Breaking the Light and Heavy Chain Linkage of Human Immunoglobulin G1 (IgG1) by Radical Reactions

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We report that the production of hydrogen peroxide by radical chain reductions of molecular oxygen into water in buffers leads to hinge degradations of a human IgG1 under thermal incubation conditions. The production of the hydrogen peroxide can be accelerated by superoxide dismutase or redox active metal ions or inhibited by free radical scavengers. The hydrogen peroxide production rate correlates well with the hinge cleavage. In addition to radical reaction mechanisms described previously, new degradation pathways and products were observed. These products were determined to be generated via radical reactions initiated by electron transfer and addition to the interchain products were determined to be generated via radical reactions.

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2 The abbreviations used are: SOD, superoxide dismutase; HC or H, heavy chain; LC or L, light chain; SEC, size exclusion chromatography; RP, reversed-phase.
FIGURE 1. Schematic illustration of the interchain disulfide bond between the heavy and light chain of an IgG1. The hinge cleavage sites that were reported previously (3–8) are indicated by the arrows. The interchain disulfide bond is connected by Cys215LC and Cys225HC. Cys215 LC is the primary radical site for hydroxyl radical attack. The amino acid sequence suggests that the Lys-C and Asp-N proteases are appropriate for peptide mapping (see “Results” for details).

In addition to the previously observed Fab fragments (8, 18), new fragments were observed (e.g. Glu1–Ser224 and Glu1–Cys225 of the heavy chain (HC) and Asp1–Glu214 and Asp1–Cys215 of the light chain (LC)). Among them, an additional sulfur group was attached to the side chain of the C-terminal Cys215LC and Cys225HC in the two fragments, respectively. In particular, a -CONH2 rather than a -COOH at the C termini of the fragments of the Glu1–Cys225, Glu1–Ser224, and Asp1–Glu214 was determined. These fragments were not the products of simple reduction of the interchain disulfide bond; rather, they were formed by radical reaction pathways that were not determined in mAbs previously. The results from analyzing the products allows us to propose new radical reactions that break the C–S bond of the side chain of Cys225HC or Cys215LC, yielding a sulfur radical adduct to the side chain of Cys215LC or Cys225HC and dehydroalanine in the counterpart. Subsequent radical reactions and hydrolysis reactions lead to cleavage of the peptide bond, yielding an amide at the new C terminus. Also, because H2O2 is the inevitable by-product of radical reactions in buffer systems, related strategies of how to improve the stability of mAbs is discussed.

EXPERIMENTAL PROCEDURES

Material—The antibody used in this study is a recombinant fully human antibody of the IgG1 subclass. The molecule was expressed in Chinese hamster ovary (CHO) cells and chromatographically purified at Genentech, Inc. Examination of the primary sequence indicates that the antibody contains Glu at the N terminus of the HC, and an Asp at the N terminus of the LC.

Thermal Incubation of the IgG1—The IgG1 was buffer-exchanged into PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) and then incubated at 37 °C, with or without 5 μM Cu2+ or Fe3+ or SOD (200 units). Catalase (1000 milliunits; Sigma) was added to quench the reaction. In some cases, EDTA (50 mM) or 5,5-dimethyl-1-pyrroline N-oxide (100 mM) was added into the sample prior to the addition of Cu2+, Fe3+, or SOD.

Detection of H2O2 Production—H2O2 production was determined using the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen), as described previously (19, 20). The buffer with or without Fe3+ or SOD was incubated at 37 °C and then mixed with Amplex Red working solution and incubated at room temperature, protected from light. The H2O2 production was measured by fluorescence signals. Fluorescence emission excited at 545 nm was detected at 590 nm. All assays were performed in six replicates, and the results were reported as the average.

SEC Analysis and Purification of the Degradation Products—The degraded products were separated by size exclusion chromatography (SEC) on TSK G3000SWxl dual columns, 7.8 × 300 mm, at a flow rate of 0.5 ml/min. Eluting protein was monitored at 280 nm. The SEC running buffer contained 250 mM potassium phosphate, 200 mM potassium chloride, pH 6.2. The cleavage was measured by the relative percentage of the integrated peak area of partial molecules. SEC fractionation was carried out on an Agilent 1200 HPLC system (Agilent Technologies) equipped with a fraction collector. Purified fractions were pooled and concentrated by centrifugation in Millipore Centricon YM-30 filter units with a 10,000 molecular weight cut-off (Millipore, Billerica, MA).

