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Effect of the Conformational Stability of the CH2 Domain on the Aggregation and Peptide Cleavage of a Humanized IgG

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Abstract To examine the effect of the conformational stability of the CH2 domain on aggregation and peptide cleavage of a humanized IgG1, we carried out size exclusion chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses of incubated sample solutions. By comparing the residual percentage of monomer after incubation at 60 and 80°C at various pH levels, we found that aggregation and peptide cleavage of the humanized IgG1 occurred during long incubation at 60°C under acidic conditions. Next, we confirmed cleavage of the Asp272-Pro273 peptide bond in the CH2 domain. Comparison of the cleavage rates of the IgG1 monomer and a peptide containing the same Asp-Pro sequence revealed that the conformational stability of the CH2 domain retards cleavage of the Asp272-Pro273 peptide bond at 60°C and pH 4.0. The finding of aggregation and peptide cleavage of the humanized IgG1 after long incubation at 60°C under acidic conditions was supported by another finding: there were lower unfolding temperatures of the CH2 domain at pH 4.0 and 5.0. We conclude that the conformational stability of the CH2 domain is closely related to aggregation and peptide cleavage of the humanized IgG1 under acidic conditions. We also found that the 2-[N-morpholino] ethane sulfonate buffer inhibits aggregation of the IgG1 at pH 4.0–5.0 and 7.0–8.0.

Keywords pH · Protein deterioration · Higher-order structure · Structural stability · Asp-Pro peptide bond · Chemical cleavage · Unfolding temperature

Introduction

Immunoglobulin G (IgG) antibodies are protein molecules with a molecular weight of about 150 kDa that consist of two H chains and two L chains connected by disulfide bonds and

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non-covalent interactions. IgG molecules have two characteristic structural features: they are multi-domain proteins comprising two Fab domains and one Fc domain, and they have rich β-sheet structures. It is reported that the multiple characteristic transitions observed during heat denaturation of IgG molecules are the result of unfolding of each of its domains, successively [1–4]. The aggregation of IgGs in concentrated solutions is a serious problem for pharmaceutical applications because such applications require high dosages (typically ≥100 mg per body). In a previous paper [5], we reported the results of a study we conducted to search for superior storage conditions for a humanized IgG1 antibody at pH 5.5 or 6.5. In that study, we found lower aggregation in some types of buffer solution (2-[N-morpholino] ethane sulfonate [MES], 3-[N-morpholino] propanesulfonic acid, acetate and imidazole buffers) than in others (phosphate and citrate buffers).

Acidic pH conditions are widely used to elute IgG from Protein A affinity columns and to inactivate viruses during the purification of therapeutic antibodies. To clarify IgG aggregation mechanisms, many researchers have studied aggregation under acidic conditions. In one study, for example, it was found that changes in secondary and tertiary structures contribute to the aggregation of an IgG2 antibody that undergoes marked aggregation at pH 4.0 to 5.0 [6]. In another study, structural changes in two humanized antibodies under acidic conditions were researched in detail using differential scanning calorimetry (DSC), circular dichroism, and analytical ultracentrifugation [7]. The results of such studies suggest that the formation of denatured intermediates that expose hydrophobic surfaces contributes to the aggregation of IgG molecules. Furthermore, it has been reported that isomerization of Asp-Gly sequences [8, 9] and cleavage of Asp-Pro sequences [10] occur under weak acidic conditions, and it is known that deamidation of Asn residues can occur under physiological conditions [11]. These chemical changes may trigger the aggregation of humanized IgGs. These findings suggest that researchers should focus not only on the mechanisms of aggregation, but also on the chemical reactions of humanized IgGs. Therefore, investigation of aggregate formation and chemical degradation in various buffer solutions at various pH levels is important in relation to increasing the stability of IgG formulations in the liquid state.

Thus, in the study reported here, we explored the deterioration of a humanized IgG1 on storage at pH levels ranging from 4.0 to 9.0 by evaluating its aggregation and degradation in two different buffers. One was phosphate buffer, which is widely used in biochemical experiments; the other was MES, which we found to be a superior buffer for storage of the humanized IgG1 at pH 5.5 and 6.5 in our previous study [5]. We also investigated the effect of structural stability on the aggregation and chemical cleavage of the humanized IgG1 by evaluating its unfolding temperature and by peptide analysis of degradation products after incubation at each pH.

Materials and Methods

Materials and Reagents

The IgG molecule investigated in this study was a humanized IgG1 monoclonal antibody to human interleukin-6 receptor donated by Chugai Pharmaceutical (Tokyo, Japan). The TSK-gel G4000SWXL column (7.8×300 mm) used was purchased from Tosoh (Tokyo, Japan).

