

1 Subject Category: Protein structure and analysis

2 **Small conformational changes in IgG1 detected as acidic charge variants by cation**
3 **exchange chromatography**

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14

15ABSTRACT

16Fully characterizing the post-translational modifications present in charge variants of therapeutic
17monoclonal antibodies (mAbs), particularly acidic variants, is challenging and remains an open area of
18investigation. In this study, to test the possibility that chromatographically separated acidic fractions of
19therapeutic mAbs contain conformational variants, we undertook a mAb refolding approach using as a case
20study an IgG1 that contains many unidentified acidic peaks with few post-translational modifications, and
21examined whether different acidic peak fractions could be generated corresponding to these variants. The
22IgG1 drug substance was denatured by guanidine hydrochloride, without a reducing agent present, and
23gradually refolded by stepwise dialysis against arginine hydrochloride used as an aggregation suppressor.
24Each acidic chromatographic peak originally contained in the IgG1 drug substance was markedly increased
25by this stepwise refolding process, indicating that these acidic variants are conformational variants.
26However, no conformational changes were detected by small-angle X-ray scattering experiments for the
27whole IgG1, indicating that the conformational changes are minor. Chromatographic, thermal and
28fluorescence analyses suggested that the conformational changes are a localized denaturation effect centred
29around the aromatic amino acid regions. This study provides new insights into the characterization of acidic
30variants that are currently not fully understood.

31

32*Keywords:*

33Acidic variant

34Cation-exchange chromatography

35Conformational change

36Monoclonal antibody

37Refolding

38

39

40Abbreviations

41ANS	1-Anilino-8-naphthalene sulfonate
42CEX	Cation-exchange chromatography
43DSF	Differential scanning fluorimetry
44DS	Drug substance
45DLS	Dynamic light scattering
46 R_h	Hydrodynamic radius
47HIC	Hydrophobic interaction chromatography
48 $D_{\max}$	Maximum dimension
49 $\lambda_{\max}$	Maximum emission wavelength
50 $p(r)$	Pair-distance distribution function
51 R_g	Radius of gyration
52SEC	Size-exclusion chromatography
53SAXS	Small-angle X-ray scattering
54 T_m	Thermal denaturation temperature
55	

561. Introduction

57 Therapeutic monoclonal antibodies (mAbs), like other proteins, are susceptible to chemical and
58 enzymatic post-translational modifications during their manufacture, resulting in a heterogeneous mix of
59 variants containing various post-translational modifications [1]. One of the most common forms of
60 heterogeneity is charge variation, and thorough characterization of charge variants is important in order to
61 identify critical quality attributes. Ion-exchange chromatography is the gold standard method for charge
62 variant analysis. Cation-exchange chromatography (CEX) is the default method because many therapeutic
63 mAbs have basic isoelectric points [2]. Isoelectric focusing is also employed, where the separation is based
64 on the net charge of the protein. However, isoelectric focusing is less sensitive to the localized charge
65 distribution on the protein surface. Charge variants with the same isoelectric point by isoelectric focusing
66 have been reported to show multiple peaks when analyzed by chromatographic techniques [3–5],
67 suggesting that net charge is not the only factor affecting the chromatographic behavior, and that localized
68 charge distribution and stereochemistry may alter the chromatographic elution profiles in subtle ways [6].

69 Charge variants are classified as either acidic or basic relative to the main variant. Acidic variants
70 exhibit an isoelectric point lower than that of the main variant and elute earlier than the main peak when
71 analyzed by CEX, whereas basic variants exhibit a higher isoelectric point and elute later. Various post-
72 translational modifications have been reported to contribute to the formation of acidic and basic variants
73 [6–9]. Post-translational modifications that can lead to the formation of acidic variants include sialylation,
74 asparagine deamidation, glycation, and C-terminal lysine cleavage. Factors that can lead to the formation of
75 basic variants include the presence of C-terminal lysine, N-terminal glutamine, C-terminal amidation,
76 isomerization, and succinimide formation. Some post-translational modifications directly alter the net
77 charge of the proteins while others induce conformational changes that affect the local charge distribution.
78 Interestingly, Ponniah et al. reported succinimide species eluting as acidic variants in anion-exchange
79 chromatography [10], contrary to previous reports of succinimide species being classified as basic variants
80 [11–13]. A difference in net charge cannot explain the elution of succinimide species as acidic variants, and
81 the authors of that report suggested that succinimide formation causes a conformational change, which
82 leads to differences in surface charge distribution.

83 Although several types of post-translational modification have already been identified in various
84 mAbs, the full characterization of post-translational modifications in charge variants of mAbs, particularly
85 acidic variants, is challenging and remains an open area of investigation [9, 14–16]. It is usually found that
86 the sum of the quantitative values of the identified acidic species does not fully account for the total acidic
87 regions in the elution profile. We previously characterized acidic variants of an IgG1 mAb internally by
88 using standard techniques, but we could hardly detect differences between the main peak fraction and the
89 acidic peak fractions (unpublished results). Briefly, sialidase digestion partially decreased the area of the
90 most prevalent acidic peak and increased the area of the main peak, indicating that the acidic peak is only
91 partially composed of IgG1 containing terminal sialic acid on the Fc N-glycans. Glycation was not
92 identified as a main attribute because the decrease in the peak areas of the acidic fractions by enzymatic
93 deglycosylation with PNGase F was limited to the effect of sialylation. The deglycosylated main and acidic
94 fractions were reduced and carboxymethylated, and then the molecular weights of their heavy and light
95 chains were determined by liquid chromatography-mass spectrometry. As a result, the two molecular
96 weights were found to be completely identical between the main and acidic fractions. Furthermore, trypsin
97 peptide mapping of the deglycosylated main and acidic fractions revealed that the acidic variants do not
98 include primary sequence modifications (such as deamidation). The most probable cause of this gap may be
99 slight conformational differences that are difficult to detect. Interestingly, Gervais et al. report that, based
100 on results of extended characterization and refolding studies, the most prevalent acidic peak of *Erwinia*
101 *chrysanthemi* L-asparaginase (a 140-kDa homotetramer in its active form) is a conformational variant with
102 no primary sequence modifications [17].

103 In this study, to test the possibility that the acidic fractions of therapeutic mAbs contain conformational
104 variants, we examined whether additional acidic peaks could be generated by a refolding process without a
105 reducing agent, using as an example the aforementioned IgG1 mAb that contains many unidentified acidic
106 peaks with few post-translational modifications. Since acidic peaks were generated by the refolding
107 process, we considered these acidic peaks as conformational variants and examined the conformational
108 changes that occur in the acidic fraction using various analytical methods.

1092. Materials and methods

1102.1 Materials

111 The drug substance (DS) used in this study was a humanized IgG1 mAb to human interleukin-6 receptor,
112 manufactured by Chugai Pharmaceutical (Tokyo, Japan). The theoretical isoelectric point of the IgG1 was
113 9.3. Cytochrome C from equine heart and ribonuclease A from bovine pancreas were purchased from
114 Merck (Darmstadt, Germany). 2-Morpholinoethanesulfonic acid monohydrate (MES) was purchased from
115 Dojindo Laboratories (Kumamoto, Japan). 1-Anilino-8-naphthalene sulfonate (ANS) was purchased from
116 Cayman Chemical (Ann Arbor, MI, USA). All other chemicals were purchased from Fujifilm Wako Pure
117 Chemical Corporation (Osaka, Japan). The protein samples were formulated in a buffer of 25 mM
118 MES/NaOH, 150 mM NaCl, pH 6.1, unless otherwise noted. The protein concentration was determined
119 using UV absorbance at 280 nm.

1202.2 Protein refolding by stepwise dialysis

121 The IgG1 DS, cytochrome C and ribonuclease A, as well as the main and acidic peak fractions isolated
122 from the IgG1 DS by preparative CEX described below were used in refolding studies without a reducing
123 agent, where all unfolding/refolding steps were maintained at pH 6.1 and performed at room temperature
124 (nominally 23°C). The protein samples were unfolded in the MES formulation buffer in the presence of 6
125 M guanidine hydrochloride at a protein concentration of 0.1 mg/mL for 1 hour. The guanidine
126 hydrochloride was gradually removed to refold the protein by stepwise dialysis using a Slide-A-Lyzer
127 dialysis cassette (20,000 MWCO for IgG1 samples; 3,500 MWCO for cytochrome C and ribonuclease A
128 samples; Thermo Fisher Scientific, Waltham, MA, USA) against 100-fold volume excess of MES
129 formulation buffer with 0.5 M arginine hydrochloride in combination with systematically decreasing
130 concentrations of guanidine hydrochloride (i.e., 3 M, 2 M, 1 M, and 0.5 M) for more than 6 hours each,
131 followed by a final dialysis step against the MES formulation buffer without arginine hydrochloride or
132 guanidine hydrochloride for more than 18 hours.

1332.3 Cation-exchange chromatography (CEX)

134 The main and acidic mAb peak fractions were isolated from the IgG1 DS by preparative CEX, using a
135 Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific) equipped with a semi-preparative column,

136Propac WCX-10 (9.0×250 mm, 10 µm, Thermo Fisher Scientific). Mobile phase A consisted of 25 mM
137MES/NaOH, pH 6.1 while mobile phase B was 25 mM MES/NaOH, 250 mM NaCl, pH 6.1. The IgG1 DS
138was diluted with mobile phase A and injected onto the column at a concentration of 22.5 mg/mL. The
139injection volume was 160 µL, where the column flow-rate was maintained at 1 mL/min throughout the ion
140exchange, and performed at 40°C. After sample injection, the column was run for 5 minutes using a mobile
141phase ratio of 63% v/v A and 37% v/v B followed by a linear elution gradient of 0.4% v/v mobile phase B
142per minute, ending at 50 minutes. The column was then washed for 10 minutes with 100% B. The protein
143elution was monitored by UV absorbance at 280 nm. Each collected sample was buffer-exchanged into the
144standard formulation buffer of 25 mM MES/NaOH, 150 mM NaCl, pH 6.1 and concentrated to over 3
145mg/mL using Amicon Ultra filters (50,000 MWCO; Merck).

146 The elution profiles of IgG1, cytochrome C, and ribonuclease A samples were analyzed by analytical
147CEX, using an Agilent 1290 Infinity HPLC system (Santa Clara, CA, USA) equipped with an analytical
148weak cation exchange column, ProPac WCX-10 (4.0×250 mm, 10 µm, Thermo Fisher Scientific). Except
149as described below, the methods were the same as those for preparative CEX. Before chromatographic
150analysis, the protein samples were buffer-exchanged into mobile phase A and concentrated as needed by
151centrifugation using Amicon Ultra filters (50,000 MWCO for IgG1 samples, 3000 MWCO for
152cytochrome C and ribonuclease A samples; Merck). The protein samples were injected at a concentration of
1530.5 mg/mL. The injection volumes were 30 µL for the IgG1 samples and 100 µL for cytochrome C and
154ribonuclease A, maintaining a flow rate of 0.5 mL/min at room temperature. For the IgG1 samples, after
155running the column for 5 minutes with 63% v/v A and 37% v/v B, a linear elution gradient of 0.4% v/v
156mobile phase B per minute was applied out to 80 minutes. For cytochrome C, a linear elution gradient of
1570.4% v/v B per minute was applied after running the column at 46% v/v A and 54% v/v B for 5 minutes,
158ending at 55 minutes. For ribonuclease A, a linear elution gradient of 0.2% v/v B per minute was used after
159running the column at 67% v/v A and 33% v/v B for 5 minutes, for a total of 100 minutes (in this case,
160mobile phase B consisted of 25 mM MES/NaOH, 500 mM NaCl, pH 6.1). In all instances, and at the
161completion of the CEX gradient, the column was washed for 5 minutes with 100% v/v of the respective
162mobile phase B.