Reversed-phase Chromatography and Time-of-flight Mass Analysis (RP-HPLC-TOF/MS)—RP-HPLC-TOF/MS was performed as described previously (7). Briefly, antibody samples were diluted to 1 mg/ml in 50 mM Tris-Cl (Sigma) at pH 8.0 with a final concentration of 3 μM guanidine hydrochloride (Mallinckrodt). RP-HPLC was performed on an Agilent 1200 HPLC system. The mobile phase included water with 0.11% trifluoroacetic acid (TFA) as solvent A and acetonitrile (Burdick Jackson) with 0.09% TFA as solvent B. A Varian PLRP-S (Varian, Inc., Palo Alto, CA), 4.6 × 50-mm, 8-μm particle size, 1000-Å pore size column was used for the RP-HPLC-TOF/MS analysis. The column eluent was analyzed by UV detection at 215 nm and then directed in-line to a TOF mass spectrometer. The initial mobile phase was 25% solvent B for 5 min, and then a two-stage gradient was applied: 2% solvent B per min from 25 to 30% solvent B, followed by 0.3% solvent B per min from 30 to 42% solvent B. The separation was performed at 75 °C at a flow rate of 0.5 ml/min. Electrospray ionization TOF/MS was performed on an Applied Biosystems QSTAR Elite XL mass spectrometer equipped with an Agilent 1200 HPLC system. The electrospray ionization mass spectra were analyzed using BioAnalyst protein deconvolution software (Applied Biosystems).

Protease Digestion and Peptide Maps—The IgG1 was denatured in the presence of 4 μM guanidine hydrochloride for 5 min at 75 °C. Prior to digestion, samples were buffer-exchanged into 50 mM Tris-HCl, pH 8.0, using Bio-Spin 6 columns (Bio-Rad) according to the manufacturer’s instructions. Recombinant sequencing grade Asp-N or Lys-C (Roche Applied Science) was added to samples at an enzyme/protein ratio of 1:10 (w/w), and the samples were digested at 37 °C overnight. Analytical peptide maps consisted of loading 50 μg of the digest onto a Phenomenex Jupiter Proteo C18 column, 2.0 × 250 mm, heated at 55 °C. The separation was performed by gradient elution on an Agilent HP 1200 HPLC system. The column was held at the initial condition of 0.5% solvent A (0.11% trifluoroacetic acid in water) at a flow rate of 0.3 ml/min for 5 min, and then the digest was eluted with a linear gradient to 60% solvent B (0.09% trifluoroacetic acid in acetonitrile) over 160 min. The peptides

at room temperature, protected from light. The H2O2 production was measured by fluorescence signals. Fluorescence emission excited at 545 nm was detected at 590 nm. All assays were performed in six replicates, and the results were reported as the average.
were identified by data-dependent tandem MS fragmentation using a Thermo LTQ Orbitrap mass spectrometer.

RESULTS

Detection of H₂O₂ as the Product of O₂ Reduction in the Buffer System—Molecular oxygen (O₂) has two unpaired electrons, one on each oxygen atom. With the electron spin restriction, the univalent reduction of O₂ to O₂⁻ is a facile process (reaction 1 in Scheme 2). In fact, the reduction of O₂ into more reactive species like H₂O₂ and •OH has been determined in a biological system (16, 17, 21). However, there is no report showing the presence of the O₂⁻ and its derivatives in a buffer system, probably due to the lack of an easy and direct assay to measure the O₂⁻ in a quantitative way.

We found that the chain reaction of the reductions of the O₂ also takes place in buffer solutions. Evidence in support of this reaction was obtained from the measurement of H₂O₂ production using a method as described previously (19, 20). The rate of the H₂O₂ production is slow, but it can be accelerated by SOD and/or Fe³⁺ to a level that can be easily detected (19, 20) (Fig. 2A). The production of H₂O₂ in PBS buffer is very limited because no evidence for a continuing accumulation of the H₂O₂ was obtained during a 3-week incubation. In addition, the level of the H₂O₂ production varies, depending on the buffer age. Similar observations were obtained from other buffers, such as Tris-HCl and acetate (not shown). We found that redox metal ions (e.g. Cu²⁺ or Fe³⁺) are required because EDTA blocks the H₂O₂ production (Fig. 2A). Collectively, these observations suggest that the chain reactions in Scheme 1 can take place via the following reactions in Scheme 2; the O₂⁻ in a buffer can be converted into the H₂O₂ under catalysis of redox metal ions (reactions 2 and 3) or by SOD (reaction 4). Consequently, the hydroxyl radical can be produced via the Fenton-like chemistry (reaction 5).

