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# β-Elimination and Peptide Bond Hydrolysis: Two Distinct Mechanisms of Human IgG1 Hinge Fragmentation upon Storage

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Long-term storage of recombinant human IgG1 monoclonal antibodies in solution leads to the generation of Fc-Fab and Fab fragments (Figure 1A). The fragmentation results from a gradual nonenzymatic cleavage within the heavy chain (HC) upper hinge sequence $C_{220}$DKTHTC[1−3] and is presumed to involve hydrolysis of the CO−NH peptide bonds. Evidence for cleavage of the adjacent $S_{219}$−$C_{220}$ bond has also been reported.[1] However, the exact mechanism of scission of the $S_{219}$−$C_{220}$ bond remains puzzling because of the complicating presence of the light-chain−heavy-chain (LC $C_{219}$−HC $C_{220}$) disulfide (Figure 1B).

Here we present experimental data supporting cleavage of the HC $S_{219}$−$C_{220}$ bond through a β-elimination mechanism, initiated by disruption of the LC−HC disulfide and followed by cleavage of the HC. We also propose that the recently characterized Fab thioether-linkage,[4] also reported for other human IgG1s,[5] occurs via a β-elimination but following a different pathway.

IgG1 formulated in phosphate buffered saline (pH adjusted between 4 and 10) was stored for 17 days at 45 °C to accelerate degradation (see Supporting Information, Table S1). Size-exclusion chromatography (SEC) of incubated samples showed partial degradation of the antibody to Fc-Fab and Fab fragments, consistent with previous reports.[1,6] The rate of degradation was highly pH-dependent, minimizing at pH 6 and rapidly increasing at both pH extremes.

SEC Fab fractions were analyzed by SDS-PAGE under nonreducing conditions (pH 8 data is shown in Figure 1C). Three bands appear: the expected ∼50 kDa Fab fragment and an unexpected pair of closely spaced bands at ∼23 kDa that were identified by in-gel digestion and mass spectrometry as a Fab HC fragment (lower band) and LC (upper band). These results suggest that in addition to the expected "covalent" Fab fragment (the 50 kDa band), IgG1 degradation also yields a "noncovalent" Fab fragment (i.e., Fab without the LC−HC disulfide linkage). The amount of noncovalent Fab was negligible at pH 4 and increased significantly with pH.

The Fab peak collected by SEC for the pH 8 sample was analyzed under nonreducing conditions by HPLC−electrospray ionization time-of-flight mass spectrometry (LC/MS). High mass accuracy (0.01%) permitted the following assignments: (1) a 47−48 kDa Fab fragment "ladder" of peaks consisting of a Fab HC fragment that is disulfide-bridged to the intact LC (and also correlates to the ∼50 kDa band shown in Figure 1C (see also Figure S1b)). The "ladder" arises from cleavage across the HC $C_{220}$DKTHTC hinge sequence;[1−3] (2) a 23 kDa Fab HC fragment that corresponds to the cleavage between $S_{219}$ and $C_{220}$ (correlating to the lower ∼23 kDa band in Figure 1C) (see also Figure S1a); and (3) several peaks corresponding to LC (correlating to the upper ∼23 kDa band in Figure 1C). LC/MS of deglycosylated (PNGase F) and reduced (DTT) Fc-Fab SEC fraction showed a ladder of peaks between 25 and 26 kDa consistent with Fc HC fragments due to cleavage across the $C_{220}$DKTHTC hinge sequence (Figure 1D). All but one of the peaks in the ladder complement the Fab fragment ladder. The missing Fc fragment (HC220−446) corresponds to cleavage between HC $S_{219}$−$C_{220}$, which is surprising because the complementary Fab fragment (HC1−219) is very strong (Figure S1a). Instead, Figure 1D contains a strong peak whose mass corresponds to cleavage between $C_{220}$ and $D_{221}$ shifted to higher mass by ∼70 Da. Such modification is unexpected and suggests that an unusual cleavage event, other than simple peptide-bond hydrolysis between $S_{219}$−$C_{220}$ may have occurred during the antibody incubation period.