1632.4 *Size-exclusion chromatography (SEC)*

164 The elution profiles of IgG1 samples were analyzed by SEC at room temperature, using an Agilent
1651290 Infinity HPLC system equipped with a size exclusion column, TSK-gel G3000SW_{XL} (7.8×300 mm, 5
166μm, Tosoh, Tokyo, Japan). The IgG1 samples were injected at a concentration of 1 mg/mL, with an
167injection volume of 40 μL. A running buffer of 50 mM sodium phosphate, 300 mM NaCl, pH 7.0 was used
168at a flow rate of 0.5 mL/min. The protein elution was monitored by UV absorbance at 280 nm.

1692.5 *Hydrophobic interaction chromatography (HIC)*

170 The elution profiles of the IgG1 samples were analyzed by HIC using an Agilent 1290 Infinity HPLC
171system equipped with a hydrophobic interaction column, ProPac HIC-10 (4.6×100 mm, 5 μm, Thermo
172Fisher Scientific). Before chromatographic analysis, the IgG1 samples were buffer-exchanged into a buffer
173of 25 mM MES/NaOH, pH 6.1 and concentrated as needed by centrifugation using Amicon Ultra filters
174(50,000 MWCO; Merck). The protein samples were injected at a concentration of 0.4 mg/mL. The injection
175volume was 10 μL. Mobile phase A was 100 mM sodium phosphate, pH 6.1. Mobile phase B was 100 mM
176sodium phosphate, 2 M (NH₄)₂SO₄, pH 6.1. The flow rate was set to 0.5 mL/min and the analysis was
177performed at room temperature. A linear elution gradient of 2% mobile phase A per minute was applied
178after running the column at 50% v/v A and 50% v/v B for 5 minutes, and ended at 20 minutes. The column
179was then washed for 10 minutes with 100% v/v A. The protein elution was monitored by UV absorbance at
180280 nm.

1812.6 *Dynamic light scattering (DLS)*

182 DLS measurements were performed using a DynaPro Plate Reader II (Wyatt Technology Corporation,
183Santa Barbara, CA, USA) to obtain the average hydrodynamic radius [R_h (nm)] at 25°C. The IgG1 samples
184were dispensed into a 384-well plate at a concentration of 0.5 mg/mL. The sample volume was 25 μL.

1852.7 *Small-angle X-ray scattering (SAXS)*

186 SAXS experiments were performed at the EMBL P12 beam line (PETRA III, DESY synchrotron,
187Hamburg, Germany) [18]. An automated robotic sample changer (BioSAXS; Arinax, Grenoble, France)
188was employed to deliver the IgG1 samples (1 mg/mL) and corresponding matched buffers at 20°C under
189continuous flow to the beam line through a 1 mm capillary of the sample exposure unit [19]. Two-

190dimensional SAXS patterns were collected using a PILATUS 6M pixel detector, a sample-detector distance
191of 3.0 m and a wavelength (λ) of 0.124 nm. A momentum transfer range of $0.026 < s < 7.288 \text{ nm}^{-1}$ (where s
192 $= 4\pi\sin\theta/\lambda$, 2θ is the scattering angle) was covered. Data reduction were performed by the automated data
193pipeline SASFLOW [20]. Briefly, the isotropic two-dimensional scattering intensities were normalized to
194the intensity of the transmitted beam and azimuthally averaged to generate one-dimensional scattering
195profiles [$I(s)$ vs s]. Data collection was divided into 20 separate frames of 50 msec each. To exclude data
196from samples potentially affected by radiation damage, automated pairwise 1D data frame comparisons and
197subsequent data-frame rejection were employed, followed by undamaged sample frame averaging and
198buffer scattering subtraction. The radius of gyration (R_g) and extrapolated scattering to zero angle [$I(0)$] was
199obtained by employing the Guinier approximation [$\ln I(s)$ vs s for $sR_g < 1.3$] as well as the calculation of the
200scattering pair-distance distribution function [$p(r)$] from which the maximum dimension ($D_{\max}$) was also
201estimated [21]. The fits to the theoretical scattering profile from the high-resolution crystal structure of a
202human IgG1 (pdb code:1hzh.pdb) were computed with CRY SOL [22].

2032.8 Differential scanning fluorimetry (DSF)

204 DSF measurements were performed using a Prometheus NT.48 (NanoTemper Technologies, Munich,
205Germany) with UV transparent capillaries. In the DSF experiments, a buffer condition of 25 mM
206citrate/NaOH, 150 mM NaCl, pH 4.1 was used in addition to the standard formulation MES buffer at pH
2076.1 because the thermal denaturation temperature (T_m) of the Fab domain at pH 6.1 exceeded this
208instrument's upper measurement temperature (i.e., 95°C). The IgG1 samples were buffer-exchanged into
209the buffer at pH 4.1 via centrifugation using Amicon Ultra filters (50,000 MWCO; Merck). The IgG1
210samples were loaded into the capillaries at a concentration of 0.5 mg/mL. The loading volume was 11 μL .
211DSF scans were performed at a rate of 1°C/min from 15°C to 95°C. Thermal denaturation was measured by
212detecting the temperature-dependent changes in the ratio of intrinsic fluorescence at emission wavelengths
213of 330 and 350 nm (350 nm/330 nm). The T_m was determined from the peaks of the first derivative of the
214fluorescence ratio. Data were analyzed with ThermControl software (NanoTemper Technologies).

2152.9 *Fluorescence measurements*

216 Fluorescence measurements were performed with a microplate spectrofluorometer Infinite M1000
217(Tecan, Mannedorf, Switzerland). For the ANS fluorescence measurements, a concentrated stock solution
218of ANS (2 mM ANS in the standard formulation MES buffer) was added to each IgG1 sample (3 mg/mL)
219to obtain a final ANS concentration of 200 μ M, and 20 μ L of the mixed sample solution was dispensed into
220a 384-well black plate. Fluorescence spectra of ANS were recorded from 450 to 600 nm with an excitation
221wavelength of 385 nm at 25°C. The ANS fluorescence spectrum of each IgG1 sample was corrected by
222subtracting the spectrum of a blank solution of standard formulation MES buffer containing 200 μ M ANS.
223For intrinsic fluorescence measurements, 20 μ L of each IgG1 sample (3 mg/mL) was dispensed into a 384-
224well black plate. Intrinsic fluorescence spectra were recorded from 300 to 360 nm with an excitation
225wavelength of 285 nm at 25°C. The intrinsic fluorescence spectrum of each IgG1 sample was corrected by
226subtracting the spectrum of the blank solution of standard formulation MES buffer.

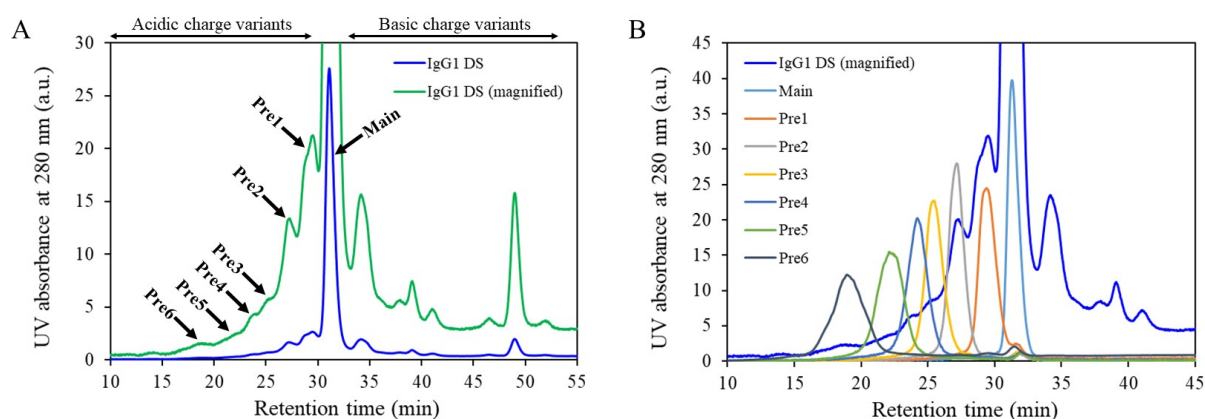


Fig. 1. (A) Representative CEX profile of the IgG1 drug substance (DS) used in this study. The acidic peaks investigated are designated *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* in order of decreasing elution time. **(B)** CEX profiles of the *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* fractions isolated from the IgG1 DS. Magnified CEX profile of the IgG1 DS is shown as a reference.

2273. Results

2283.1 Purity check of isolated acidic and main fractions

229 A representative CEX profile of the IgG1 DS is shown in Figure 1A. A number of acidic/basic charge
 230 variants elute before/after the main peak (hereafter referred to as *Main*), which elutes at around 31 minutes.
 231 In this study, we focused on the physicochemical characterization of six acidic peaks, which, in order of
 232 decreasing elution time, are referred to here as *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6*, respectively. The
 233 *Main* and *Pre* fractions were isolated from the DS by preparative CEX, buffer-exchanged, and concentrated
 234 to over 3 mg/mL. These collected fractions were re-injected for analytical CEX to check the purity (Fig.
 235 1B). The isolated *Main* fraction was found to be free of acidic and basic peaks. Each isolated *Pre* fraction
 236 still contained a small amount of *Main* peak, but was subjected to subsequent characterization without
 237 further purification.

