pH 4.0, protein was eluted from ProA column with 100 mM citrate buffer, pH 4.0, followed by hold in the same buffer for 1 hr before neutralization; (c) pH 3.5, protein was eluted from ProA column with 100 mM citrate buffer, pH 3.5, followed by hold in the same buffer for 1 hr before neutralization.

Table 2. Affinity and Tm comparison of wild type antibodies IgG4 CDR-X, IgG1 CDR-X, and their mutants IgG4 CDR-X(L445P), IgG1 CDR-X(P445L) by SPR and VPDSC

| Samples               | $k_a$ [1/(M·s)] | $k_d$ [1/s] | $K_D$ (M) | Fab | $T_m$ (°C) |
|-----------------------|-----------------|-------------|-----------|-----|------------|
| IgG4 CDR-X            | 6.6 x 10^4      | 2.3 x 10^-4 | 3.4 x 10^-9 | 69.8 |
| IgG4 CDR-X(L445P)     | 5.9 x 10^4      | 2.1 x 10^-4 | 3.6 x 10^-9 | 70.2 |
| IgG1 CDR-X            | 6.1 x 10^4      | 2.2 x 10^-4 | 3.5 x 10^-9 | 82.0 |
| IgG1 CDR-X(P445L)     | 6.2 x 10^4      | 2.2 x 10^-4 | 3.5 x 10^-9 | 82.8 |

Table 2. Affinity and Tm comparison of wild type antibodies IgG4 CDR-X, IgG1 CDR-X, and their mutants IgG4 CDR-X(L445P), IgG1 CDR-X(P445L) by SPR and VPDSC
to IgG4 Fc instability under acidic condition. Mutating L\textsuperscript{445} to P, the corresponding residue in the IgG1 C-terminal, significantly improves IgG4 stability by significantly reducing both cleavage and aggregation in acidic conditions.

IgG\textsubscript{4} Fc stability is influenced by possible interactions between Fab and Fc

In order to examine whether the effect of Leu\textsuperscript{445}, which destabilizes IgG\textsubscript{4} CDR-X in acidic conditions, is applicable to other IgG\textsubscript{4} antibodies, two IgG\textsubscript{4} antibodies (IgG\textsubscript{4} CDR-Y and IgG\textsubscript{4} CDR-Z) targeting the same ligand as IgG\textsubscript{4} CDR-X but with very different CDRs were tested under the same low pH stress described above (sodium citrate, pH 3.5). SE-HPLC chromatograms of each sample, after 0, 1, and 24 hours, are shown in Figure 5. After 24 hrs of stress, soluble aggregation increased to ~12% in both IgG\textsubscript{4} CDR-Y and IgG\textsubscript{4} CDR-Z, which is comparable to the 16.1% of aggregation seen in IgG\textsubscript{4} CDR-X. However, precipitation of IgG\textsubscript{4} CDR-Y and IgG\textsubscript{4} CDR-Z was negligible, while IgG\textsubscript{4} CDR-X experienced 37.4% protein loss (Table 4). Subsequent mass analysis of IgG\textsubscript{4} CDR-Y and IgG\textsubscript{4} CDR-Z revealed that C-terminal truncation did not occur upon low pH stress. Since CDR

Figure 4. SE-HPLC monomer peak change and protein loss of IgG\textsubscript{4} CDR-X, IgG\textsubscript{1} CDR-X and their mutants IgG\textsubscript{4} CDR-X(L\textsuperscript{445}P), IgG\textsubscript{1} CDR-X(P\textsuperscript{445}L) after acidic stress. The purified protein was buffer exchanged into 100 mM citrate buffer, pH 3.5, and incubated for 1 and 24 hrs at room temperature before neutralization. (a) Overlaid SE-HPLC chromatograms of T0, T1 hr and T24 hr; (b) SE-HPLC monomer peak area change; (c) Protein loss measured by A280.
sequences are the only differences between these three IgG antibodies, the different behavior of heavy chain C-terminal cleavage and subsequent aggregation/precipitation of the antibodies suggests that the CDR sequence has a role in Fc stability, and may indicate a potential interaction of Fab with CH2-CH3 domain.