Preparation of the IgG1 Monomer Sample Solutions

We prepared IgG1 sample solutions from an IgG1 stock solution (50 mg/mL IgG1 in 15 mM sodium phosphate buffer, pH 6.5) by dialysis, pH adjustment, and dilution. The

prepared sample solutions contained 10 mg/mL (65 μM) of IgG1 monomer in 15 mM phosphate or MES buffer with pH values ranging from 4.0 to 9.0.

Assessment of IgG1 Stability in the Sample Solutions

To evaluate the stability of the IgG1 monomer in the sample solutions, the solutions were incubated at 60°C for 4 weeks or 80°C for 2 h. After incubation, the residual percentage of the IgG1 monomer and the percentage of soluble aggregates and degradates formed were evaluated by size exclusion chromatography equipped to high-performance liquid chromatography (SEC-HPLC) as follows: The unincubated sample solutions and the supernatants of incubated sample solutions (after centrifugation at 12,000 rpm for 5 min) were diluted with water to 1 mg/mL, applied to an HPLC system (Waters) with a TSK-gel G4000SWXL column, and then eluted with 50 mM phosphate buffer, pH 7.0, containing 0.3 M NaCl.

The purity of the IgG1 monomer in the sample solutions was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli [12] with the following modifications: The unincubated sample solutions and uncentrifuged incubated sample solutions, with or without 2-mercaptoethanol (a reducing agent), were diluted with buffer containing 10% SDS to obtain a final protein concentration of 100 μg/mL. The protein bands were stained with Coomassie brilliant blue. The stained gel was scanned using a photo scanner (Seiko Epson, Japan) to produce a high-resolution gray-scale image. The staining intensity of the monomer band in each sample was evaluated using "Scion Image" image analysis software (Scion Corporation, MD, USA) and the percentage of recovered monomer (unchanged monomer plus monomer released in the presence of SDS) in the sample solution was calculated from the ratio of the staining intensity in the incubated sample solution to the staining intensity in the corresponding unincubated sample solution.

N-terminal Amino Acid Sequence Analysis

We analyzed the N-terminal amino acid sequences of unknown bands in the reducing SDS-PAGE electropherograms by the Edman degradation method using Protein Sequencer 473A (Applied Biosystems, CA, USA), after transferring the bands to a PVDF membrane.

Evaluation of Asp-Pro Cleavage in the IgG and a Corresponding Peptide

A 10 mg/mL IgG1 solution in 15 mM acetate buffer (pH 4.0) was incubated for 1, 2, and 4 weeks. The intact H chain content was determined by densitometry of scanned SDS-PAGE electropherograms as described above, and the residual percentage of intact H chain was calculated.

A 15-residue peptide with the sequence, VDVSHEDPEVKFNWY, was prepared by Fmoc solid-phase peptide synthesis. This peptide was dissolved in 15 mM acetate buffer to give a concentration of 66.7 μM, which is of the same molar concentration as the 10 mg/mL IgG1 solution. The residual percentage of the peptide after incubation for 6, 12, 18, 24, and 36 h at 60°C was found by RP-HPLC using an HPLC system (Hitachi, Japan) with a TSK-gel ODS-120T (250 mm×4.6 mm-i.d.) column (Tosoh, Japan). The compositions of mobile phases A and B were (A) 1% acetonitrile and 0.05% hydrochloric acid and (B) 60% acetonitrile and 0.05% hydrochloric acid. Peaks were found by detection at 350 nm. A sample solution containing 3.3 nmol of peptide was injected, and the residual percentage of intact peptide was found from the ratio of the intact peptide peak areas in incubated and unincubated samples.

Differential Scanning Calorimetry

DSC was carried out using a VP-DSC calorimeter (MicroCal). Each unincubated IgG1 monomer sample solution was diluted to 0.5 mg/mL by adding the same buffer solution, and then a DSC scan was performed at a rate of 0.5°C/min from 40 to 105°C. Data were analyzed using Origin software (MicroCal).

Results

Aggregation of the IgG1 Monomer During Incubation

We evaluated the aggregation of the humanized IgG1 in phosphate or MES buffer solutions with pH values ranging from 4.0 to 9.0 after incubation at 60°C for 4 weeks or at 80°C for 2 h. After incubation, any insoluble aggregates that had formed were removed by centrifugation, and the solutions were analyzed by SEC-HPLC.