2383.2 *Generation of acidic peaks following refolding*

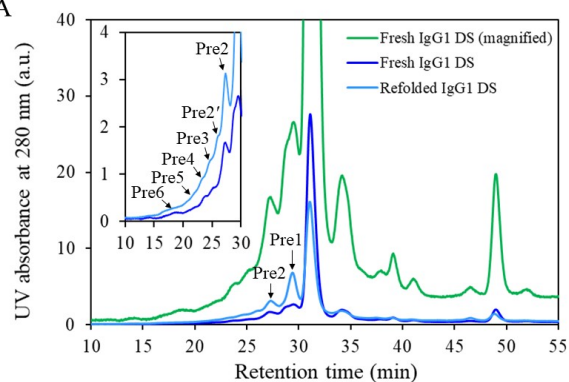
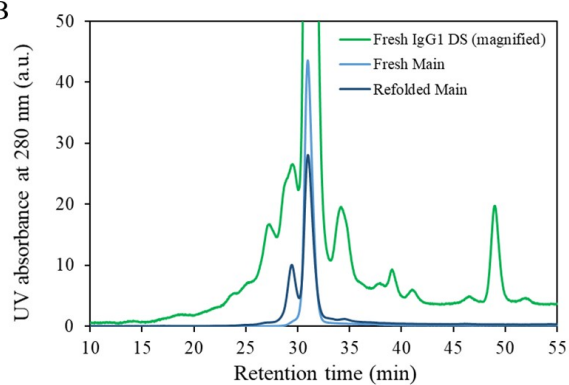
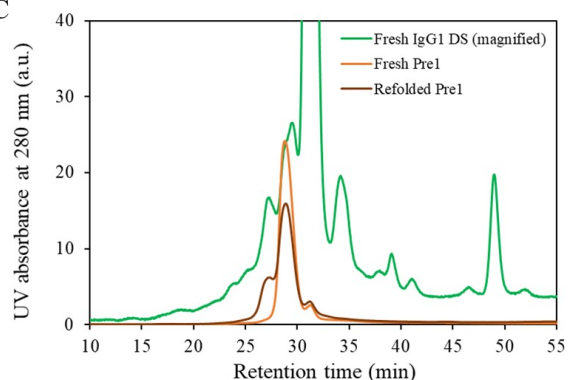
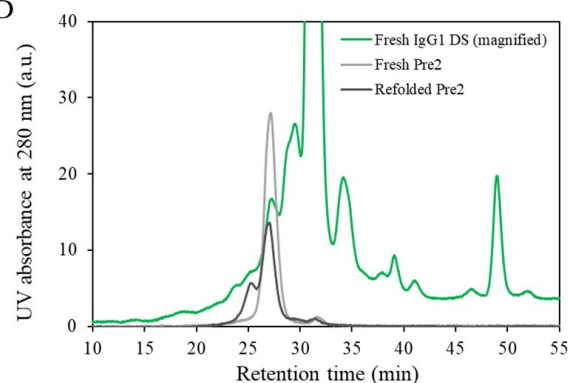
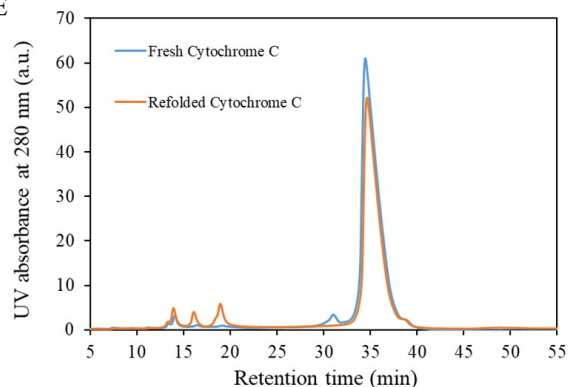
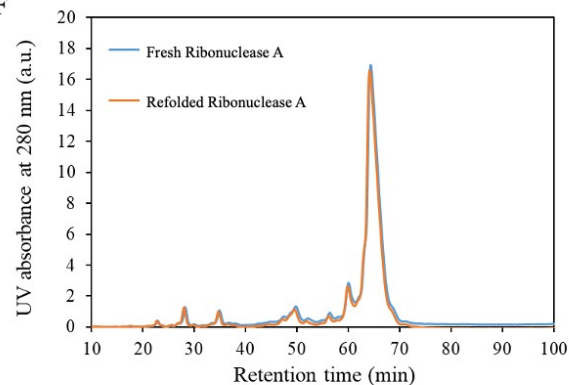
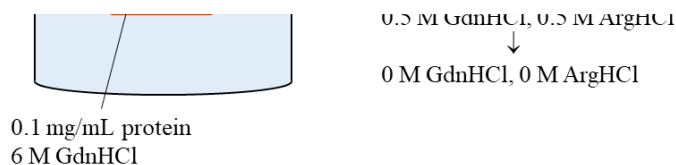
239 **A****B****C****D****E****F**

Fig. 3. CEX profiles of fresh (i.e., non-treated) and refolded **(A)** IgG1 drug substance (DS), **(B)** Main, **(C)** Pre1, **(D)** Pre2, **(E)** cytochrome C, and **(F)** ribonuclease A. The magnified CEX profile of the IgG1 DS is shown in **A**, **B**, **C**, and **D** as a reference. Inset in **A** depicts magnified elution profiles from 10 to 30 minutes. The refolding procedure is outlined in Figure 2.



dialysis (GdnHCl: guanidine hydrochloride, ArgHCl: arginine hydrochloride).

240 To clarify whether the *Pre* peaks corresponded to conformational variants, we performed refolding
241 experiments without a reducing agent. The IgG1 DS and isolated fractions were subjected to denaturation
242 using 6 M guanidine hydrochloride, and were then refolded by stepwise dialysis as outlined in Figure 2.
243 The concentration of guanidine hydrochloride was gradually reduced and 0.5 M arginine hydrochloride was
244 utilized to suppress the formation of aggregates during the refolding process [23, 24]. The protein
245 concentration was set as low as possible (i.e., 0.1 mg/mL) to avoid the formation of aggregates.

246 CEX profiles of fresh (i.e., non-treated) and refolded IgG1 DS are shown in Figure 3A. Following
247 refolding, the area of the *Main* peak decreased, and that of each *Pre* peak increased. The longer the
248 retention time of *Pre*, the greater the increase. A new peak that was not detected in the fresh IgG1 DS was

249 generated between the *Pre2* and *Pre3* peaks (depicted as *Pre2'* in Fig. 3A) after refolding. The total peak
250 area of the samples was not changed during the refolding process (the ratio of refolded to fresh is 1.01),
251 suggesting that almost no aggregates were generated under the refolding conditions. The isolated *Main* and
252 *Pre* fractions were also each subjected to the refolding process to examine the changes in CEX profile. The
253 CEX profiles of fresh and refolded *Main* fractions are shown in Figure 3B. The peak area of *Main*
254 decreased, and *Pre1* and a small amount of *Pre2* were generated. *Pre1* generated from the *Main* fraction by
255 the refolding process exhibited a symmetrical peak shape, whereas the *Pre1* peak in the fresh IgG1 DS was
256 asymmetric, suggesting that the *Pre1* fraction contains multiple components with conformational variant
257 being just one of them. Similarly, the isolated *Pre1* and *Pre2* fractions were refolded and analyzed by CEX
258 (Fig. 3C and 3D). *Pre2* and *Pre3* peaks were generated from the *Pre1* and *Pre2* fractions, respectively. A
259 *Main* peak was not generated from the *Pre1* and *Pre2* fractions.

260 To investigate whether the generation of acidic peaks by refolding is a general phenomenon that could
261 occur in other proteins as well, we subjected cytochrome C and ribonuclease A to the same refolding
262 process and CEX analysis (Fig. 3E and 3F). In cytochrome C, as in the IgG1 DS, the area of the main peak
263 decreased, and the areas of the acidic peaks increased. An acidic peak eluting at around 31 minutes
264 disappeared. In ribonuclease A, the CEX profile was not changed by the refolding process.

265 These results suggest that the acidic peaks in the IgG1 DS are conformational variants and that such
266 variants could be generated in other proteins. To elucidate the conformational changes occurring in the *Pre*
267 fractions, we characterized them using the following methods.

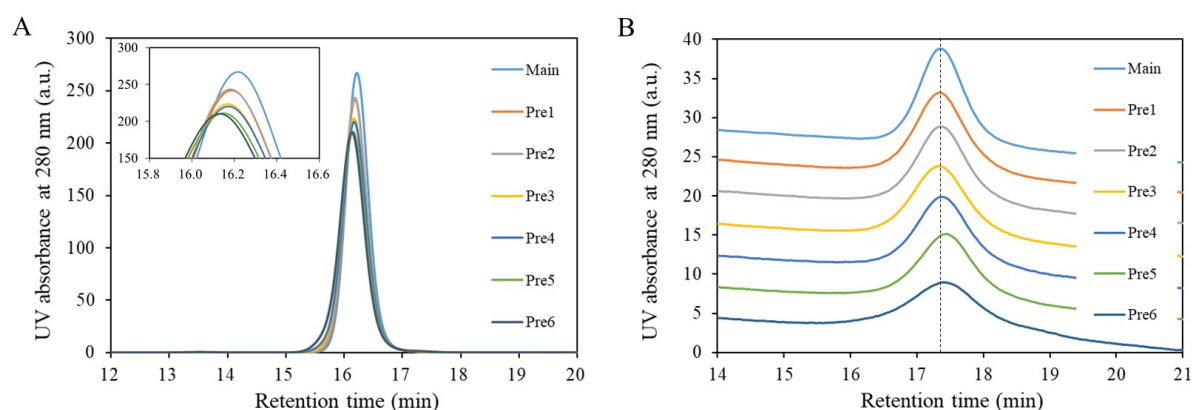


Fig. 4. (A) SEC and (B) HIC profiles of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* fractions. Inset in A depicts magnified elution profiles from 15.8 to 16.6 minutes. Dotted line in B shows the retention time of peak of *Main*.

2683.3 SEC and HIC analysis

269 The isolated *Main* and *Pre* fractions were analyzed by SEC. All samples eluted as a single mAb
 270 monomer peak and no oligomers or aggregates such as dimers were detected (Fig. 4A). The shorter the
 271 elution time of *Pre* in CEX, the broader the monomer peak and the slightly shorter the elution time in SEC,
 272 suggesting that the conformational change of *Pre* variants altered their interaction with the SEC column
 273 resin or that the conformation of the *Pre* variants was slightly expanded compared to that of the *Main*
 274 variant. The isolated *Main* and *Pre* fractions were also analyzed by HIC (Fig. 4B). Similar to the SEC
 275 profiles, the shorter the elution time of *Pre* in CEX, the broader the peak in HIC. The retention times of
 276 *Pre4*, *Pre5*, and *Pre6* were slightly greater than that of *Main*, indicating that these *Pre* variants were slightly
 277 more hydrophobic than the *Main* variant.

Table 1 Hydrodynamic radius [R_h (nm)], radius of gyration [R_g (nm)], maximum dimension [D_{max} (nm)], and χ^2 of fit to the theoretical SAXS profile of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*,

2783.4 DLS and SAXS analysis

279 The R_h values of each fraction were determined by DLS measurements (Table 1). No difference in
 280 hydrodynamic properties were detected by DLS, and all samples showed the same R_h values (i.e., 5.3 nm),
 281 which is consistent with the R_h values reported for an IgG1 monomer [25]. SAXS profiles and the $p(r)$
 282 functions of each fraction obtained at 20°C are displayed in Figure 5A and Figure 5B, respectively. The

*^a obtained by DLS measurements at a concentration of 0.5 mg/mL at 25°C. Data
 represent the mean $\pm$ SD of three experiments.

*^b obtained by SAXS measurements at a concentration of 1 mg/mL at 20°C.

*^c obtained by fitting to the theoretical scattering profile from the crystal structure of a
 human IgG1 (pdb code:1hzh.pdb) with the ATSAS program CRY SOL [22].