Target binding is another approach used to assess the potential impact of the Fab on Fc stability. The same acidic stress experiment was applied to a complex of IgG4_CDR-X and its target in parallel with IgG4_CDR-X alone (Table 5). No precipitation was observed for the complex, but it was observed for the antibody alone with 37.4% protein loss (Table 4). Soluble aggregates of the complex were measured to be 28.7% after 24 hrs of low pH exposure. Mass analysis showed only 28.7% cleavage of ~57 Da, which we suspected was caused by the low pH hold step (pH 3.5 for 1 hr). We studied the effects of buffer type, pH and the duration of the low pH hold step, and found that L-glycine buffer appeared to stabilize the IgG4_CDR-X molecule, whereas both acetate and citrate buffers appeared to destabilize the molecule, with citrate resulting in more extensive cleavage. It has been reported that two types of modification (cleavage at Lys447 and amidation either on P445 in IgG1 or L445 in IgG4 molecules) are observed on the HC C-terminal region of recombinant IgG1 and

Discussion

Mass analysis of the ProA-purified IgG4_CDR-X showed a complete C-terminal Gly446 cleavage of ~57 Da, which we suspected was caused by the low pH hold step (pH 3.5 for 1 hr). We studied the effects of buffer type, pH and the duration of the low pH hold step, and found that L-glycine buffer appeared to stabilize the IgG4_CDR-X molecule, whereas both acetate and citrate buffers appeared to destabilize the molecule, with citrate resulting in more extensive cleavage. It has been reported that two types of modification (cleavage at Lys447 and amidation either on P445 in IgG1 or L445 in IgG4 molecules) are observed on the HC C-terminal region of recombinant IgG1 and

Figure 5. Overlaid SE-HPLC chromatograms of IgG4_CDR-Y and IgG4_CDR-Z after acidic stress.
Table 3. (A) Heavy chain C-terminal cleavage and (B) soluble aggregation and precipitation of IgG4_CDR-X, IgG1_CDR-X and their mutants IgG4_CDR-X(L465P), IgG1_CDR-X(P466L) after acidic stress

A

| Samples          | Theoretical Mass (Da) | Observed Mass (Da) | HC modifications and cleavages determined by MS analysis |
|------------------|-----------------------|--------------------|--------------------------------------------------------|
| IgG4_CDR-X       |                       |                    |                                                        |
| T0               | 48944.9               | 50245.5            | HC + G0F + PyroQ – Lys                                   |
| T24hr supernatant| 48923.5               | 49228.5            | HC + G1F + PyroQ – TQKS…LGK                              |
| T24hr Pellet     | 49458.5               | 49453.0            | HC + G2F + PyroQ – KLSL…LGK                              |
| IgG4_CDR-X(L465P)| T0                    | 48928.9            | 50229.0 HC + G0F + PyroQ – Lys                           |
| T24hr supernatant| 49330.0               | 50353.5            | HC + G0F + PyroQ – QKLS…PGK                            |
| T24hr Pellet     | 50387.5               | 50593.5            | HC + G1F + PyroQ – Gly - Lys                             |
| IgG1_CDR-X       | T0                    | 49132.3            | 50330.0 HC + G0F + PyroQ – Lys                           |
| T24hr supernatant| 50330.0               | 50592.0            | HC + G1F + PyroQ – Lys                                  |
| T24hr Pellet     | 50341.0               | 50594.0            | HC + G0F + PyroQ – Lys                                  |
| IgG1_CDR-X(P466L)| T0                    | 49148.3            | 50450.0 HC + G0F + PyroQ – Lys                           |
| T24hr supernatant| 49864.5               | 50609.0            | HC + G0F + PyroQ – Lys (<1%)                            |
| T24hr Pellet     | 49864.5               | 50609.0            | HC + G0F + PyroQ – Lys (<1%)                            |

*Percentage of HC + G0F + PyroQ was ~40% based on MS data.

B

| Incubation time | IgG4_CDR-X | IgG4_CDR-X (L465P) | IgG1_CDR-X | IgG1_CDR-X(P466L) |
|-----------------|------------|--------------------|------------|-------------------|
| T0              | 0          | 0/n/a              | 0          | 0/n/a             |
| T1hr            | 2.2        | 0                  | 0          | 0/n/a             |
| T24hr           | 16.1       | 37.4               | 13.9       | 28.4              |

*Soluble aggregation as determined by SE-HPLC.