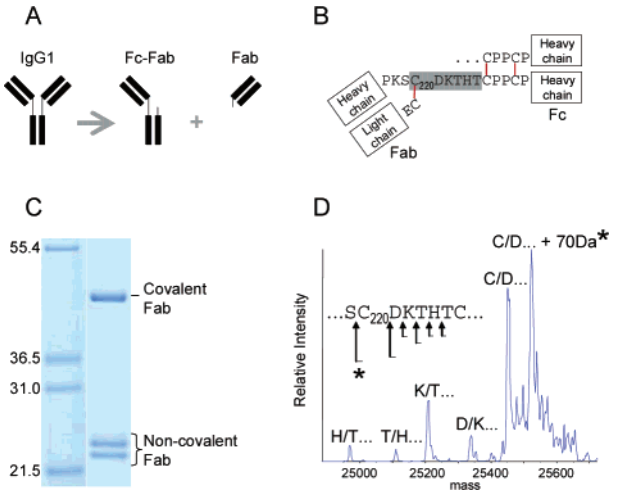


***Figure 1.*** Schematic diagram of IgG1 showing cleavage in the hinge region (thin lines) (A). Detail of IgG1 hinge peptide sequence; region that undergoes cleavage is shaded, disulfide bridges are indicated in red (B). Fab analysis: SDS-PAGE of nonreduced SEC Fab peak (right lane) and molecular markers (C). Fc analysis: mass spectrum detail (LC/MS) of Fc HC fragments from deglycosylated and reduced SEC Fc-Fab peak. The T/C fragment was too weak to be observed (D).

Further support for the unusual cleavage mechanism between $S_{219}$−$C_{220}$ was obtained by peptide mapping. In-gel digestion of the Fab HC fragment SDS-PAGE (reducing conditions) band showed the expected ladder of fragments resulting from cleavage across the hinge sequence (Figure 2). Note the good complementation of the relative intensity patterns between the Fc and Fab ladders (cf. Figures 1D and 2). All but one of the peaks in the Fab ladder exhibited masses consistent with the presence of a C-terminal hydroxyl group, reflecting and confirming their derivation via peptide-bond hydrolysis. However, the peptide corresponding to cleavage between $S_{219}$−$C_{220}$ was missing, instead replaced by a strong peak 1 Da lower in mass (at *m*/*z* 957; ±0.1 Da). Tandem-MS confirmed this peak to be the missing peptide but with a C-terminal amide group (Figure S2). Likewise, peptide mapping indicated that a 70.00 ± 0.02 Da modification was attached to the HC221−446 fragment (see Supporting Information). Tandem MS further showed that this modification was at the amino-terminus of the fragment (i.e., $D_{221}$) and was consistent with being a pyruvoyl group ($C_3H_3O_2$) (Figures S4 and S5).



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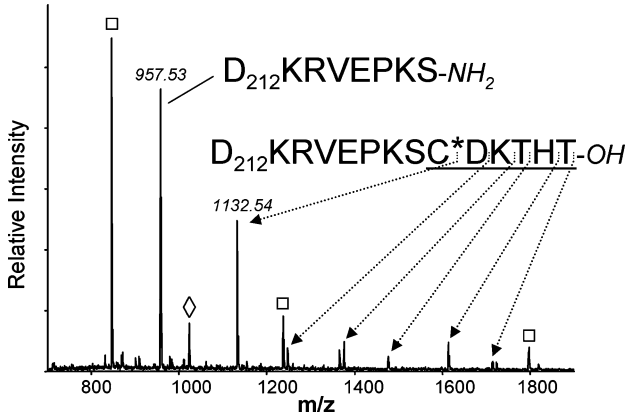
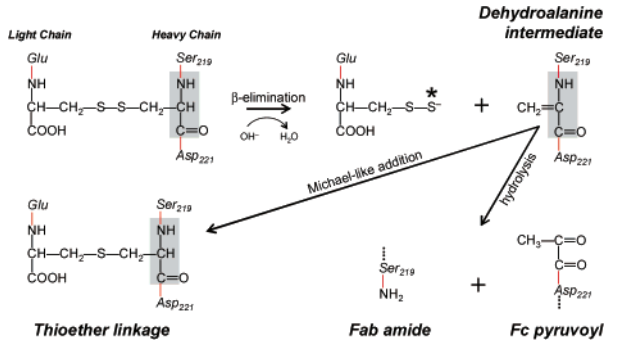



***Figure 2.*** C-terminal amide confirmed by MALDI-TOF-MS; Asp-N digest of the Fab HC fragment; portion of the spectrum showing the peptide $D_{212}$−$S_{219}$ with C-terminal amide and peptide "ladder" of $D_{212}$−$C_{220}$ to $D_{212}$−$T_{225}$ with C-terminal hydroxyl. Mass values on peaks are monoisotopic $(M + H)^+$. C* = Cys modified with acrylamide; □ = other HC Asp-N peptides; ◇ = Asp-N autolysis peptide.