As in our earlier study [5], we found both a higher molecular weight peak (soluble aggregates) and a lower molecular weight peak (degradates) after incubation at 60°C, but only a higher molecular weight peak after incubation at 80°C (Fig. 1). The residual percentage of monomer and the percentage of soluble aggregates after incubation at 80°C

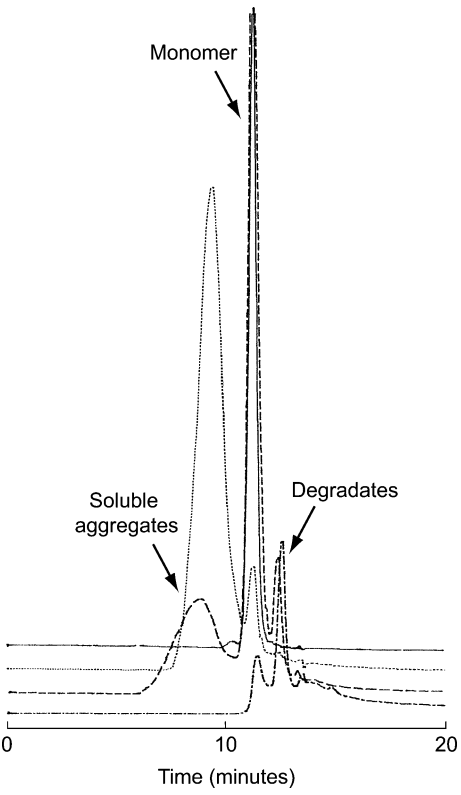

Fig. 1 Typical size exclusion chromatograms of the humanized IgG1 in 15 mM MES buffer (pH 4.0 or 6.5) before and after incubation. The *solid line* shows results found before incubation (pH 6.5); the *other lines* show results found after incubation under the following conditions: *dotted* 80°C for 2 h (pH 6.5); *broken* 60°C for 4 weeks (pH 6.5); *dashed* 60°C for 4 weeks (pH 4.0)

are shown in Fig. 2. The residual percentage of monomer and the percentages of soluble aggregates and degradates after incubation at 60°C are shown in Fig. 3.

After incubation at 80°C for 2 h, the residual percentage of monomer was relatively high at pH 4.0 and 5.0 but negligible at pH≥7.0. In contrast, the pH profile of the percentage of soluble aggregates was bell-shaped, with a maximum between pH 6.0 and 7.0. The highest residual percentage of monomer was about 90% at pH 4.0 and 5.0 in MES buffer.

After incubation at 60°C for 4 weeks, on the other hand, the pH profiles of the residual percentage of monomer and the percentage of soluble aggregates both showed maxima between pH 6.0 and 7.0 in both types of buffer solution. Furthermore, insoluble aggregates were observed in both types of buffer solution. This is consistent with the fact that the total percentages of monomer, soluble aggregates, and degradates do not add up to 100% at low or high pH levels.

SDS-PAGE Analysis of Incubated IgG1 Solutions

To evaluate whether the soluble aggregates form by covalent bonds and to find which IgG1 peptide bond(s) is cleaved, the incubated IgG1 solutions were analyzed by reducing and

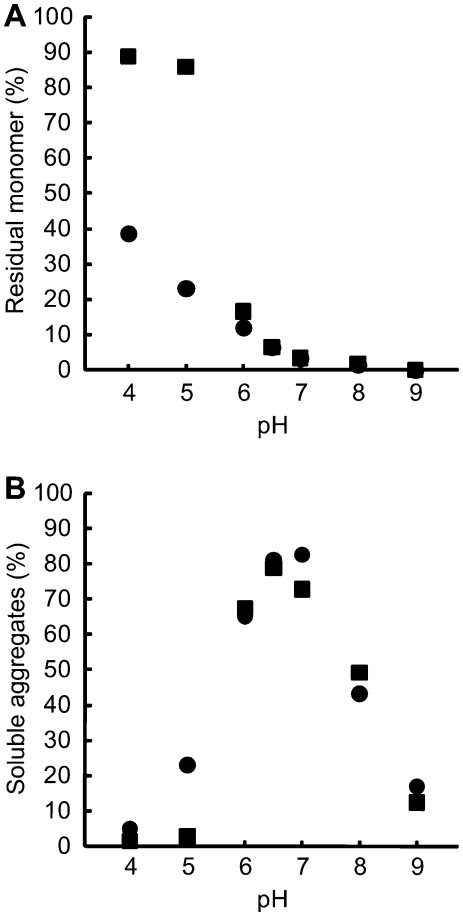

Fig. 2 The pH profiles of the residual percentage of monomer (**a**) and the soluble aggregate content (**b**) by SEC-HPLC after incubation of the humanized IgG1 in MES buffer (*filled square*) or phosphate buffer (*filled circle*) at 80°C for 2 h