Table 4. Soluble aggregation and precipitation of IgG4_CDR-Y and IgG4_CDR-Z compared with IgG4_CDR-X after acidic stress

| Incubation time | IgG4_CDR-X | IgG4_CDR-Y | IgG4_CDR-Z |
|-----------------|------------|------------|------------|
| T0              | 0          | 0          | 0          |
| T1hr            | 2.2        | 2.3        | 3.5        |
| T24hr           | 16.1       | 11.2       | 2.2        |

*Soluble aggregation as determined by SE-HPLC

Table 5. Comparison of acid-induced soluble aggregation, precipitation and heavy chain C-terminal cleavages of IgG4_CDR-X in antigen-complexed and uncomplexed form

| Incubation time | Aggregates IgG4_CDR-X complex | HC cleavage IgG4_CDR-X complex | Aggregates IgG4_CDR-X | HC cleavage IgG4_CDR-X |
|-----------------|-------------------------------|------------------------------|-----------------------|------------------------|
| T0              | 0.0                           | 0.0                          | 0.0                   | 0.0                    |
| T1hr            | 1.8                           | 45                           | 2.2                   | 45                     |
| T24hr           | 28.7                          | 88                           | 16.1                  | 88                     |

*Soluble aggregation as determined by SE-HPLC

IgG4 molecules. One possible Gly1446 cleavage is due to amidation of L445 on the C-terminal region (–LGK) of IgG4 HC, but this would lead to a 58 Da loss from the molecule after Lys447 had already been cleaved. It is also documented that amidation of Leu1445 is an enzymatic process and occurs only at the CHO expression stage. Our results with and without low pH hold
indicate that the mass loss occurred after expression. Lys-C peptide mapping revealed a mass of 619.4 Da corresponding to -SLSLSL in IgG4_CDR-X with low pH hold and 676.4 Da corresponding to peptide -SLSLSLG in the antibody without low pH hold. This observation confirms that the 57 Da mass loss is from Gly\textsuperscript{446} cleavage at HC C-terminal. This is the first reported instance of Gly\textsuperscript{446} cleavage under an acidic condition. The cleavage is likely due to acid-induced hydrolysis, as the main difference between the samples was the use of a low pH hold.\textsuperscript{17,18}

IgG\textsubscript{4} is prone to aggregate and less stable than IgG\textsubscript{1} despite their subtle differences in structure and sequence.\textsuperscript{4} Mutating certain IgG\textsubscript{4} Fc residues to corresponding residues in IgG\textsubscript{1} has been reported to stabilize IgG\textsubscript{4} molecules. The most notable mutants are S\textsuperscript{228}P and R\textsuperscript{409}K, which stabilize the hinge region and reduce aggregation, respectively.\textsuperscript{1,3} However, those studies all showed that heavy chain C-terminal residues have little or no impact on IgG\textsubscript{4} stability and function. Here, we are the first to report that Leu\textsuperscript{445} plays an important role in IgG\textsubscript{4} instability (aggregation and fragmentation) under acidic conditions. Mutating Leu\textsuperscript{445} to Pro (the corresponding residue in IgG\textsubscript{1}) in IgG\textsubscript{4}_CDR-X can significantly improve IgG\textsubscript{4} stability. In contrast, mutating Pro\textsuperscript{445} to Leu in IgG\textsubscript{1}_CDR-X reduces IgG\textsubscript{1} stability, revealing the Pro could play an important role in stabilizing the IgG\textsubscript{4} heavy chain. However, the effect of Leu\textsuperscript{445} in IgG\textsubscript{4} was not observed with two other IgG\textsubscript{4} molecules evaluated, IgG\textsubscript{4}_CDR-Y and IgG\textsubscript{4}_CDR-Z. These antibodies bind to the same target protein and share the same sequence in HC constant regions (CH1, hinge, CH2, and CH3) and LC, but only have CDR sequences different from that of CDR-X. These data indicate that heavy chain C-terminal cleavage is influenced by the IgG\textsubscript{4}’s CDR sequences, and thus suggests a potential impact of the Fab on the stability of the CH2-CH3 domain. Furthermore, antigen binding significantly improves the stability of IgG\textsubscript{4} _CDR-X. These results indicate that the C-terminal stability of the IgG\textsubscript{4} HC is affected by the CDR sequence and Fab conformation. Studies utilizing X-ray and neutron scattering on solution structures indicate that a unique aspect of the IgG\textsubscript{4} Fab is that it is situated close to the Fc region, especially at high protein concentrations.\textsuperscript{8} This spatial proximity may result in greater Fc stability depending on the conformation of the Fab, influenced by the CDR and antigen binding. Both our data and antibody structures by X-ray and neutron scattering suggest the possible presence of interactions between Fab and CH2-CH3 domains that influence the stability of HC C-terminal region on IgG\textsubscript{4} and IgG\textsubscript{4} antibodies.