***Scheme 1.*** Proposed Pathways Leading to the Degradation of the LC−HC Disulfide by $\beta$-elimination.[a]




[a] Shading denotes the HC $C_{220}$ backbone; ∗ = the exact nature of the LC following the $\beta$-elimination/hydrolysis pathway is under investigation.

Thus our data confirm peptide-bond hydrolysis of the hinge but point to a different pathway leading to scission of the HC $S_{219}$−$C_{220}$ bond upon antibody storage. The C-terminal amide (on the released Fab fragment) and the pyruvoyl group (on the complementary Fc fragment) are recognized hydrolysis products of the unsaturated amino acid dehydroalanine,[7−11] a residue not known to be present in the native antibody but likely derived from a $\beta$-elimination mechanism.

Under appropriate conditions disulfide bonds can undergo $\beta$-elimination, transforming one of the participating cysteine residues into dehydroalanine.[11−14] Dehydroalanine is relatively unstable and susceptible to electrophilic addition reactions.[12,15] Alternatively, hydrolysis of the dehydroalanine ∼NH-αC∼ bond can occur yielding two products: an N-terminal peptide amide fragment and C-terminal peptide fragment beginning with a pyruvoyl group (Scheme 1).[7−11] Our observations thus far conform to the latter mechanism: (1) $\beta$-Elimination rupturing of the LC−HC disulfide and subsequent hydrolysis of the dehydroalanine would explain the presence of the noncovalent Fab fragment (Figure 1C). (2) Two products expected from (and unique to) the hydrolysis of dehydroalanine were detected: an N-terminal Fab fragment ending with $S_{219}$ amide (Figure 2) and a C-terminal Fc fragment with a pyruvoyl group in place of the original HC $C_{220}$ (Figures 1D and S5). (3) $\beta$-Elimination is initiated by deprotonation of the Cys α-carbon by hydroxide ion so is favored at elevated pH. Our results are consistent with this trend since the extent of cleavage of the $S_{219}$−$C_{220}$ bond (compared to the other hinge cleavages) was significantly higher at elevated pH.

Another feature observed from the incubation study is the detection of a thioether-linked antibody (Figure S3). This linkage was recently elucidated to be between LC $Cys_{219}$ and HC $Cys_{220}$[4] though its mechanism of formation remains unspecified. Our pH studies also showed the extent of thioether-linked antibody significantly elevated at higher pH. We thus propose that the thioether linkage originates from a $\beta$-elimination occurring at the LC−HC disulfide followed by dehydroalanine formation. However, unlike in the case of the $S_{219}$−$C_{220}$ cleavage, the fate of the dehydroalanine follows a more common pathway associated with this residue, namely a Michael addition, and in this instance, with the adjacent LC C-terminal Cys sulfhydryl to form a nonreducible lanthionine (thioether) linkage (Scheme 1).[12,15]

Therapeutic monoclonal antibodies represent a rapidly growing field with sales projected to reach $30 billion by 2010.[16] Detailed analytical characterization and understanding of their stability and degradation processes is a fundamental part of their successful development. Biophysical methods combined with mass spectrometry allowed us to resolve puzzling questions concerning the hinge degradation of IgG1 antibodies, revealing a central role of the $\beta$-elimination mechanism. Judicious use of these powerful analytical technologies should bring even deeper insight into the degradation of these large macromolecules and lead to improved modalities of their formulation.

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**Supporting Information Available:** Materials and methods; LC/MS of the Fab and thioether fragments; tandem-MS of the Fab amide fragment and the Fc fragments. This material is available free of charge via the Internet at http://pubs.acs.org.

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