Thermal Incubation Generates Hinge Cleavage of an IgG1—Critical evidence linking the presence of O₂⁻/H₂O₂ to the hinge cleavage of an IgG1 was obtained from analysis of an IgG1 incubated in phosphate-buffered saline at 37 °C, using the same conditions as above. The products of the hinge cleavage were measured by SEC. As shown in Fig. 2B, conditions under which more H₂O₂ was produced resulted in more cleavage products. Incubation of the IgG1 in PBS for 15 days induced a 3.7% level of hinge cleavage, whereas the addition of 5 μM FeCl₃ into the reaction resulted in an increase of cleavage to 9.7%. A similar increase in cleavage was observed with the addition of CuSO₄ (not shown). In addition, SOD was also capable of accelerating the hinge cleavage. Incubation of the IgG1 with both Fe³⁺ and SOD resulted in a cleavage level of ~17%, and inclusion of Cu²⁺ further increased the cleavage to ~32%. However, EDTA was found capable of inhibiting >95% of the metal ion-induced cleavage, implying an involvement of the transitional metal ions in the reaction. In addition, all cleavage reactions could be stopped by catalase or blocked in the presence of 5,5-dimethyl-1-pyrroline N-oxide, a spin radical trap. Collectively, these results suggest an involvement of H₂O₂ in the reactions and that the radical reaction mechanism may be responsible for the hinge cleavage, as described previously (8).

An IgG molecule is itself capable of generating H₂O₂ (19, 20), which may lead to radical reactions that result in hinge cleavage. However, the self-produced H₂O₂ has been determined to generate at the interface of the heavy and light chains with a

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\begin{align*}
O_2 + e^- &\rightarrow O_2^- \quad (1) \\
O_2^- + Fe^{3+} &\rightarrow O_2 + Fe^{2+} \quad (2) \\
O_2^- + Fe^{2+} + 2H^+ &\rightarrow H_2O_2 + Fe^{3+} \quad (3) \\
2O_2^- + 2H^+ &\rightarrow H_2O_2 + O_2 \quad (4) \\
Fe^{2+} + H_2O_2 &\rightarrow \cdot OH + \cdot OH + Fe^{3+} \quad (5)
\end{align*}
\]

SCHEME 2. Reactions to produce hydroxyl radical.

FIGURE 2. The correlation between H₂O₂ production and hinge cleavage. A, H₂O₂ production in PBS buffer at pH 7.4 and 37 °C for 15 days. For clarity, the H₂O₂ production in the presence of EDTA (20 μM) + SOD (200 units) was set as a reference base level. The production of H₂O₂ was determined by the Amplex Red method using fluorescence signals (19, 20). B, hinge cleavage of an IgG1 under the same incubation conditions as in A. The cleavage was shown as a relative percentage of the peak area of the products to the whole molecule at 37 °C for 15 days in the PBS buffer. The cleavage can be accelerated by the addition of Fe³⁺ (5 μM), or SOD (200 units) but inhibited by 5,5-dimethyl-1-pyrroline N-oxide (10 μM) or EDTA (20 μM). Results represent the mean of six replicates.
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FIGURE 3. Analysis of the hinge cleavage products. The cleavage products were generated by incubating of the IgG1 in PBS buffer with 5 μM FeCl3 at 37 °C for 15 days, and then the Fab and partial molecule were purified by SEC. A, SEC separation profile with three separated peaks, labeled Main, Partial, and Fab. B, RP-HPLC UV profile from analysis of the Fab under non-reducing conditions. Peaks L (1), Fd (2), and Fab (3) were characterized by TOF/MS (Fig. 4), and the results were summarized in Table 1.

relative steady production rate (19, 22–24). Thus, the fact that redox metal ions and/or SOD mediate the H2O2 production and the hinge cleavage excluded the possibility that the self-produced H2O2 was the main force to drive the hinge cleavage.