L-glycine is a popular excipient used in formulations as a protein stabilizer.\textsuperscript{19} In our study, 20 mM glycine is sufficient to completely inhibit C-terminal cleavage and subsequent aggregation of IgG\textsubscript{4}_CDR-X, which is not stable in citrate or acetate buffers at low pH. Although the stabilizing mechanism of L-glycine is not clear, two factors could be considered. First, L-glycine could stabilize the protein by increasing its thermal stability.\textsuperscript{19} As glycine is a zwitterionic molecule, it can directly interact with the protein via both its negative and positive charged groups due to its small size. This direct interaction with the charged side chains and peptide backbone of the protein plays an important role in protein stabilization. Glycine can also compete for water between the unfolding protein and the cosolute, thus increasing the energy required to hydrate the unfolding protein. Secondly, the C-terminal Gly\textsuperscript{446} cleavage is caused by acid-induced hydrolysis,\textsuperscript{18} and as glycine is one of the products of this hydrolysis reaction, addition of glycine can inhibit the reaction, abating IgG cleavage via a product feedback inhibition mechanism. At the 20 mM glycine concentration used in our studies, the molar ratio of protein to glycine reaches 1:600. It is reasonable to hypothesize that such an inundation of glycine is sufficient to inhibit glycine cleavage to an undetectable level. Since glycine completely inhibits heavy chain C-terminal fragmentation and subsequent aggregation of IgG\textsubscript{4}_CDR-X under acidic conditions, it is likely that Gh\textsuperscript{446} cleavage significantly destabilizes the IgG\textsubscript{4}_CDR-X Fc and becomes the first step towards further upstream truncations fragmentation.

We also evaluated both acetate and citrate buffers at pH 3.5 for the low pH hold, and the data showed that citrate induces significantly more IgG\textsubscript{4}_CDR-X HC C-terminal fragmentation and aggregation than acetate. These results are consistent with other studies regarding the potential effects of citrate and acetate buffer on IgG aggregation.\textsuperscript{20} Citrate is a trivalent chemical containing three carboxylic acids with pKas of 3.1, 4.8, and 6.4, which results in high ionic strength. Acetate has only one carboxyl group with a pKa of about 4.8, which results in weaker ionic strength. Both citrate and acetate\textsuperscript{19} interact with proteins through their negative charge, thus the effect of their specific ion on protein stability is due to differences in ion strength. It was proposed\textsuperscript{20} that citrate ions preferentially accumulate near the surface of an antibody more than acetate ions, decreasing apparent conformational stability and increasing aggregation rates. Alternatively, accumulation of citrate ions is causing weaker electrostatic repulsions between proteins under low ionic strength environment, resulting in increased aggregation. Therefore, we promote use of acetate buffer rather than citrate in ProA purification processes.

In conclusion, HC C-terminal Gly\textsuperscript{446} cleavage was found in IgG\textsubscript{4}_CDR-X at low pH, and likely occurs through an acid-induced hydrolysis. Gly\textsuperscript{446} cleavage triggers further C-terminal cleavages. Its aggregation is induced by HC C-terminal cleavage. Two mitigating approaches are found to improve IgG\textsubscript{4} stability. Glycine buffer prevents cleavage of Gly\textsuperscript{446}, while the L\textsuperscript{445}P mutation provides greater stability to the HC C-terminus. Furthermore, IgG\textsubscript{4} has a unique spatial orientation, with the Fab located near the Fc region. Certain mutations in the CDR and antigen binding can also help to stabilize the CH2-CH3 domains, which suggests the presence of interaction between the Fab and CH2-CH3 domains. These key factors should be considered when IgG\textsubscript{4} is exposed to manufacturing conditions.

Materials and methods

Antibody construct generation, expression and protein purification

The constructs used in this study were designed using Benchling software (Benchling, San Francisco, CA) with backbone of pTT5 and are described in Table 6. DNA was prepared with Qiagen Mega Prep kit (Qiagen, Gaithersburg, MD) and transfected into CHO cells for either transient expression or stable CHOZN* pool development. Antibodies were purified using a Protein A HiTrap column (GE Healthcare, cat. #}
11–0034-95). Purified monoclonal antibodies (mAbs) were analyzed by SDS-PAGE, SE-HPLC, and MS to confirm purity and amino acid sequences.

**Low pH hold during the antibody purification process**

Low pH hold is a routine practice during manufacturing, where therapeutic antibodies are held at pH 3.5 for 1 hr for viral inactivation. Study of the effects of buffer pH (pH 3.5, 4.0, and 7.0) on IgG stability was performed with IgG _CDR-X_ expressed from a stable CHO cell pool. The experiments were carried out by eluting IgG _CDR-X_ from a ProA column with 100 mM citrate buffers at pH 3.5 and 4.0. Eluted samples were incubated for one hr at room temperature before being neutralized with 1 M Tris. pH 7.0 samples were immediately neutralized after pH 3.5 elution. SE-HPLC and MS analysis was performed on all samples after neutralization.