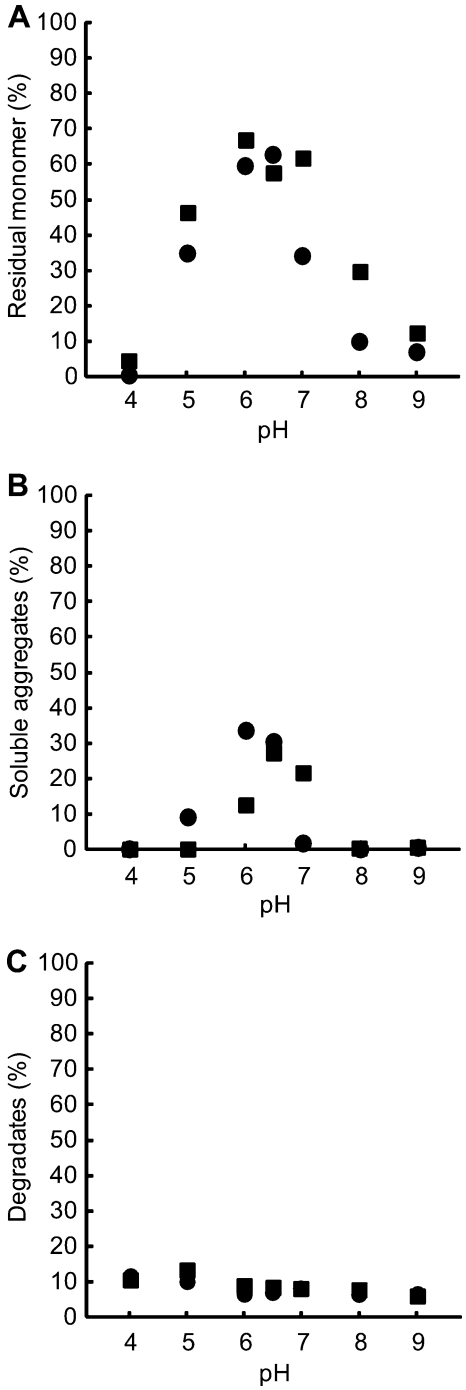

Fig. 3 The pH profiles of the residual percentage of monomer (**a**), the soluble aggregate content (**b**), and the degradate content (**c**) by SEC-HPLC after incubation of the humanized IgG1 in MES buffer (*filled square*) or phosphate buffer (*filled circle*) at 60°C for 4 weeks

nonreducing SDS-PAGE. Typical electropherograms are shown in Fig. 4. The IgG1 monomer band was detected at the same position as that of the molecular weight marker at about 200 kDa because of the presence of sugar chains.

After incubation at 80°C for 2 h, nonreducing SDS-PAGE revealed several bands at higher molecular weights than the monomer. Next, the percentage of recovered monomer was evaluated by densitometry. The percentage of recovered monomer decreased with increasing pH (Fig. 5). At pH 8.0 and 9.0, the overall band intensity decreased because of the formation of insoluble aggregates that remained insoluble even in the presence of SDS. From the viewpoint of difference in buffer types, in contrast to the SEC-HPLC, the percentages of recovered monomer contents in the MES and phosphate sample solutions were comparable. From these results, we concluded that the aggregates present after incubation at 80°C for 2 h in MES or phosphate buffer had mainly formed by non-covalent interactions rather than covalent bonds and that aggregates formed under acidic conditions were soluble in the presence of SDS. We also found that MES retarded aggregation during incubation at 80°C at pH 4.0 and 5.0. However, a single band (Unknown-1) was observed on the high molecular weight side of the H chain at pH 8.0 and 9.0 in reducing SDS-PAGE, suggesting the formation of nondisulfide covalent bonds.