283 SAXS profiles and $p(r)$ were typical of monomeric IgG1 [26] and no marked differences were detected
 284 between the *Main* and *Pre* fractions. The scattering profiles were compared to the theoretical scattering
 285 profile from the crystal structure of a human IgG1 (pdb code:1hzh.pdb) with the ATSAS program
 286 CRY SOL. The obtained χ^2 values are listed in Table 1 and range 1.3 to 1.6 suggesting a good agreement
 287 between the solution structure and model. The value of R_g and D_{max} were within the error estimates for
 288 different fractions (Table 1). These results suggest that the conformational changes occurring in the *Pre*
 289 variants are minor.

2903.5 DSF analysis

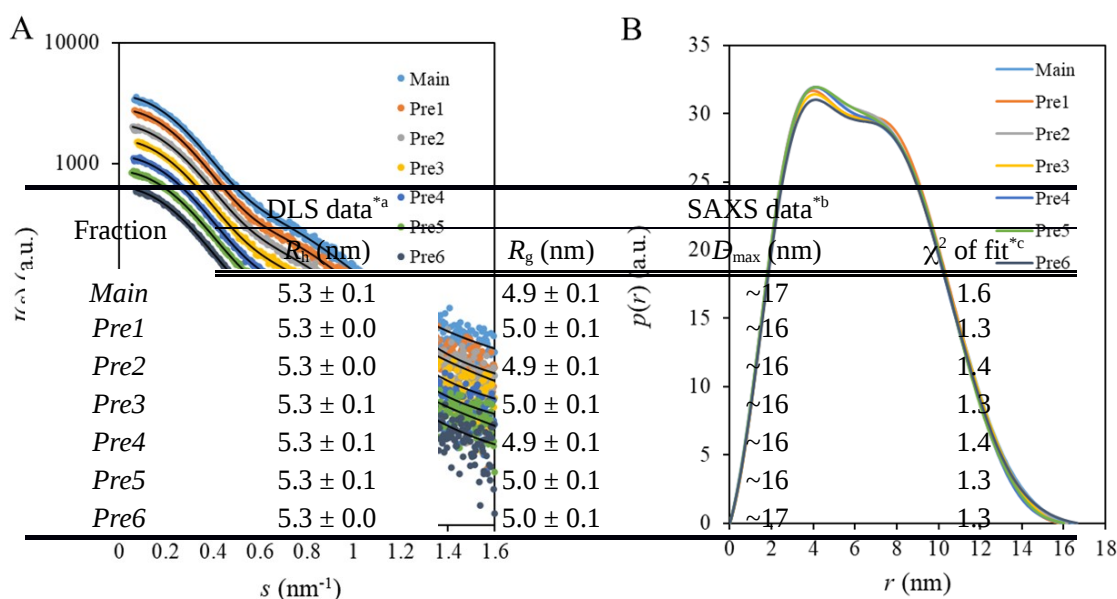


Fig. 5. (A) SAXS profiles of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* fractions (1 mg/mL) at 20°C. The scattering data of *Pre* fractions were shifted downwards in logarithmic scale to avoid overlapping. Solid lines are the result of fitting by indirect Fourier transformation. **(B)** the pair-distance distribution function [$p(r)$].

291 For the IgG1 used in this study, thermal denaturation of the CH2, CH3, and Fab domains occurs
 292 separately in order of increasing temperature [27]. To clarify which domains potentially undergo
 293 conformational changes in the *Pre* variants, the T_m of each domain of the *Main* and *Pre* fractions was
 294 determined by DSF and compared. DSF thermograms of each fraction at pH 6.1 and pH 4.1 are shown in
 295 Figure 6A and Figure 6B, respectively. At pH 6.1, the T_m of the Fab domain exceeded the upper
 296 temperature limit of the instrument, and consequently the thermal denaturation of only the two domains of
 297 CH2 and CH3 could be detected; however, because the T_m of these domains decreases with the reduction in
 298 pH, the thermal denaturation of the three domains of CH2, CH3, and Fab could be detected at pH 4.1. The
 299 thermal denaturation curves almost overlapped between the samples, but slight differences between the
 300 *Main* and *Pre* fractions were detected in the CH2 and Fab domains. The T_m of the CH2 and CH3 domains at
 301 pH 6.1 are shown in Figure 6C and 6D, and the T_m of the CH2, CH3, and Fab domains at pH 4.1 are shown
 302 in Figure 6E, 6F, and 6G. The T_m of the CH2 domain of the *Pre* fractions was slightly lower than that of the

303 *Main* fraction at both pH values, but no marked difference was detected between the *Main* and *Pre*
304 fractions in the CH3 domain. The T_m of Fab domain tended to be lower in the *Pre* fractions than in the
305 *Main* fraction at pH 4.1, but the difference was extremely small. These results suggest that the
306 conformational changes of the *Pre* variants are minor and occur at multiple sites, at least across the CH2
307 and Fab domains.

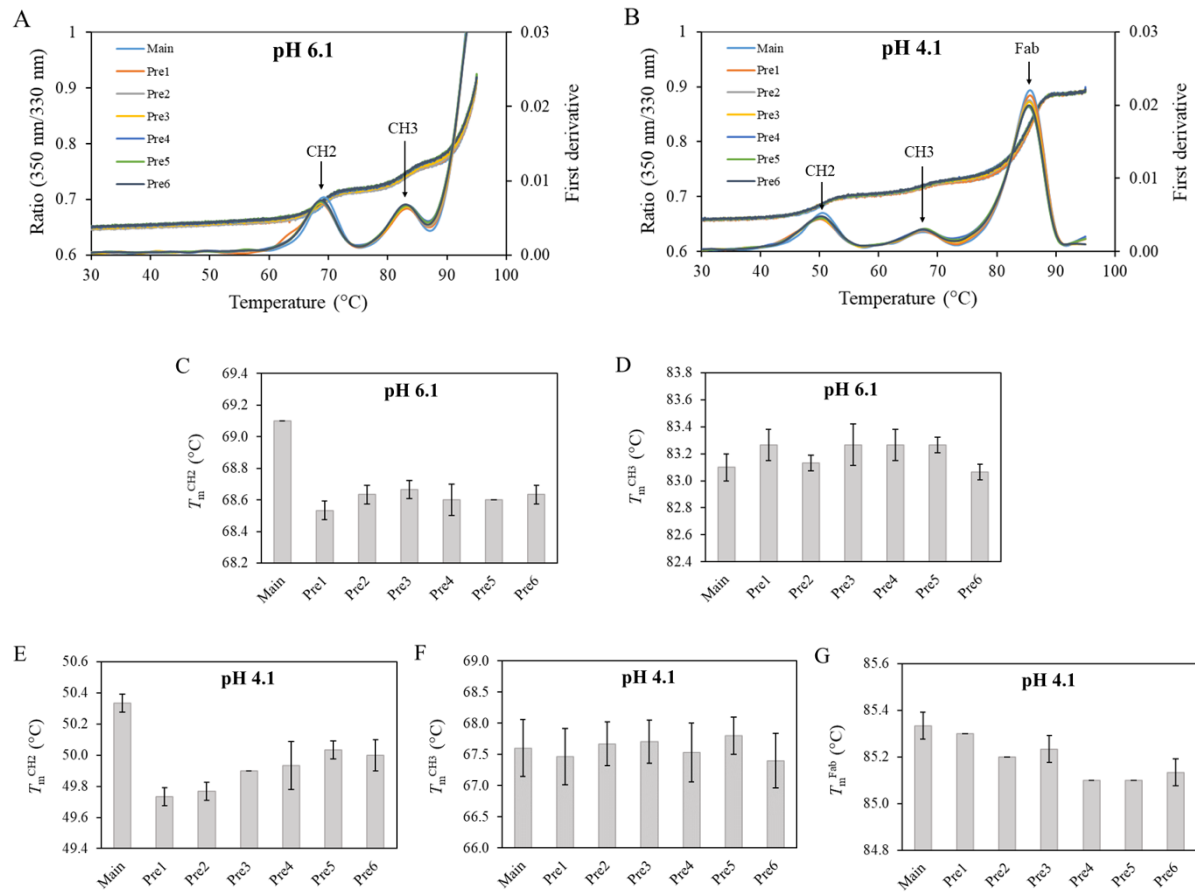
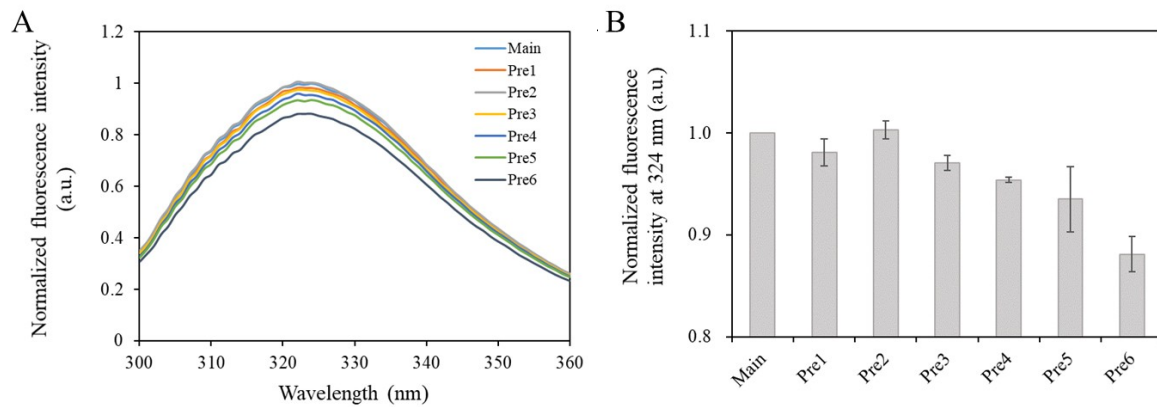


Fig. 6. DSF thermograms of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* fractions (0.5 mg/mL) at (A) pH 6.1 and (B) pH 4.1. The y-axes represent the ratio of intrinsic fluorescence at emission wavelengths of 330 and 350 nm (350 nm/330 nm) and the first derivative. Each curve represents the mean of three experiments. The peaks of the first derivative correspond to the thermal denaturation of CH2, CH3, and Fab domains in order of increasing temperature. The T_m (°C) of (C) CH2 and (D) CH3 domains at pH 6.1. The T_m of (E) CH2, (F) CH3, and (G) Fab domains at pH 4.1. Each column represents the mean $\pm$ SD of three experiments.

3083.6 ANS and intrinsic fluorescence analysis

309 To further investigate the conformational changes occurring in the *Pre* variants, the hydrophobicity of
 310 each fraction was evaluated by ANS, a fluorescent probe that binds to hydrophobic regions. The ANS
 311 fluor^{Fig. 7.} (A) ANS fluorescence spectra of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* *Pre*
 312 fract^{fractions} at 25°C. The concentrations of ANS and IgG1 were 200 μ M and 2.7 mg/mL, respectively.
 313 inten^{The} The excitation wavelength was 385 nm. The fluorescence intensity was normalized to the intensity I_m
 of the *Main* sample at 494 nm. Each curve represents the mean of three experiments. (B)
 Normalized fluorescence intensity at 494 nm and (C) λ_{max} (nm). Each column represents the mean
 $\pm$ SD of three experiments.