**Low pH stress in different buffers**

Purified antibodies were buffer exchanged into 100 mM pH 3.5 sodium citrate or acetate buffer, using Zeba spin desalting column (Thermo Scientific, Waltham, MA). Protein concentrations were immediately determined by A280 on a Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA) using an absorptivity of 1.46 mL·mg⁻¹·cm⁻¹, and then were diluted to 2 mg/mL into the same buffer. Appearance test and SE-HPLC were performed immediately (T0) for each sample. After 1, 6, and 24 hours of incubation at room temperature, aliquots were taken for appearance, A280, and SE-HPLC, and liquid chromatography-mass spectrometry (LC-MS) analysis (detailed LC-MS procedures are described below). If precipitation was observed, precipitate was separated from supernatant by centrifugation. Both supernatant and pellet (reconstituted in 6 M guanidine HCl) were analyzed by LC-MS.

**Size exclusion-high performance liquid chromatography**

SE-HPLC was conducted using a Wyatt SEC 7.8 × 300 mm column (WTC-03055, Wyatt, Santa Barbara CA) on an Agilent HPLC 1260 Infinity system (Agilent Technologies, Santa Clara, CA). Samples (20 to 30 µg per injection) were injected and phosphate-buffered saline (pH 7.4) was used as the mobile phase. The flow rate was 1 mL/min and run time was 15 mins. UV absorbances were monitored at both 214 and 280 nm. The content of aggregates (high-molecular weight (HMW) species) was determined by peak integration using Agilent HPLC Chemstation Software.

**Differential scanning calorimetry**

Thermal stability was investigated using Malvern MicroCal VP-Capillary DSC (Malvern Panalytical, Westborough, MA). Samples (400 µL at 0.4–0.5 mg/mL) were loaded into a 96-well plate along with a reference buffer. The thermal scan was performed from 20 to 100°C at a scan rate of 90°C/hr. Data was analyzed using Origin 7.0 software from Malvern Panalytical to obtain the thermal transition midpoint (T_m).

**Liquid chromatography-mass spectrometry**

Mass was analyzed with Waters Micromass LCT Premier Mass Spectrometer (Waters Corporation, Milford, MA). Each sample was denatured in 100 mM Tris, pH 7.5, 6 M guanidine HCl (GuHCl), reduced with 50 mM dithiothreitol at 37°C for 30 mins, and then acidified with 0.5% (v/v) trifluoroacetic acid (TFA). Approximately 30 µg of each sample were loaded onto a Tosoh TSKgel Phenyl 5PW (7.5 mm × 7.5 cm, 10 µm) column (P/N- 07573, King of Prussia PA). Mobile phase A was composed of 0.05% (v/v) TFA in water and mobile phase B was composed of 0.05% TFA in 95% acetonitrile (ACN). The ACN gradient (10 to 37.5% B) was delivered from 2 to 18 mins at a flow rate of 1.0 mL/min. UV absorbance was monitored at 280 nm. On-line mass measurement was performed in W positive ion mode. Capillary and cone voltages were set at 3000 V and 100 V, respectively. Desolvation and source temperatures were 200°C and 100°C, respectively, and the nitrogen gas flow was at 400 L per hour. Deconvoluted mass spectra were calculated from multiply-charged raw data using Waters MaxEnt1 software. All pellet samples were solubilized in 6 M guanidine HCl. Deglycosylated samples were prepared by PNGase F treatment (New England Biolabs, cat. # P0704S) following the vendor’s instructions. Fc/2 was generated by IdeS (GENOVIS, cat. # A0-FRI-020) digestion.

**Binding affinity by surface plasmon resonance**

Antigen (made in house) binding activity of the antibody samples was analyzed using SPR Mass-2 (Sierra Sensors Billerica, MA). The capturing antibody, goat anti-human Fc (Cat. # 109–005-098, Jackson ImmunoResearch Lab, West Grove, PA), was immobilized to a high capacity amine sensor through amine coupling. After a mAb sample (ligand) was captured, seven 2.5x serial dilutions of antigen (analyte), from 100 nM to 0.41 nM, were flowed through the sensor chip for binding kinetics measurements. Sierra Analyzer software from Sierra Sensors was used to determine association and dissociation rate constants (k_a and k_d), as well as the equilibrium dissociation constant (K_D).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interests was reported by the authors.
Abbreviations

mAb monoclonal antibody
HC heavy chain
LC light chain
CDR complementarity-determining region
Fab antigen binding fragment
ProA protein A
MES 2-(N-morpholino) ethanesulfonic acid
MS mass Spectrometry
TFA trifluoroacetic acid
ACN acetonitrile

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