Analysis of the Cleaved Fab by RP-HPLC-TOF/MS—Although the radical mechanism may be responsible for the hinge cleavage, it remains to be determined if the thermal induced degradation generates the same products as those produced by H2O2 mediated reactions. Garrison (14), Shimazu and Tappel (25), and Buxton et al. (26) had demonstrated that the products of radical reactions could be quite different under different reaction conditions. Characterization of the products would help to address these questions. To this end, cleavage products of the partial IgG1 and Fab were purified by SEC (Fig. 3A), and the Fab was first analyzed by RP-HPLC-TOF/MS under non-reducing conditions. Fig. 3B shows the RP-HPLC profile with three major products labeled by peaks 1, 2, and 3. TOF/MS analysis revealed that the peak 1 eluting at 16.5 min derived from the LC, which was characterized by multiple peaks with masses of 23,256.2, 23,360.5, 23,392.1, 23,408.4, 23,424.2, and 23,439.8 Da, respectively. The 23,256.2 Da corresponds to the mass of a fragment of Asp1–Ser214, and the other peaks correspond to the masses of the LC, the LC with +32, +48, +64, and +80 Da adducts, respectively (Fig. 4A and Table 1). However, under reducing conditions, the L showed four peaks with molecular masses of 23,364.0, 23,379.4, 23,396.0, and 23,412.7 Da, respectively (Fig. 4B), which correspond to the fragment of Glu1–Ser224, the LC, and the LC with +16 Da, +32 Da, and 48 Da adducts. The absence of the +64 and +80 Da adducts under the reducing conditions suggests that these two adducts were disulfide bond-related, implying that the +32 Da adduct observed under non-reducing conditions may be the result of gaining a sulfur group that was attached to the side chain of Cys. The peptide mapping confirmed this conclusion (see below). It has been known that atmospheric oxygen is capable of oxidizing the free -SH group of Cys (27, 28). Earlier studies indicated that Cys-SH is heat-labile and can be oxidized into Cys-SOH and Cys-SO2H; these two products have been found to be stable and can be converted into Cys-SO3H as the ultimate product (29, 30). It is possible that the observed adducts of +16, +32, and +48 Da derive from oxidation during RP-HPLC-TOF/MS or sample processing, and these adducts were added on to the extra sulfur group on the LC.

Analyzing the Fab portion of the H (Fd) under non-reducing conditions revealed very similar results. The Fd species eluting at 23.3 min was a mixture of two components with masses of 23,375.7 and 23,557.4 Da (Fig. 4C), corresponding to Glu1–Ser224 and Glu1–Cys225 with a +78 Da adduct, respectively. Peptide mapping analysis indicated that the +78 Da extra mass was attributable to an additional SO3 group at the side chain and a -CONH2 at the C terminus of Cys225 (see below).

Unlike peaks 1 and 2, the Fab peak 3 eluting at 24 min in the RP-HPLC profile showed a ladder of cleavage sites with the upper hinge residues of Cys225–Asp226–Lys227–Thr228–His229 at the C-terminal ends (Fig. 4D). The UV signal-based peak integration revealed a 1:1 ratio for the peak 3 (Fab) to the sum of the peaks 1 (LC) and 2 (Fd), suggesting that half of the products were generated by the expected breakage of the H-L bond under the thermal incubation conditions.

RP-HPLC-TOF/MS Analysis of the Fab after Additional H2O2 Treatment—Compared with the H2O2-mediated radical reactions that take place under high oxygen tension, thermal incubation-induced degradations take place under low oxygen tension due to the very slow rates of the production of H2O2 and consumption of O2 to produce the H2O2. Thus, it remained to be determined if the H-L bond could be directly attacked by hydroxyl radical, leading to breakage of the interchain linkage at the side chain of Cys. To explore this possibility, the effect of H2O2 on the purified Fab was examined to determine if any additional breakage of the H-L or cleavage of residues around C-terminal Cys occurred. The Fab at −0.47 mg/ml was treated with 20 mM H2O2 in a molar ratio of H2O2/Fab at 2000:1 at 25 °C for 6 days and then analyzed by RP-HPLC-TOF/MS. No difference in the UV profile or the results of TOF/MS analysis was observed before and after the H2O2 treatment (not shown), implying that Cys225HC/Cys215LC in the Fab may not be the direct target for H2O2-mediated radical reaction.

Characterization of the Products of the Hinge Cleavage by Peptide Maps—Finding fragments of Glu1–Ser224 and Glu1–Cys225 with an additional sulfur group at Cys225HC and of Asp1–Glu214 and Asp1–Cys215 of the LC with an additional sulfur group at Cys215LC highlights the unique degradation pathways around the H-L bond. If these fragments are derived from radical reactions, the C-terminal residues at some prod-
ucts should be an amide (-NH₂) rather than -COOH according to radical reaction mechanisms (8, 18). To this end, Lys-C and Asp-N peptide mapping were conducted under non-reducing conditions to monitor the C-terminal peptides at the LC and Fd. As expected, no adducts of 11001 or 11003 Da were detected at residues except for the C-terminal Cys from the LC or Fd. As shown in Fig. 5A, three forms of the C-terminal peptide of the LC elute at /H11011 21, 22, and 23 min in the Lys-C map, which correspond to SFNRGEC215 with a 11001 adduct at 21 min, the same peptide with a 11003 Da adduct at 22 min, and the peptide SFNRGE214 at 23 min, respectively. The observation of the series of b ions (b4, b5, and b6) and y ions (y4 and y5) provides the necessary data to assign the location of the 11001 adduct at the side chain of Cys215, as shown in Fig. 6A. Similarly, the 11003 Da was assigned to the side chain of Cys215 by the b ions (b2, b4, and b6) and y ions (y2 and y5) (Fig. 6B). Combining the results from RP-HPLC-TOF/MS, the 11001 and 11003 Da adducts could be explained by a -SOH and -S group at the side chain of Cys215. Fig. 6C shows the MS/MS spectrum of the peptide SFNRGE214, a truncated form of SFNRGEC215. The observed y ions (y3, y4, and y5) clearly indicate a CONH₂ group, rather than COOH, at the C terminus. Among these three forms of the