B

fractions at 25°C. The concentration of IgG1 was 3 mg/mL. The excitation wavelength was 285 nm. The fluorescence intensity was normalized by the maximum intensity of the *Main* sample. Each curve represents the mean of three experiments. **(B)** Normalized fluorescence intensity at 324 nm. Each column represents the mean $\pm$ SD of three experiments.

314 315 316 317 318

emission wavelength (λ_{max}) of ANS in the *Pre* fractions was slightly blue-shifted compared to that of the *Main* fraction. Subsequently, environmental changes around aromatic residues were investigated by intrinsic fluorescence measurements. The intrinsic fluorescence spectra under excitation at 285 nm are shown in Figure 8A. The fluorescence intensity tended to decrease slightly as the *Pre*-fraction number increased (Fig. 8B).

3194. Discussion

320 As previous characterization studies on charge variants of therapeutic mAbs have shown, the full
321 characterization of post-translational modifications in acidic variants of mAbs is challenging and remains
322 an open area of investigation [9, 14–16]. It is usually found that quantitative measurements of identified
323 acidic species leave some gaps, and that the sum of the identified acidic species does not fully account for
324 the total acidic regions in the elution profile. Neill et al. reported that the basic variants of a mAb detected
325 by CEX could be all fully accounted for by identified post-translational modifications, whereas the acidic
326 variants could only be partially explained by deamidation and isomerization [9]. Dada et al. characterized
327 both the acidic and basic regions of imaged capillary isoelectric focusing profile of an IgG1 mAb by using

328preparative immobilized pH gradient isoelectric focusing fractionation and reported that unlike the basic
329variants where the detected modifications are discrete and exclusive, all acidic peaks to a varying degree
330shared similar modifications [16].

331 As described in Introduction section, the acidic fractions of the IgG1 mAb used in this study have also
332been extensively characterized previously by various techniques. However, interestingly, most of them
333showed no differences from the *Main* fraction (unpublished results). Since few post-translational
334modifications were detected, we speculated that the main attributes characterizing these unidentified acidic
335variants would be alterations in their higher order structure. Interestingly, Gervais et al. report that, on the
336basis of capillary isoelectric focusing, liquid chromatography-mass spectrometry, and liquid
337chromatography-tandem mass spectrometry data, the acidic variants of L-asparaginase do not include
338primary sequence modifications (such as deamidation), and they concluded on the basis of SAXS and ion
339mobility-mass spectrometry data showing slight conformational differences between the acidic and main
340peaks that the most prevalent acidic peak is a conformational variant [17]. That consideration was further
341supported by the results of refolding experiments demonstrating that the acidic peak could be converted
342into the main peak after unfolding using 8M urea and 2.2 M N-ethylurea and refolding by dialysis against
343water [17]. Even such a small conformational change may alter the overall surface charge distribution,
344owing to changes in the local distribution of charged residues. With reference to the study by Gervais et al.,
345in this study we investigated whether conformational changes occur in the unidentified acidic variants of
346IgG1, and we analyzed the conformational changes occurring in the acidic fractions in more detail.

347 First, we examined whether refolding without a reducing agent could generate the acidic variants of
348IgG1. By applying stepwise dialysis, the use of arginine hydrochloride during refolding [23, 24], and a low
349concentration of the IgG1, we found conditions under which the IgG1 could be refolded without
350aggregation formation and under which the acidic variants could be generated. The results of the refolding
351studies revealed that the unidentified acidic variants of IgG1 would be conformational variants.
352Interestingly, *Pre1* was generated from *Main*, *Pre2* was generated from *Pre1*, and *Pre3* was generated from
353*Pre2* by the refolding process, suggesting that the acidity of the IgG1 molecular surface increased in a
354stepwise pattern as certain conformational changes accumulated in the *Main* molecule and these variants
355were eluted as multiple *Pre* peaks depending on the degree of accumulation of the conformational changes.
356The fact that only a small amount of *Pre2* and no *Pre3* were generated from *Main* is likely due to the low

357 probability of multiple conformational changes occurring simultaneously within a single molecule. With L-
358 asparaginase, the refolding process generated the main peak from the acidic peak [17], but this was not the
359 case for the IgG1 used in this study, presumably because the activation energy barrier for changing from
360 *Pre* variants to the *Main* variant is very high in IgG1. The *Pre1* peak generated from *Main* by refolding
361 process was symmetric. On the other hand, the *Pre1* peak originally contained in the IgG1 DS was
362 asymmetric, which is likely due to the presence of sialylated IgG1 as described in Introduction section in
363 addition to the conformational variant.

364 SAXS experiments were performed to investigate the conformational changes occurring in the *Pre*
365 variants, but no marked differences in SAXS profiles were detected between the *Main* and *Pre* fractions,
366 unlike in the case of L-asparaginase [17]. As well, no difference was detected between the *Main* and *Pre*
367 fraction's R_h measured by DLS. These results indicate that the conformational changes occurring in the *Pre*
368 variants are likely localized/minor and do not greatly affect the overall global disposition of the IgG1 in
369 solution. Since IgG1 is a multi-domain/modular protein that is present in solution as a population-state
370 ensemble, the minor conformational changes occurring in the *Pre* variants may be difficult to detect by
371 SAXS due to the net averaging of conformational fluctuations. By isolating the Fc and Fab domains after
372 fragmentation by enzyme treatment (such as papain digestion) and comparing the SAXS data between the
373 *Main* and *Pre* fractions, it may be possible to identify those domains in which the conformational changes
374 occur. However, isolating the Fc and Fab domains of the *Pre* fractions for SAXS experiments is time-
375 consuming and labor-intensive; therefore, in this study, the SAXS experiments were limited to the
376 measurement of the whole IgG1. Online CEX-SAXS, which can obtain SAXS data while isolating and
377 purifying each domain using enzyme-treated IgG1, may be a suitable tool for detecting conformational
378 changes occurring in the *Pre* variants [28–30]. Further studies will be required in order to detect the
379 conformational changes in the *Pre* variants using SAXS.

380 In the SEC, HIC, DSF, and fluorescence analyses, slight differences were observed between the *Main*
381 fraction and the *Pre* fractions, however, the differences detected were extremely small. The results of HIC
382 and ANS fluorescence showed that the *Pre* variants were slightly more hydrophobic than the *Main* variant.
383 In DSF, the T_m of the CH2 and Fab domains were slightly decreased in the *Pre* fractions, suggesting that the
384 conformational changes in the *Pre* variants occur at multiple sites, at least across these two domains. The
385 intrinsic fluorescence intensity of the *Pre* fractions decreased in a CEX retention time-dependent manner,

suggesting that the minor conformational changes of the *Pre* variants occur around aromatic residue regions. Wen et al. reported that microconformational changes within aromatic hydrophobic regions of several representative proteins could be induced by incubation with amine or guanidine additives [31]. Taking into account the results of that study, it could be assumed that, in the *Pre* variants, the side chains of aromatic residues are exposed to water due to local denaturation around the aromatic side chains, resulting in a decrease in the intrinsic fluorescence intensity and an increase in the hydrophobicity of the molecular surface. In SEC, the elution time of the monomer peak was slightly shorter for the *Pre* fractions; considering the results of HIC, fluorometry, and DSF, this was presumably due to the slightly expanded conformation of the *Pre* variants compared to that of the *Main* variant due to local denaturation. This hypothesis is consistent with the results of a previous study, in which SAXS measurements detected a slightly expanded state in the acidic peak of L-asparaginase [17].

For cytochrome C, acidic peaks were also generated by the refolding process. It is interesting to note that conformational variants resulting from the refolding process were detected as acidic peaks rather than basic peaks both in IgG1 and in cytochrome C. One possible explanation is that the exposure of aromatic amino acid side chains to water is induced by local denaturation, resulting in altered interactions with the column resin and the subsequent detection as acidic peaks. However, further studies are needed to clarify the mechanism of this phenomenon. On the other hand, refolding of ribonuclease A did not change the CEX profile, and no acidic peaks were generated. The stability of ribonuclease A is well known. The classical method for purification of ribonuclease A from bovine pancreas relies on its high stability that can maintain its integrity and solubility under severe conditions [32]. Conformational changes such as those seen in this study may be less likely to occur for highly stable proteins such as ribonuclease A.

5. Conclusion

Charge variation is one of the most common forms of heterogeneity in therapeutic mAbs, and thorough characterization of charge variants is important to identify critical quality attributes. However, it is not unusual that the sum of the quantitative values of the identified acidic species does not fully account for the total acidic regions in the elution profile. In this study, acidic variants of IgG1, which had been rarely identified in previous characterizations, were found to be generated by the process of refolding without a

413reducing agent, indicating that these acidic variants are conformational variants. On the other hand, no
414conformational changes were detected by SAXS experiments for the intact IgG1, indicating that the
415conformational changes are minor. The results of HIC and ANS fluorescence showed that the *Pre* variants
416were slightly more hydrophobic than the *Main* variant. In DSF, the T_m of the CH2 and Fab domains were
417slightly decreased in the *Pre* fractions, suggesting that the minor conformational changes in the *Pre* variants
418occur at multiple sites, at least across these two domains. The intrinsic fluorescence intensity of the *Pre*
419fractions decreased in a CEX retention time–dependent manner, suggesting that the minor conformational
420changes of the *Pre* variants occur around aromatic residue regions. Taken together, these results suggest
421that, in the *Pre* variants, the side chains of aromatic residues are exposed to water due to local denaturation
422around the aromatic side chains, resulting in a decrease in the intrinsic fluorescence intensity and an
423increase in the hydrophobicity of the molecular surface. For cytochrome C, acidic peaks were also
424generated by the refolding process. One possible explanation is that the exposure of aromatic amino acid
425side chains to water is induced by local denaturation, resulting in altered interactions with the column resin
426and the subsequent detection as acidic peaks. Further studies are needed to elucidate in more detail the
427conformational changes, their formation mechanisms, and the mechanisms by which they are detected as
428acidic variants. This study provides new insights into the characterization of acidic variants of therapeutic
429mAbs that are not currently fully understood.

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7 Supervision, Writing – review and editing. **Yuji Yamanaka:** Project administration, Writing – review and
8 editing.