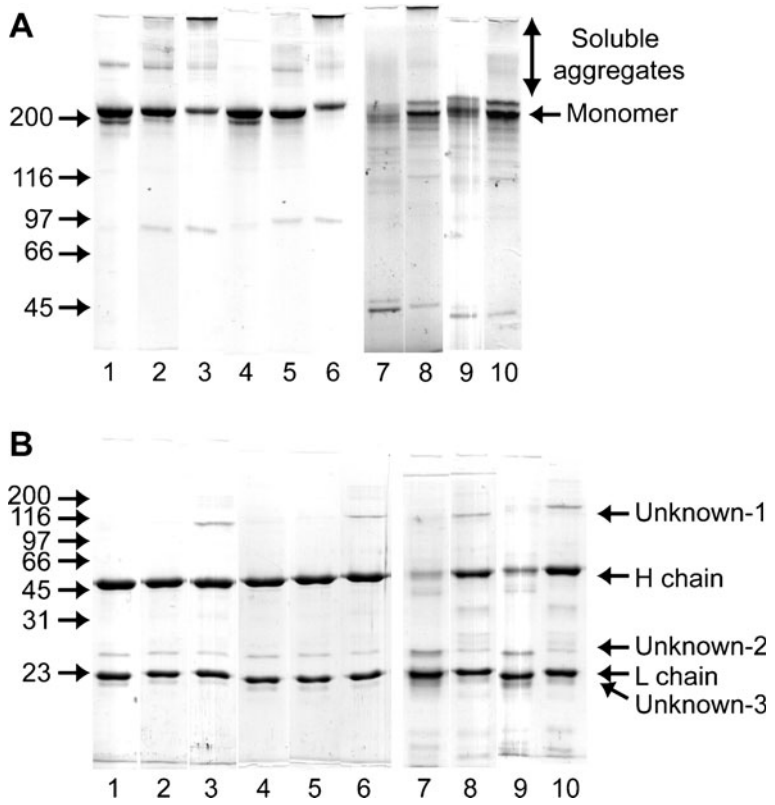

Fig. 4 Nonreducing (**a**) and reducing (**b**) SDS-PAGE electropherograms after incubation of the humanized IgG1. *Lanes 1–3* incubation at 80°C for 2 h in phosphate buffer at pH 4.0 (*L1*), 6.0 (*L2*), and 8.0 (*L3*). *Lanes 4–6* incubation at 80°C for 2 h in MES buffer at pH 4.0 (*L4*), 6.0 (*L5*), and 8.0 (*L6*). *Lanes 7 and 8* incubation at 60°C for 4 weeks in phosphate buffer at pH 4.0 (*L7*) and 6.0 (*L8*). *Lanes 9 and 10* incubation at 60°C for 4 weeks in MES buffer at pH 4.0 (L9) and 6.0 (L10)

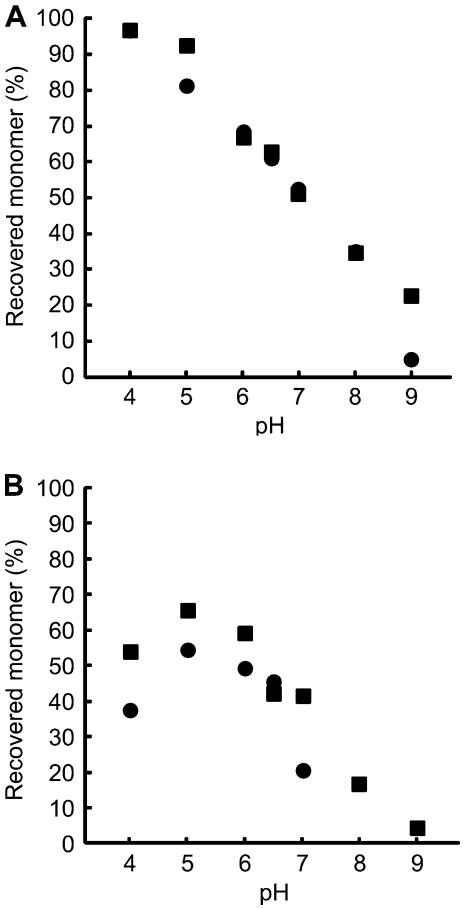

Fig. 5 The pH profiles of the recovered percentage of monomer by densitometry of scanned nonreducing SDS-PAGE electropherograms after incubation of the humanized IgG1 in MES buffer (*filled square*) or phosphate buffer (*filled circle*) at 80°C for 2 h (**a**) or at 60°C for 4 weeks (**b**)

After incubation at 60°C for 4 weeks, nonreducing SDS-PAGE showed a broad band on the high molecular weight side of the monomer. Densitometry revealed maximum monomer content in the pH range of 5.0 to 6.0 in both types of buffer (Fig. 5). At higher pH levels, most of the bands disappeared because of the formation of insoluble aggregates that remained insoluble even in the presence of SDS, as with incubation at 80°C for 2 h. At pH 4.0 and 5.0, on the other hand, the percentage of recovered monomer content in both types of buffer was higher than that detected by SEC-HPLC, indicating that the soluble and insoluble aggregates present after incubation at 60°C for 4 weeks at pH 4.0 and 5.0 had mostly formed by non-covalent interactions that could be broken by the addition of SDS, releasing monomer.