| Residues (start-end) | Theoretical mass | Observed mass | Difference | Proposed modification |
|----------------------|------------------|---------------|------------|-----------------------|
| Asp¹–Cys215          | 23,359.9         | 23,360.5      | 0.6        | +S                    |
|                     |                  | 23,392.1      | 32.3       | +SOH                  |
|                     |                  | 23,408.4      | 48.5       | +SO₂H                 |
|                     |                  | 23,424.2      | 64.3       | +SO₃H                 |
|                     |                  | 23,439.8      | 79.9       |                       |
| Asp¹–Ser214          | 23,256.0         | 23,256.2      | 0.2        |                       |
| Glu¹–Ser224          | 23,376.1         | 23,375.7      | −0.4       |                       |
| Glu¹–Cys225          | 23,479.4         | 23,557.4      | 78.0       | +SO₃                  |
| L + Glu¹–Cys225      | 46,839.2         | 46,839.1      | −0.1       |                       |
| L + Glu¹–Asp²26      | 46,954.3         | 46,954.0      | −0.3       |                       |
| L + Glu¹–Lys²27      | 47,082.5         | 47,082.1      | −0.4       |                       |
| L + Glu¹–Thr²28      | 47,183.6         | 47,182.9      | −0.7       |                       |
| L + Glu¹–His²29      | 47,320.7         | 47,319.6      | −1.1       |                       |
| Fab                  |                  |               |            |                       |
| Asp¹–Cys215          | 23,363.9         | 23,364.0      | 0.1        | -OH                   |
|                     |                  | 23,379.4      | 15.5       |                       |
|                     |                  | 23,396.0      | 32.1       | O₂H                  |
|                     |                  | 23,412.7      | 48.8       |                       |
|                     |                  | 23,260.0      | 0.0        |                       |

*Masses listed are for fragments with -CONH₂ at the C terminus.

FIGURE 4. TOF/MS analysis of the cleaved Fab. The deconvoluted mass spectra of peaks 1–3 from Fig. 3A are presented in A, C, and D, respectively. B, the deconvoluted mass spectrum of the reduced LC. Each major peak is labeled with the observed mass, and corresponding fragments are summarized in Table 1.

TABLE 1
The average masses of the Fab fragments analyzed by RP-HPLC-TOF/MS

| Residues (start-end) | Theoretical mass | Observed mass | Difference | Proposed modification |
|----------------------|------------------|---------------|------------|-----------------------|
| Non-reducing conditions |
| LC                   |                  |               |            |                       |
| Asp¹–Cys215          | 23,359.9         | 23,360.5      | 0.6        | +S                    |
|                     |                  | 23,392.1      | 32.3       | +SOH                  |
|                     |                  | 23,408.4      | 48.5       | +SO₂H                 |
|                     |                  | 23,424.2      | 64.3       | +SO₃H                 |
|                     |                  | 23,439.8      | 79.9       |                       |
| Asp¹–Ser214          | 23,256.0         | 23,256.2      | 0.2        |                       |
| Glu¹–Ser224          | 23,376.1         | 23,375.7      | −0.4       |                       |
| Glu¹–Cys225          | 23,479.4         | 23,557.4      | 78.0       | +SO₃                  |
| Fab                  |                  |               |            |                       |
| Asp¹–Cys215          | 23,363.9         | 23,364.0      | 0.1        | -OH                   |
|                     |                  | 23,379.4      | 15.5       |                       |
|                     |                  | 23,396.0      | 32.1       | O₂H                  |
|                     |                  | 23,412.7      | 48.8       |                       |
| LC (reducing conditions) |
| Asp¹–Cys215          | 23,260.0         | 23,260.0      | 0.0        |                       |
C-terminal peptide of the LC, it appears that the truncated form accounts for $\sim$25%, whereas the full-length modified forms account for $\sim$75% of the C-terminal peptide (Table 2).