1 Subject Category: Protein structure and analysis

2 **Small conformational changes in IgG1 detected as acidic charge variants by cation**
3 **exchange chromatography**

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14

15ABSTRACT

16Fully characterizing the post-translational modifications present in charge variants of therapeutic
17monoclonal antibodies (mAbs), particularly acidic variants, is challenging and remains an open area of
18investigation. In this study, to test the possibility that chromatographically separated acidic fractions of
19therapeutic mAbs contain conformational variants, we undertook a mAb refolding approach using as a case
20study an IgG1 that contains many unidentified acidic peaks with few post-translational modifications, and
21examined whether different acidic peak fractions could be generated corresponding to these variants. The
22IgG1 drug substance was denatured by guanidine hydrochloride, without a reducing agent present, and
23gradually refolded by stepwise dialysis against arginine hydrochloride used as an aggregation suppressor.
24Each acidic chromatographic peak originally contained in the IgG1 drug substance was markedly increased
25by this stepwise refolding process, indicating that these acidic variants are conformational variants.
26However, no conformational changes were detected by small-angle X-ray scattering experiments for the
27whole IgG1, indicating that the conformational changes are minor. Chromatographic, thermal and
28fluorescence analyses suggested that the conformational changes are a localized denaturation effect centred
29around the aromatic amino acid regions. This study provides new insights into the characterization of acidic
30variants that are currently not fully understood.

31

32*Keywords:*

33Acidic variant

34Cation-exchange chromatography

35Conformational change

36Monoclonal antibody

37Refolding

38

39

40Abbreviations

41ANS	1-Anilino-8-naphthalene sulfonate
42CEX	Cation-exchange chromatography
43DSF	Differential scanning fluorimetry
44DS	Drug substance
45DLS	Dynamic light scattering
46 R_h	Hydrodynamic radius
47HIC	Hydrophobic interaction chromatography
48 $D_{\max}$	Maximum dimension
49 $\lambda_{\max}$	Maximum emission wavelength
50 $p(r)$	Pair-distance distribution function
51 R_g	Radius of gyration
52SEC	Size-exclusion chromatography
53SAXS	Small-angle X-ray scattering
54 T_m	Thermal denaturation temperature
55	

561. Introduction

57 Therapeutic monoclonal antibodies (mAbs), like other proteins, are susceptible to chemical and
58 enzymatic post-translational modifications during their manufacture, resulting in a heterogeneous mix of
59 variants containing various post-translational modifications [1]. One of the most common forms of
60 heterogeneity is charge variation, and thorough characterization of charge variants is important in order to
61 identify critical quality attributes. Ion-exchange chromatography is the gold standard method for charge
62 variant analysis. Cation-exchange chromatography (CEX) is the default method because many therapeutic
63 mAbs have basic isoelectric points [2]. Isoelectric focusing is also employed, where the separation is based
64 on the net charge of the protein. However, isoelectric focusing is less sensitive to the localized charge
65 distribution on the protein surface. Charge variants with the same isoelectric point by isoelectric focusing
66 have been reported to show multiple peaks when analyzed by chromatographic techniques [3–5],
67 suggesting that net charge is not the only factor affecting the chromatographic behavior, and that localized
68 charge distribution and stereochemistry may alter the chromatographic elution profiles in subtle ways [6].

69 Charge variants are classified as either acidic or basic relative to the main variant. Acidic variants
70 exhibit an isoelectric point lower than that of the main variant and elute earlier than the main peak when
71 analyzed by CEX, whereas basic variants exhibit a higher isoelectric point and elute later. Various post-
72 translational modifications have been reported to contribute to the formation of acidic and basic variants
73 [6–9]. Post-translational modifications that can lead to the formation of acidic variants include sialylation,
74 asparagine deamidation, glycation, and C-terminal lysine cleavage. Factors that can lead to the formation of
75 basic variants include the presence of C-terminal lysine, N-terminal glutamine, C-terminal amidation,
76 isomerization, and succinimide formation. Some post-translational modifications directly alter the net
77 charge of the proteins while others induce conformational changes that affect the local charge distribution.
78 Interestingly, Ponniah et al. reported succinimide species eluting as acidic variants in anion-exchange
79 chromatography [10], contrary to previous reports of succinimide species being classified as basic variants
80 [11–13]. A difference in net charge cannot explain the elution of succinimide species as acidic variants, and
81 the authors of that report suggested that succinimide formation causes a conformational change, which
82 leads to differences in surface charge distribution.

83 Although several types of post-translational modification have already been identified in various
84 mAbs, the full characterization of post-translational modifications in charge variants of mAbs, particularly
85 acidic variants, is challenging and remains an open area of investigation [9, 14–16]. It is usually found that
86 the sum of the quantitative values of the identified acidic species does not fully account for the total acidic
87 regions in the elution profile. We previously characterized acidic variants of an IgG1 mAb internally by
88 using standard techniques, but we could hardly detect differences between the main peak fraction and the
89 acidic peak fractions (unpublished results). Briefly, sialidase digestion partially decreased the area of the
90 most prevalent acidic peak and increased the area of the main peak, indicating that the acidic peak is only
91 partially composed of IgG1 containing terminal sialic acid on the Fc N-glycans. Glycation was not
92 identified as a main attribute because the decrease in the peak areas of the acidic fractions by enzymatic
93 deglycosylation with PNGase F was limited to the effect of sialylation. The deglycosylated main and acidic
94 fractions were reduced and carboxymethylated, and then the molecular weights of their heavy and light
95 chains were determined by liquid chromatography-mass spectrometry. As a result, the two molecular
96 weights were found to be completely identical between the main and acidic fractions. Furthermore, trypsin
97 peptide mapping of the deglycosylated main and acidic fractions revealed that the acidic variants do not
98 include primary sequence modifications (such as deamidation). The most probable cause of this gap may be
99 slight conformational differences that are difficult to detect. Interestingly, Gervais et al. report that, based
100 on results of extended characterization and refolding studies, the most prevalent acidic peak of *Erwinia*
101 *chrysanthemi* L-asparaginase (a 140-kDa homotetramer in its active form) is a conformational variant with
102 no primary sequence modifications [17].

103 In this study, to test the possibility that the acidic fractions of therapeutic mAbs contain conformational
104 variants, we examined whether additional acidic peaks could be generated by a refolding process without a
105 reducing agent, using as an example the aforementioned IgG1 mAb that contains many unidentified acidic
106 peaks with few post-translational modifications. Since acidic peaks were generated by the refolding
107 process, we considered these acidic peaks as conformational variants and examined the conformational
108 changes that occur in the acidic fraction using various analytical methods.

1092. Materials and methods

1102.1 Materials

111 The drug substance (DS) used in this study was a humanized IgG1 mAb to human interleukin-6 receptor,
112 manufactured by Chugai Pharmaceutical (Tokyo, Japan). The theoretical isoelectric point of the IgG1 was
113 9.3. Cytochrome C from equine heart and ribonuclease A from bovine pancreas were purchased from
114 Merck (Darmstadt, Germany). 2-Morpholinoethanesulfonic acid monohydrate (MES) was purchased from
115 Dojindo Laboratories (Kumamoto, Japan). 1-Anilino-8-naphthalene sulfonate (ANS) was purchased from
116 Cayman Chemical (Ann Arbor, MI, USA). All other chemicals were purchased from Fujifilm Wako Pure
117 Chemical Corporation (Osaka, Japan). The protein samples were formulated in a buffer of 25 mM
118 MES/NaOH, 150 mM NaCl, pH 6.1, unless otherwise noted. The protein concentration was determined
119 using UV absorbance at 280 nm.

1202.2 Protein refolding by stepwise dialysis

121 The IgG1 DS, cytochrome C and ribonuclease A, as well as the main and acidic peak fractions isolated
122 from the IgG1 DS by preparative CEX described below were used in refolding studies without a reducing
123 agent, where all unfolding/refolding steps were maintained at pH 6.1 and performed at room temperature
124 (nominally 23°C). The protein samples were unfolded in the MES formulation buffer in the presence of 6
125 M guanidine hydrochloride at a protein concentration of 0.1 mg/mL for 1 hour. The guanidine
126 hydrochloride was gradually removed to refold the protein by stepwise dialysis using a Slide-A-Lyzer
127 dialysis cassette (20,000 MWCO for IgG1 samples; 3,500 MWCO for cytochrome C and ribonuclease A
128 samples; Thermo Fisher Scientific, Waltham, MA, USA) against 100-fold volume excess of MES
129 formulation buffer with 0.5 M arginine hydrochloride in combination with systematically decreasing
130 concentrations of guanidine hydrochloride (i.e., 3 M, 2 M, 1 M, and 0.5 M) for more than 6 hours each,
131 followed by a final dialysis step against the MES formulation buffer without arginine hydrochloride or
132 guanidine hydrochloride for more than 18 hours.

1332.3 Cation-exchange chromatography (CEX)

134 The main and acidic mAb peak fractions were isolated from the IgG1 DS by preparative CEX, using a
135 Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific) equipped with a semi-preparative column,

136Propac WCX-10 (9.0×250 mm, 10 µm, Thermo Fisher Scientific). Mobile phase A consisted of 25 mM
137MES/NaOH, pH 6.1 while mobile phase B was 25 mM MES/NaOH, 250 mM NaCl, pH 6.1. The IgG1 DS
138was diluted with mobile phase A and injected onto the column at a concentration of 22.5 mg/mL. The
139injection volume was 160 µL, where the column flow-rate was maintained at 1 mL/min throughout the ion
140exchange, and performed at 40°C. After sample injection, the column was run for 5 minutes using a mobile
141phase ratio of 63% v/v A and 37% v/v B followed by a linear elution gradient of 0.4% v/v mobile phase B
142per minute, ending at 50 minutes. The column was then washed for 10 minutes with 100% B. The protein
143elution was monitored by UV absorbance at 280 nm. Each collected sample was buffer-exchanged into the
144standard formulation buffer of 25 mM MES/NaOH, 150 mM NaCl, pH 6.1 and concentrated to over 3
145mg/mL using Amicon Ultra filters (50,000 MWCO; Merck).

146 The elution profiles of IgG1, cytochrome C, and ribonuclease A samples were analyzed by analytical
147CEX, using an Agilent 1290 Infinity HPLC system (Santa Clara, CA, USA) equipped with an analytical
148weak cation exchange column, ProPac WCX-10 (4.0×250 mm, 10 µm, Thermo Fisher Scientific). Except
149as described below, the methods were the same as those for preparative CEX. Before chromatographic
150analysis, the protein samples were buffer-exchanged into mobile phase A and concentrated as needed by
151centrifugation using Amicon Ultra filters (50,000 MWCO for IgG1 samples, 3000 MWCO for
152cytochrome C and ribonuclease A samples; Merck). The protein samples were injected at a concentration of
1530.5 mg/mL. The injection volumes were 30 µL for the IgG1 samples and 100 µL for cytochrome C and
154ribonuclease A, maintaining a flow rate of 0.5 mL/min at room temperature. For the IgG1 samples, after
155running the column for 5 minutes with 63% v/v A and 37% v/v B, a linear elution gradient of 0.4% v/v
156mobile phase B per minute was applied out to 80 minutes. For cytochrome C, a linear elution gradient of
1570.4% v/v B per minute was applied after running the column at 46% v/v A and 54% v/v B for 5 minutes,
158ending at 55 minutes. For ribonuclease A, a linear elution gradient of 0.2% v/v B per minute was used after
159running the column at 67% v/v A and 33% v/v B for 5 minutes, for a total of 100 minutes (in this case,
160mobile phase B consisted of 25 mM MES/NaOH, 500 mM NaCl, pH 6.1). In all instances, and at the
161completion of the CEX gradient, the column was washed for 5 minutes with 100% v/v of the respective
162mobile phase B.