In reducing SDS-PAGE, as in samples incubated at 80°C, a single band (Unknown-1) was detected on the high molecular weight side of the H chain band. In samples incubated at 60°C for 4 weeks at pH 4.0, however, two further bands (Unknown-2 and Unknown-3) were detected, one on each side of the L chain (Fig. 4). The residual percentages of H and L chains in solutions incubated at pH 4.0, 5.0, 6.0, and 6.5 for 4 weeks at 60°C are shown in Fig. 6. The residual percentage of H chain decreased with decreasing pH in both types of buffer, whereas the residual percentage of L chain showed quantitative recovery at pH 4.0

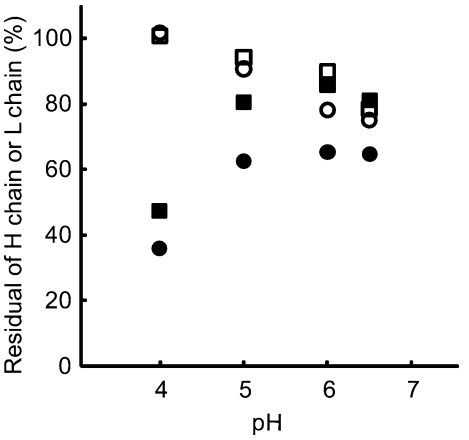

Fig. 6 The pH profiles of the residual percentages of H and L chains by densitometry of scanned reducing SDS-PAGE electropherograms after incubation of the humanized IgG1 at 60°C for 4 weeks. *Squares* indicate MES buffer (*filled square* H chain, *empty square* L chain). *Circles* indicate phosphate buffer (*filled circle* H chain, *empty circle* L chain)

and gradually decreased with increasing pH. These results suggested that Unknown-2 and Unknown-3 are derived from the H chain.

Cleavage Site During Incubation at 60°C for 4 Weeks at pH 4.0

To identify Unknown-2 and Unknown-3, we analyzed their peptide sequences using a peptide sequencer. The N-terminal sequence of the peptide in Unknown-2 was Pro-Glu-Val-Lys-Phe, but that of the peptide in Unknown-3 could not be determined. Based on the amino acid sequence of the IgG1 H chain, we identified the cleavage site as the Asp272-Pro273 peptide bond in the IgG1 CH2 domain.

The N-terminal residue of the IgG1 H chain is glutamine, which can readily cyclize as pyroglutamic acid. Pyroglutamic acid cannot be sequenced by the method we used (Edman degradation), so failure to determine the N-terminal sequence in Unknown-3 suggests the formation of N-terminal pyroglutamic acid in Unknown-3 during incubation at 60°C for 4 weeks at pH 4.0.

Kinetics of Asp-Pro Cleavage During Incubation at 60°C at pH 4.0

To examine the effect of the conformational stability of the IgG1 CH2 domain on cleavage of the Asp-Pro peptide bond, we incubated IgG1 H chain and a 15-residue peptide (VDVSHEDPEVKFNWY, corresponding to Val266-Tyr280 of the IgG1 H chain) at 60°C at pH 4.0 in 15 mM acetate buffer (in which the 15-residue peptide has higher solubility than in MES or phosphate). After incubation, we evaluated the residual percentage of intact IgG1 H chain by reducing SDS-PAGE, and the residual percentage of intact 15-residue peptide by RP-HPLC (Fig. 7). The residual percentage of the 15-residue peptide was 50% after only half a day, whereas the residual percentage of the IgG1 H chain was still above 75% after a week. That suggests that the conformational stability of the IgG1 molecule retards non-enzymatic cleavage of the Asp272-Pro273 peptide bond in the IgG1 CH2 domain.

Unfolding Temperatures of the IgG1 Molecule

We evaluated the influence of pH on the unfolding temperature of the IgG1 in phosphate or MES buffer by DSC. The unfolding of the IgG1 can be divided into three steps, as we

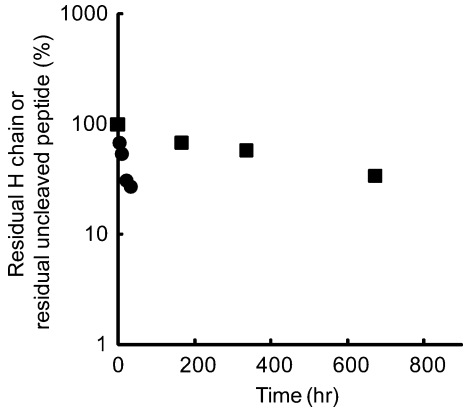

Fig. 7 Time courses of the residual percentage of H chain (*filled square*) in an IgG1 solution and the residual percentage of uncleaved peptide (*filled circle*) in a solution of a peptide corresponding to the IgG1 H chain Asp-Pro sequence, during incubation at 60°C. Both solutions were prepared using 15 mM acetate buffer (pH 4.0)

reported previously [5]. The first two steps involve unfolding of the Fc domain; the third step involves unfolding of the Fab domain.