In contrast to the LC, the C-terminal peptide of FD contains more of the truncated form DKKVEPKS, approximately $\sim$75%, which eluted at 17.8 min in the Asp-N map, as shown in Fig. 5B. The minor form of the C-terminal peptide DKKVEPKSC, eluting at 20.7 min, accounted for $\sim$25%, with a +79 Da adduct. The MS/MS spectrum provides clear evidence that the +79 Da adduct (-SO$^3_3$) is located at Cys$^{225}$. The most dominant ion of m/z 557.33 was observed in the full spectrum, which corresponds to the [MH-SO$^3_3$]$^{2+}$ form. In addition, the observed b ions (b5 and b7) and y ions (y2, y3, and y7 ions and y4-SO$^3_3$, y6-SO$^3_3$, and y7-SO$^3_3$) indicate that the -SO$^3_3$ group is located at the side chain of Cys$^{225}$ with a -CONH$_2$ at the C terminus (Fig. 6D). Fig. 6E shows the MS/MS spectrum of the truncated peptide DKKVEPKS, and a series of y ions (y3 and y5–y7) and b ions (b4–b7) clearly indicate an amide at the C terminus.

Table 2 summarizes the quantitative information of each form of the C-terminal peptides. The fact that more truncated forms of the L corresponded to more Fd fragments with an extra -S at Cys$^{225}$, or vice versa, suggests that these products were generated through a loss or gain of the sulfur group at the side chain between these two Cys residues. In other words, a fragment without Cys in the C terminus could be the result of the gain of a sulfur group at the side chain of Cys in the counterpart. The truncated fragment may be explained by hydrolysis reactions at the dehydroalanine formed by the loss of the sulfur group at Cys as described previously (14). Finding a mixture of -SOH and -S adducts at the side chain of Cys$^{215}$ implied that these products were probably generated by limited oxidation under low oxygen tension. Collectively, these observations indicated that half of the products are the disulfide-bonded population with the ladder cleavages of the C-terminal residues in the Fd, as described previously under high oxygen tension (7), and the other half are non-disulfide-bonded LC and Fd fragments that were generated by the unique breakage of the H-L bond under low oxygen tension.

Analysis of the Partial IgG1 and Main Peak Purified from SEC Isolation by RP-HPLC-TOF/MS — The H$_2$O$_2$-mediated radical reactions under high oxygen tension also release the LC species by the reduction of the H-L bond (8). If such reduction takes place under the thermal incubation conditions, a released LC from the partial molecule or the main peak would be expected. To this end, the partial IgG1 and main peak were analyzed under non-reducing conditions by RP-HPLC-TOF/MS. As shown in Fig. 7A, the non-disulfide-bonded LC eluted at 16 min in the RP-HPLC profile. The level of the LC varied in the samples; the control sample (non-treated sample) showed a level of 1.6%, whereas the main peak and the partial IgG1 showed an increase to $\sim$2.3 and 2.5%, respectively. TOF/MS analysis indicated molecular masses of 23,360.6 and 23,392.0 Da for the control sample, corresponding to a reduced form of the LC and the LC with a +32 Da adduct (Fig. 7B). The main peak revealed three species with masses of 23,256.9, 23,360.6, and 23,391.7 Da, corresponding to Asp$^1$-Ser$^{114}$, a reduced form of the L, and the reduced LC with sulfur adduct, respectively (Fig. 7C). However, the partial IgG1 showed multiple species that are similar to the LC of the cleaved Fab, with the main component as the reduced form of the LC (Fig. 7D). The additional species in the partial IgG1 and main peak that are similar to those in the cleaved Fab suggested that the thermal incubation-induced radical breakage of the H-L linkage could occur without any cleavage of the backbone.

**DICUSSIONS**

Radical Reaction Mechanism for the Thermal Induced Hinge Degradation — Many different physiological and environmental processes can lead to the formation of H$_2$O$_2$ and O$_2^\cdot$, H$_2$O$_2$ has been found responsible for degradation of many proteins through radical reaction mechanisms (31–33). However, the O$_2^\cdot$ is a relatively unreactive species whose major type of reaction is reduction (i.e. donation of an electron) (21, 34). Estimated in vivo concentrations of H$_2$O$_2$ and O$_2^\cdot$ have been reported as 10$^{-7}$ to 10$^{-9}$ M and 10$^{-11}$ M, respectively (35–37), whereas these two species do not interact to produce hydroxyl radical (OH$^\cdot$) (38). In the chain reaction of the reduction of oxygen into water, the overall reaction rate is proportional to collision frequency, so O$_2^\cdot$ and H$_2$O$_2$ fluxes depend directly upon the ambient concentration of oxygen (15–17, 39). Thus, low oxygen tension may allow thermal incubation-induced radical reactions to take place via different routes and generate products of a different nature from those generated under high oxygen tension.