1632.4 *Size-exclusion chromatography (SEC)*

164 The elution profiles of IgG1 samples were analyzed by SEC at room temperature, using an Agilent
1651290 Infinity HPLC system equipped with a size exclusion column, TSK-gel G3000SW_{XL} (7.8×300 mm, 5
166μm, Tosoh, Tokyo, Japan). The IgG1 samples were injected at a concentration of 1 mg/mL, with an
167injection volume of 40 μL. A running buffer of 50 mM sodium phosphate, 300 mM NaCl, pH 7.0 was used
168at a flow rate of 0.5 mL/min. The protein elution was monitored by UV absorbance at 280 nm.

1692.5 *Hydrophobic interaction chromatography (HIC)*

170 The elution profiles of the IgG1 samples were analyzed by HIC using an Agilent 1290 Infinity HPLC
171system equipped with a hydrophobic interaction column, ProPac HIC-10 (4.6×100 mm, 5 μm, Thermo
172Fisher Scientific). Before chromatographic analysis, the IgG1 samples were buffer-exchanged into a buffer
173of 25 mM MES/NaOH, pH 6.1 and concentrated as needed by centrifugation using Amicon Ultra filters
174(50,000 MWCO; Merck). The protein samples were injected at a concentration of 0.4 mg/mL. The injection
175volume was 10 μL. Mobile phase A was 100 mM sodium phosphate, pH 6.1. Mobile phase B was 100 mM
176sodium phosphate, 2 M (NH₄)₂SO₄, pH 6.1. The flow rate was set to 0.5 mL/min and the analysis was
177performed at room temperature. A linear elution gradient of 2% mobile phase A per minute was applied
178after running the column at 50% v/v A and 50% v/v B for 5 minutes, and ended at 20 minutes. The column
179was then washed for 10 minutes with 100% v/v A. The protein elution was monitored by UV absorbance at
180280 nm.

1812.6 *Dynamic light scattering (DLS)*

182 DLS measurements were performed using a DynaPro Plate Reader II (Wyatt Technology Corporation,
183Santa Barbara, CA, USA) to obtain the average hydrodynamic radius [R_h (nm)] at 25°C. The IgG1 samples
184were dispensed into a 384-well plate at a concentration of 0.5 mg/mL. The sample volume was 25 μL.

1852.7 *Small-angle X-ray scattering (SAXS)*

186 SAXS experiments were performed at the EMBL P12 beam line (PETRA III, DESY synchrotron,
187Hamburg, Germany) [18]. An automated robotic sample changer (BioSAXS; Arinax, Grenoble, France)
188was employed to deliver the IgG1 samples (1 mg/mL) and corresponding matched buffers at 20°C under
189continuous flow to the beam line through a 1 mm capillary of the sample exposure unit [19]. Two-

190dimensional SAXS patterns were collected using a PILATUS 6M pixel detector, a sample-detector distance
191of 3.0 m and a wavelength (λ) of 0.124 nm. A momentum transfer range of $0.026 < s < 7.288 \text{ nm}^{-1}$ (where s
192 $= 4\pi\sin\theta/\lambda$, 2θ is the scattering angle) was covered. Data reduction were performed by the automated data
193pipeline SASFLOW [20]. Briefly, the isotropic two-dimensional scattering intensities were normalized to
194the intensity of the transmitted beam and azimuthally averaged to generate one-dimensional scattering
195profiles [$I(s)$ vs s]. Data collection was divided into 20 separate frames of 50 msec each. To exclude data
196from samples potentially affected by radiation damage, automated pairwise 1D data frame comparisons and
197subsequent data-frame rejection were employed, followed by undamaged sample frame averaging and
198buffer scattering subtraction. The radius of gyration (R_g) and extrapolated scattering to zero angle [$I(0)$] was
199obtained by employing the Guinier approximation [$\ln I(s)$ vs s for $sR_g < 1.3$] as well as the calculation of the
200scattering pair-distance distribution function [$p(r)$] from which the maximum dimension ($D_{\max}$) was also
201estimated [21]. The fits to the theoretical scattering profile from the high-resolution crystal structure of a
202human IgG1 (pdb code:1hzh.pdb) were computed with CRY SOL [22].

2032.8 Differential scanning fluorimetry (DSF)

204 DSF measurements were performed using a Prometheus NT.48 (NanoTemper Technologies, Munich,
205Germany) with UV transparent capillaries. In the DSF experiments, a buffer condition of 25 mM
206citrate/NaOH, 150 mM NaCl, pH 4.1 was used in addition to the standard formulation MES buffer at pH
2076.1 because the thermal denaturation temperature (T_m) of the Fab domain at pH 6.1 exceeded this
208instrument's upper measurement temperature (i.e., 95°C). The IgG1 samples were buffer-exchanged into
209the buffer at pH 4.1 via centrifugation using Amicon Ultra filters (50,000 MWCO; Merck). The IgG1
210samples were loaded into the capillaries at a concentration of 0.5 mg/mL. The loading volume was 11 μL .
211DSF scans were performed at a rate of 1°C/min from 15°C to 95°C. Thermal denaturation was measured by
212detecting the temperature-dependent changes in the ratio of intrinsic fluorescence at emission wavelengths
213of 330 and 350 nm (350 nm/330 nm). The T_m was determined from the peaks of the first derivative of the
214fluorescence ratio. Data were analyzed with ThermControl software (NanoTemper Technologies).

2152.9 *Fluorescence measurements*

216 Fluorescence measurements were performed with a microplate spectrofluorometer Infinite M1000
217(Tecan, Mannedorf, Switzerland). For the ANS fluorescence measurements, a concentrated stock solution
218of ANS (2 mM ANS in the standard formulation MES buffer) was added to each IgG1 sample (3 mg/mL)
219to obtain a final ANS concentration of 200 μ M, and 20 μ L of the mixed sample solution was dispensed into
220a 384-well black plate. Fluorescence spectra of ANS were recorded from 450 to 600 nm with an excitation
221wavelength of 385 nm at 25°C. The ANS fluorescence spectrum of each IgG1 sample was corrected by
222subtracting the spectrum of a blank solution of standard formulation MES buffer containing 200 μ M ANS.
223For intrinsic fluorescence measurements, 20 μ L of each IgG1 sample (3 mg/mL) was dispensed into a 384-
224well black plate. Intrinsic fluorescence spectra were recorded from 300 to 360 nm with an excitation
225wavelength of 285 nm at 25°C. The intrinsic fluorescence spectrum of each IgG1 sample was corrected by
226subtracting the spectrum of the blank solution of standard formulation MES buffer.

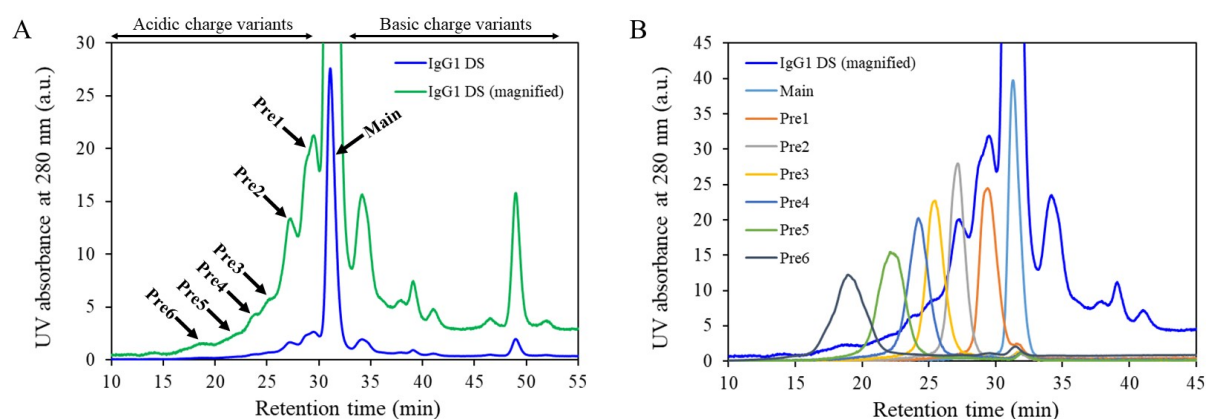


Fig. 1. (A) Representative CEX profile of the IgG1 drug substance (DS) used in this study. The acidic peaks investigated are designated *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* in order of decreasing elution time. **(B)** CEX profiles of the *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* fractions isolated from the IgG1 DS. Magnified CEX profile of the IgG1 DS is shown as a reference.

2273. Results

2283.1 Purity check of isolated acidic and main fractions

229 A representative CEX profile of the IgG1 DS is shown in Figure 1A. A number of acidic/basic charge
 230 variants elute before/after the main peak (hereafter referred to as *Main*), which elutes at around 31 minutes.
 231 In this study, we focused on the physicochemical characterization of six acidic peaks, which, in order of
 232 decreasing elution time, are referred to here as *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6*, respectively. The
 233 *Main* and *Pre* fractions were isolated from the DS by preparative CEX, buffer-exchanged, and concentrated
 234 to over 3 mg/mL. These collected fractions were re-injected for analytical CEX to check the purity (Fig.
 235 1B). The isolated *Main* fraction was found to be free of acidic and basic peaks. Each isolated *Pre* fraction
 236 still contained a small amount of *Main* peak, but was subjected to subsequent characterization without
 237 further purification.