We found that the unfolding temperatures in the first and second steps were lower at pH 4.0 and 5.0 in both types of buffer solution (Fig. 8). In particular, the unfolding temperature in the first step was markedly lower at those pH levels: 60–63 and 63–65°C in phosphate and MES buffer, respectively.

In addition, an exothermic signal appeared during the third unfolding step at higher pH levels, suggesting that aggregation of the unfolded intermediate of IgG1 occurs at that point. The exothermic signal was found at pH ≥6.0 in phosphate buffer, but only at pH 8.0 and 9.0 in MES buffer.

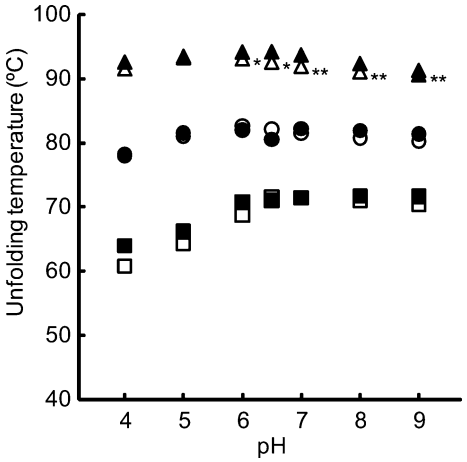

Fig. 8 The pH profile of the unfolding temperature of each unfolding step of the humanized IgG1 molecule. *Solid symbols* indicate MES buffer (*filled square* first step, *filled circle* second step, *filled triangle* third step). *Empty symbols* indicate phosphate buffer (*empty square* first step, *empty circle* second step, *empty triangle* third step). *Asterisks* (* or **) indicate that an exothermic signal was observed during the third unfolding step, either in phosphate buffer only (*) or in both buffer types (**). These exothermic signals suggest aggregation of the humanized IgG1

Discussion

Effect of the Conformational Stability of the CH2 Domain on the Aggregation and Peptide Cleavage of the Humanized IgG

Acidic conditions are crucial for the purification of humanized IgGs in Protein A affinity columns and for virus inactivation. To examine the aggregation and peptide bond cleavage of the humanized IgG1 investigated in this study under both acidic and weakly alkaline conditions, we used SEC-HPLC and SDS-PAGE to evaluate the residual percentage of monomer in solutions that had been incubated in phosphate or MES buffer at pH levels ranging from 4.0 to 9.0.

After incubation at 80°C for 2 h, nonreducing SDS-PAGE showed gradual decrease in the residual percentage of monomer with an increase in incubation pH. In our previous paper [5], we reported that aggregation of the IgG1 was initiated by denaturation of the Fc region. Moreover, a recent report indicates that the IgG1 CH2 domain has the lowest stability among human immunoglobulin domains [3]. Therefore, since the CH2 domain unfolds at temperatures below 80°C in the pH range of 4.0 to 9.0 (the first unfolding step in Fig. 8), IgG1 incubated at 80°C for 2 h should form an unfolded intermediate in which the CH2 region is denatured. Moreover, since the isoelectric point of IgG1 is estimated to be around 9, severe aggregation of the IgG1 monomer should tend to occur with increasing storage pH. This suggests that the pH dependency of the monomer content by nonreducing SDS-PAGE after incubation at 80°C for 2 h (Fig. 4) is caused by severe IgG1 aggregation that produces aggregates that remain insoluble even in the presence of SDS. We therefore conclude that unfolded IgG1 intermediate is deeply involved in aggregate formation. Although such pH-dependent aggregation is found in many proteins, including IgG, different patterns are seen in different proteins. For example, when LA298 (a humanized IgG1 antibody of the same subclass as the IgG investigated in this study) was stored at 25°C, aggregation occurred only in phosphate buffer at pH 7.0 or 8.0 and was not detectable in citrate buffer at pH 4.0 to 7.0, although the low IgG1 concentration (0.5 mg/mL) may have inhibited aggregation [13]. On the other hand, the pH profile of the aggregation rate of a 100 mg/mL IgG2 solution was inverted by changing the storage temperature. That is, the pH profile of the aggregation rate was pH 5.5<pH 5.2<pH 5.0 on storage at 37°C, but was pH 5.0<pH 5.2<pH 5.5 on storage at 29°C [14]. Moreover, IgG4 aggregation rates are affected by exposure to the acidic condition of pH 2.7, because of change in the higher-order structure of the IgG4 molecule [7].