Like all chemical reactions that generate a product, the accumulation of the product (e.g. H$_2$O$_2$) results in inhibition of its further production. Even in the presence of a catalyst (e.g. SOD or redox metal ion), the production of H$_2$O$_2$ would be limited unless there is a continuous supply of oxygen and sequential consumption of the H$_2$O$_2$. In Reactions 1–5 of Scheme 2, the
regeneration of oxygen and H₂O₂ enables the degradation of an IgG1 hinge, which in turn consumes the H₂O₂ and 'OH. This may explain why an accumulation of the cleavage products was observed, although the buffer itself has a limited capacity to produce H₂O₂.

New Pathways for the Breakage of the Light and Heavy Chain Linkage—Observing the L and Fd with an extra sulfur at Cys²¹⁵ or Cys²²⁵ and fragments without these two Cys residues sheds light on new radical reaction pathways for the thermal incubation-induced degradation of the heavy-light chain linkage. It is reasonable to believe that the unique degradation of the H-L linkage also initiates by breaking the first hinge disulfide bond, and radical formation in one of the first hinge Cys and electron transfer leads to localization of the electron to the H-L bond. Although long range electron transfer has been observed in some proteins, none has shown an electron transfer capable of bypassing a disulfide bond.

FIGURE 6. MS/MS analysis of the peptide derivatives a, b, and c from Lys-C (A) and a and b from Asp-N (B) peptide maps. A, the C-terminal peptide (860.32 Da) of the LC with a SOH at the side chain of Cys²¹⁵ (SFNRGEC). B, the C-terminal peptide (844.32 Da) of the LC comprising a sulfur at the side chain of Cys²¹⁵ (SFNRGEC). C, truncated peptide (708.32 Da) of the LC(SFNRGE). D, truncated peptide (929.54 Da) of Fd with a CONH₂ at its C terminus (DKKVEPKS²⁴⁴). E, the C-terminal peptide (1113.50 Da) of Fd with a +80 Da adduct at the side chain of Cys²²⁵ and a CONH₂ at its C terminus (DKKVEPKSC) of Fd from the Asp-N peptide map.
bond (40–42). Thus, these fragments must be generated by new pathways via breakage of the H-L bond linkage and cleavage of the backbone. Based on the previous work described by others (13, 14, 43, 44), reaction of cystine (a disulfide bond) with an electron is known to be extremely rapid, with the reaction rate constant of $1.6 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$, faster than with \(\cdot \text{OH} \) at $2.1 \times 10^9 \text{M}^{-1} \text{s}^{-1}$ (13, 26). Similar to other proteins described previously (45, 46), an electron with elevated energy transferred from Cys^{231} to the H-L of an IgG1 results in reduction of the H-L bond under high oxygen tension (18). However, under low oxygen tension, electron addition to a residue leads to the formation of a radical anion that can undergo a number of reactions depending on the conditions and substrate (47, 48). Although the chemical consequences of e\(^{-}\) attachment to the disulfide bonds of proteins are not fully understood (14), it has been demonstrated that electron addition to the thiol groups of Cys and Met leads to efficient cleavage of the C-S bond with elimination of the -SH group to give a carbon-centered radical and a thiol anion (13, 26, 49). Such reactions are slow, with low yields of minor products arising from cleavage of the C-S-C link, but produce significant amounts of cleavage products under low oxygen tensions (13).

Based on the results presented in this study, we propose the following pathways for the degradation of the H-L linkage in an

### TABLE 2

| Peptide     | Adduct | Domain | C terminus | Percentage |
|-------------|--------|--------|------------|------------|
| SFNRGEC\(^{215}\) | SOH    | LC     | ND         | ~50        |
| SFNRGEC\(^{215}\) | -S     | LC     | ND         | ~25        |
| SFNRE\(^{214}\)   | LC     | -CONH\(^2\) | ~25        |
| DKKVEPKSC\(^{225}\) | SO\(_3\) | Fd  | -CONH\(^2\) | ~25        |
| DKKVEPKS\(^{224}\) |        | Fd  | -CONH\(^2\) | ~75        |

**FIGURE 7.** RP-HPLC-TOF/MS analysis of the partial IgG1 and main peak purified by SEC. A, RP-HPLC UV profile of the partial IgG1 and main peak under non-reducing conditions in comparison with the control reference sample (Ref, Partial, the partial IgG1; Main, the main peak purified by SEC. The LC was eluted at ~16 min, as indicated by an arrow. B, the deconvoluted spectrum of the LC from the control. C, the deconvoluted spectrum of the LC from the partial IgG1. D, the deconvoluted spectrum of the LC from the main peak.
IgG1. In the first step, electron addition to the H-L bond results in the formation of a disulfide bond radical anion (Mechanism 1, reaction 1). Decomposition of the anion results in breakage of the C–S bond of the side chain of Cys^{225}HC or Cys^{215}LC that is in the H-L bond (Mechanism 1, reactions 2a–2d). With the low oxygen tension, some sulfur groups are oxidized, and the rest remain in a reduced form. In the counterpart, the Cys that lost the sulfur group forms a carbon-centered radical that is the.