On the other hand, in the present study, the pH profile of the residual percentage of monomer by nonreducing SDS-PAGE after incubation at 80°C for 2 h was different from that seen after incubation at 60°C for 4 weeks. Namely, the percentage of recovered monomer in the presence of SDS was higher after incubation at 80°C for 2 h at pH 4.0 and 5.0 than after incubation at 60°C for 4 weeks at the same pH levels. This difference may be the result of cleavage of the Asp272-Pro273 peptide bond in the IgG1 H chain under acidic conditions, as stated above. That is a reasonable inference because cleavage of Asp-Pro under acidic conditions is well-known [10], and the only Asp-Pro peptide bond in the IgG is the Asp272-Pro273 peptide bond in the H chain.

Since, however, the difference between the monomer contents found by SEC-HPLC and nonreducing SDS-PAGE at pH 4.0 cannot be explained by the degradate contents found by SEC-HPLC at pH 4.0 alone, IgG1 nicked at the Asp-Pro bond should be present among the insoluble aggregates formed after incubation at 60°C for 4 weeks at pH 4.0. Interestingly, the unfolding temperatures of the first transition in the IgG1 were 60–63°C at pH 4.0 and

63–65°C at pH 5.0, respectively (Fig. 8). The first transition is the result of denaturation of the IgG1 CH2 domain [3]. As shown above, we found that the Asp-Pro peptide bond is hydrolyzed at pH 4.0 in the absence of the conformational stability of the IgG1 CH2 domain. Therefore, we concluded that the Asp272-Pro273 peptide bond cleavage during long incubation at 60°C at pH 4.0 and 5.0 occurred in unfolded intermediate in which the CH2 domain is denatured. The finding that the preferential cleavage of the Asp272-Pro273 peptide bond was inhibited by the conformational stability of the CH2 domain in a humanized IgG1 is novel.

Significance of Our Findings for the Production of Humanized IgGs

As described in the "Introduction", elution of IgGs from Protein A affinity columns during purification of therapeutic antibodies is carried out at pH 4.0 to 5.0. Since the Asp-Pro sequence in the H chain CH2 domain is conserved in humanized IgGs, our findings have implications for the treatment of therapeutic antibodies using Protein A columns. Humanized IgGs induce very little immune response in the human body. Therapeutic antibodies are used in patients who have their own individual human leukocyte antigen systems. As a result, the cleavage of the peptide bond at Asp272 and Pro273 in humanized IgGs could induce autoantibodies because peptide fragmentation of self-protein has been reported to induce cryptic determinants that trigger the production of autoantibodies [15]. Therefore, since even small amounts of foreign proteins or peptides generally cause immune reactions, it is important to pay close attention to impurities in humanized antibodies resulting from cleavage of the H chain Asp-Pro peptide bond.

Inhibitory Effect of MES Buffer on Aggregation of Humanized IgG1 over a Wide pH Range

We have previously reported the inhibitory effect of MES buffer solution on the aggregation at pH 5.5 and 6.5 of the IgG1 examined in this study [5]. In the present study, comparison of the results in phosphate and MES buffer solutions shows that MES buffer gives high stability regardless of pH and incubation conditions. In particular, the residual percentage of monomer after incubation at 80°C at pH 4.0 to 5.0 and after incubation at 60°C at pH 7.0 to 8.0 differed markedly depending on the buffer types. These differences suggest that MES inhibits aggregation of the IgG1 over a wide pH range. As described in several previous papers, IgG1 aggregation is initiated by denaturation of the Fc region [3, 5]. Based on the thermal stability of the IgG1 at various solution pH levels (Fig. 8), unfolded intermediates of the IgG1 with a partially or fully denatured Fc region will be formed under the storage conditions we used (incubation at 80°C for 2 h or incubation at 60°C for 4 weeks). Under these conditions, interactions between MES and unfolded intermediates may retard aggregation of the IgG1. Recently, NMR analysis has revealed that an interaction between human liver fatty acid uniting protein and the MES molecule occurs in the order of microseconds to milliseconds [16]. The MES molecule consists of a hydrophilic group and a hydrophobic group. Additional experiments using techniques such as NMR would be needed to elucidate the interaction between the MES molecule and polypeptide chains. For the present, we have demonstrated in this study that MES inhibits aggregation of the IgG1 in the pH ranges, 4.0 to 5.0 and 7.0 to 8.0.

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