**Proposed pathways for the degradation of the H-L linkage in an IgG1.**

1. Electron addition to the H-L bond results in the formation of a disulfide bond radical anion.
2. Decomposition of the anion results in breakage of the C–S bond of the side chain of Cys^{225}HC or Cys^{215}LC.
3. With the low oxygen tension, some sulfur groups are oxidized, and the rest remain in a reduced form.
4. In the counterpart, the Cys that lost the sulfur group forms a carbon-centered radical.
same species as a result of hydrogen abstraction from the side chain of Ala, forming unstable dehydroalanine. Hydrolysis of the dehydropeptide yields an amide at the N-1 position of the new C terminus (Mechanism 1, reaction 3).

Meanwhile, as demonstrated by Garrison (14), the addition of $e^-$ to the carbonyls (-C=ONH-) of peptides in oxygen free solutions occurs in competition with $e^-$ addition to the disulfide bond (14); such competition results in the formation of a new intermediate radical species, C44(OH)NH-, at Cys (Mechanism 1, reaction 4). Dissociative deamination of this new radical species occurs along the N-C1 bond that cleaves the peptide bond to generate a -CONH2 at new C terminus (Mechanism 1, reaction 5). Such mechanisms have been described by others (25, 26, 49–52).

It should be pointed out that reaction 2d (Mechanism 1) only releases the LC without any cleavage of the HC because no radical remains at the side chain of Cys225 after decomposition of the radical anion. This reaction could also rationalize the observation of the LC and LC fragment in the partial IgG1 and main peak as shown in Fig. 7. Based on reactions 2a–2c (Mechanism 1), a theoretical ratio of 2:1 for the fragments of Glu1–Ser224 to Glu1–Cys225 in the Fd and Asp1–Glu215 to Asp1–Cys214 in the LC would be expected, which is not in good agreement with the observed ratio of 3:1. A possible explanation could be that, as indicated by Garrison (14), an electron radical addition to γ-carbon sites on the side chain of certain residues could occur and also lead to the formation of the dehydropeptide. In such a case, the side chain of Cys225HC would be the target for the electron addition, and the formed dehydroalanine would be hydrolyzed by reaction 3 (Mechanism 1), leading to backbone cleavage and producing a new amide at the N-1 position.

As indicated by Davies and Delsignore (34), radical attack results in gross distortions of secondary and tertiary structure of a protein, and such changes had been observed in an IgG1 under high oxygen tension (18). It is reasonable to believe that radical reactions under the thermal incubation conditions also alter local conformation or conformational dynamics of an IgG1. Although the distance between Cys231 and the H-L bond is a typical electron tunneling distance of 14 Å (18), the new local conformation or conformational dynamic in the upper hinge region could bring the H-L bond more proximal to Cys231, which would allow interaction between the electron and the H-L bond or electron transfer from the transient radical center His229 to the H-L bond (18). Collectively, our results demonstrated that the thermal incubation-induced degradation of the IgG1 hinge follows radical reaction mechanisms with a combination of pathways taking place under high and low oxygen tension that results in different products.

**Implication of H2O2 and O2 in the Development of mAb Therapeutics**—The thermal incubation-induced hinge degradation presents great challenges for the stability programs in the development of mAb therapeutics. Given the highly conserved hinge sequence in IgG1 molecules, radical reaction mechanisms that may compromise the stability, safety, and efficacy of an IgG1 need to be better understood and controlled appropriately. Because O2 and H2O2 species are the inevitable by-products of the reduction chain reaction of oxygen into water, new strategies are needed to minimize the impact of these species on the stability of an IgG1. Preventing metal ions from redox cycling is one mechanism to inhibit the production of hydrogen peroxide in the formulation development and during storage; this can be done by using reagents that have the least content of redox metal ions or by using metal-chelating reagents. Alternatively, substitution of the “hot spot” residues in the upper hinge is an attractive means of resisting radical-induced fragmentation based on our previous substitution results (18). Further investigation of the influence of the upper hinge residues and evaluation of some promising mutants could enrich our understanding of mAb degradation and provide new insights into how to engineer a new generation of therapeutic IgG1 that is capable of resisting such degradation around the H-L bond.

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**Breaking the Light and Heavy Chain Linkage of Human *IgG*1**

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