2383.2 *Generation of acidic peaks following refolding*

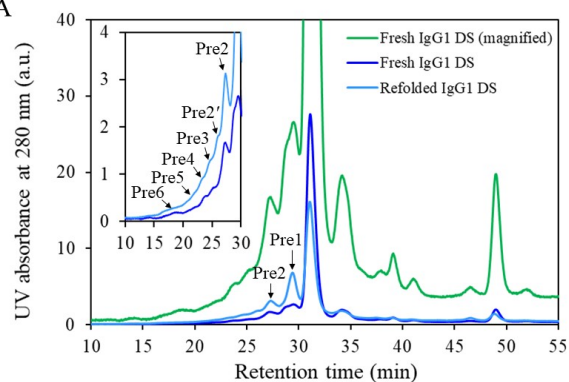
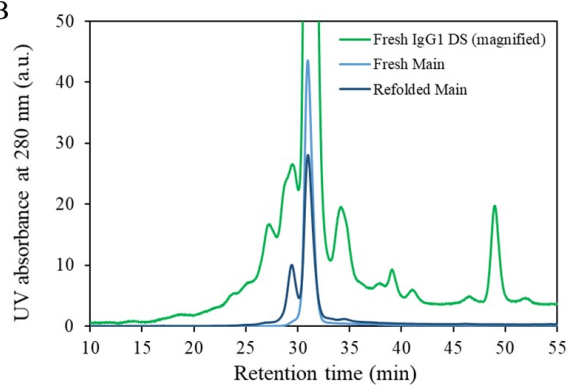
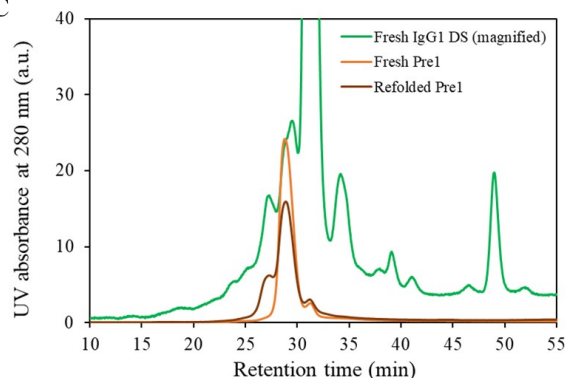
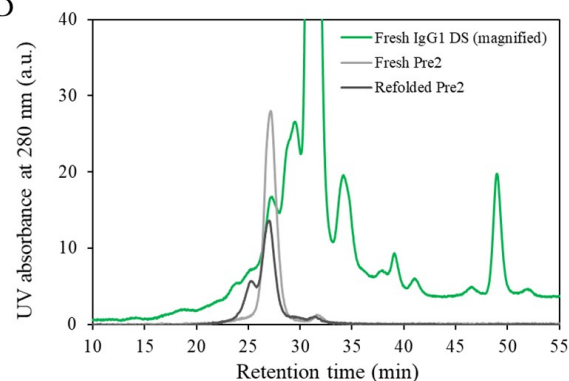
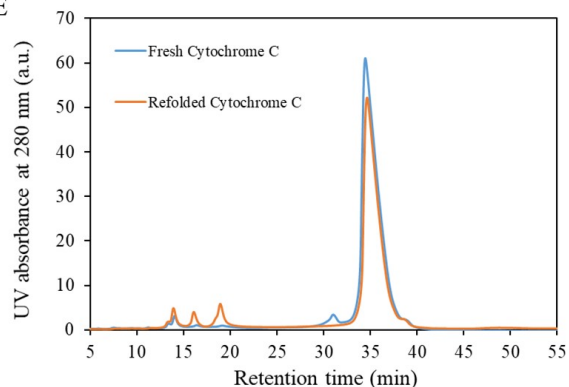
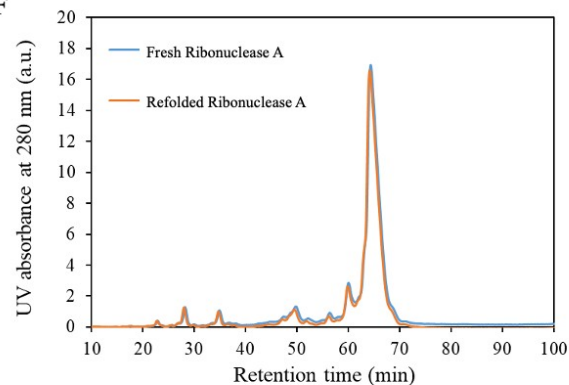
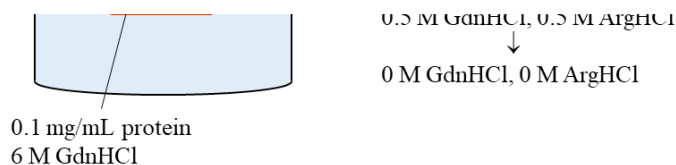
239 **A****B****C****D****E****F**

Fig. 3. CEX profiles of fresh (i.e., non-treated) and refolded **(A)** IgG1 drug substance (DS), **(B)** Main, **(C)** Pre1, **(D)** Pre2, **(E)** cytochrome C, and **(F)** ribonuclease A. The magnified CEX profile of the IgG1 DS is shown in **A**, **B**, **C**, and **D** as a reference. Inset in **A** depicts magnified elution profiles from 10 to 30 minutes. The refolding procedure is outlined in Figure 2.



dialysis (GdnHCl: guanidine hydrochloride, ArgHCl: arginine hydrochloride).

240 To clarify whether the *Pre* peaks corresponded to conformational variants, we performed refolding
241 experiments without a reducing agent. The IgG1 DS and isolated fractions were subjected to denaturation
242 using 6 M guanidine hydrochloride, and were then refolded by stepwise dialysis as outlined in Figure 2.
243 The concentration of guanidine hydrochloride was gradually reduced and 0.5 M arginine hydrochloride was
244 utilized to suppress the formation of aggregates during the refolding process [23, 24]. The protein
245 concentration was set as low as possible (i.e., 0.1 mg/mL) to avoid the formation of aggregates.

246 CEX profiles of fresh (i.e., non-treated) and refolded IgG1 DS are shown in Figure 3A. Following
247 refolding, the area of the *Main* peak decreased, and that of each *Pre* peak increased. The longer the
248 retention time of *Pre*, the greater the increase. A new peak that was not detected in the fresh IgG1 DS was

249 generated between the *Pre2* and *Pre3* peaks (depicted as *Pre2'* in Fig. 3A) after refolding. The total peak
250 area of the samples was not changed during the refolding process (the ratio of refolded to fresh is 1.01),
251 suggesting that almost no aggregates were generated under the refolding conditions. The isolated *Main* and
252 *Pre* fractions were also each subjected to the refolding process to examine the changes in CEX profile. The
253 CEX profiles of fresh and refolded *Main* fractions are shown in Figure 3B. The peak area of *Main*
254 decreased, and *Pre1* and a small amount of *Pre2* were generated. *Pre1* generated from the *Main* fraction by
255 the refolding process exhibited a symmetrical peak shape, whereas the *Pre1* peak in the fresh IgG1 DS was
256 asymmetric, suggesting that the *Pre1* fraction contains multiple components with conformational variant
257 being just one of them. Similarly, the isolated *Pre1* and *Pre2* fractions were refolded and analyzed by CEX
258 (Fig. 3C and 3D). *Pre2* and *Pre3* peaks were generated from the *Pre1* and *Pre2* fractions, respectively. A
259 *Main* peak was not generated from the *Pre1* and *Pre2* fractions.

260 To investigate whether the generation of acidic peaks by refolding is a general phenomenon that could
261 occur in other proteins as well, we subjected cytochrome C and ribonuclease A to the same refolding
262 process and CEX analysis (Fig. 3E and 3F). In cytochrome C, as in the IgG1 DS, the area of the main peak
263 decreased, and the areas of the acidic peaks increased. An acidic peak eluting at around 31 minutes
264 disappeared. In ribonuclease A, the CEX profile was not changed by the refolding process.

265 These results suggest that the acidic peaks in the IgG1 DS are conformational variants and that such
266 variants could be generated in other proteins. To elucidate the conformational changes occurring in the *Pre*
267 fractions, we characterized them using the following methods.

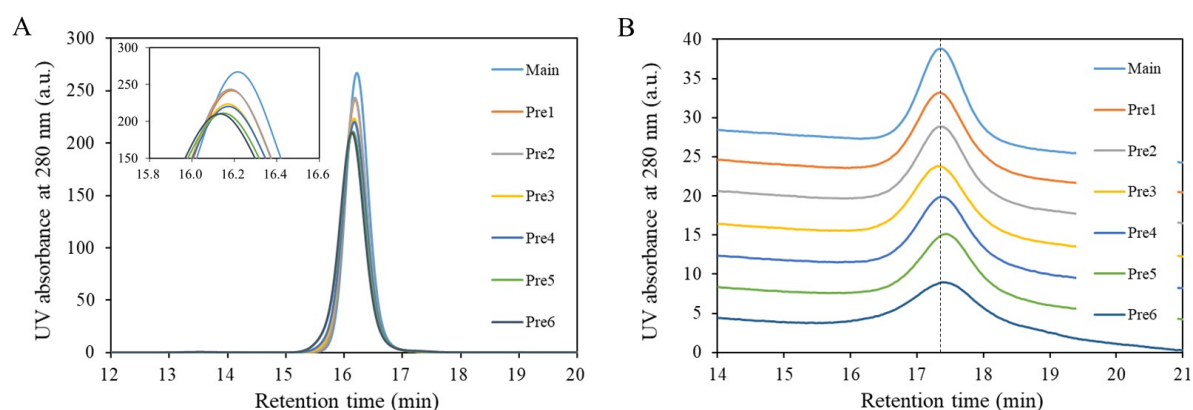


Fig. 4. (A) SEC and (B) HIC profiles of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* fractions. Inset in A depicts magnified elution profiles from 15.8 to 16.6 minutes. Dotted line in B shows the retention time of peak of *Main*.

2683.3 SEC and HIC analysis

269 The isolated *Main* and *Pre* fractions were analyzed by SEC. All samples eluted as a single mAb
 270 monomer peak and no oligomers or aggregates such as dimers were detected (Fig. 4A). The shorter the
 271 elution time of *Pre* in CEX, the broader the monomer peak and the slightly shorter the elution time in SEC,
 272 suggesting that the conformational change of *Pre* variants altered their interaction with the SEC column
 273 resin or that the conformation of the *Pre* variants was slightly expanded compared to that of the *Main*
 274 variant. The isolated *Main* and *Pre* fractions were also analyzed by HIC (Fig. 4B). Similar to the SEC
 275 profiles, the shorter the elution time of *Pre* in CEX, the broader the peak in HIC. The retention times of
 276 *Pre4*, *Pre5*, and *Pre6* were slightly greater than that of *Main*, indicating that these *Pre* variants were slightly
 277 more hydrophobic than the *Main* variant.

Table 1 Hydrodynamic radius [R_h (nm)], radius of gyration [R_g (nm)], maximum dimension [D_{max} (nm)], and χ^2 of fit to the theoretical SAXS profile of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*,

2783.4 DLS and SAXS analysis

279 The R_h values of each fraction were determined by DLS measurements (Table 1). No difference in
280 hydrodynamic properties were detected by DLS, and all samples showed the same R_h values (i.e., 5.3 nm),
281 which is consistent with the R_h values reported for an IgG1 monomer [25]. SAXS profiles and the $p(r)$
282 functions of each fraction obtained at 20°C are displayed in Figure 5A and Figure 5B, respectively. The

*^a obtained by DLS measurements at a concentration of 0.5 mg/mL at 25°C. Data
represent the mean $\pm$ SD of three experiments.

*^b obtained by SAXS measurements at a concentration of 1 mg/mL at 20°C.

*^c obtained by fitting to the theoretical scattering profile from the crystal structure of a
human IgG1 (pdb code:1hzh.pdb) with the ATSAS program CRY SOL [22].

283 SAXS profiles and $p(r)$ were typical of monomeric IgG1 [26] and no marked differences were detected
284 between the *Main* and *Pre* fractions. The scattering profiles were compared to the theoretical scattering
285 profile from the crystal structure of a human IgG1 (pdb code:1hzh.pdb) with the ATSAS program
286 CRY SOL. The obtained χ^2 values are listed in Table 1 and range 1.3 to 1.6 suggesting a good agreement
287 between the solution structure and model. The value of R_g and D_{max} were within the error estimates for
288 different fractions (Table 1). These results suggest that the conformational changes occurring in the *Pre*
289 variants are minor.

2903.5 DSF analysis

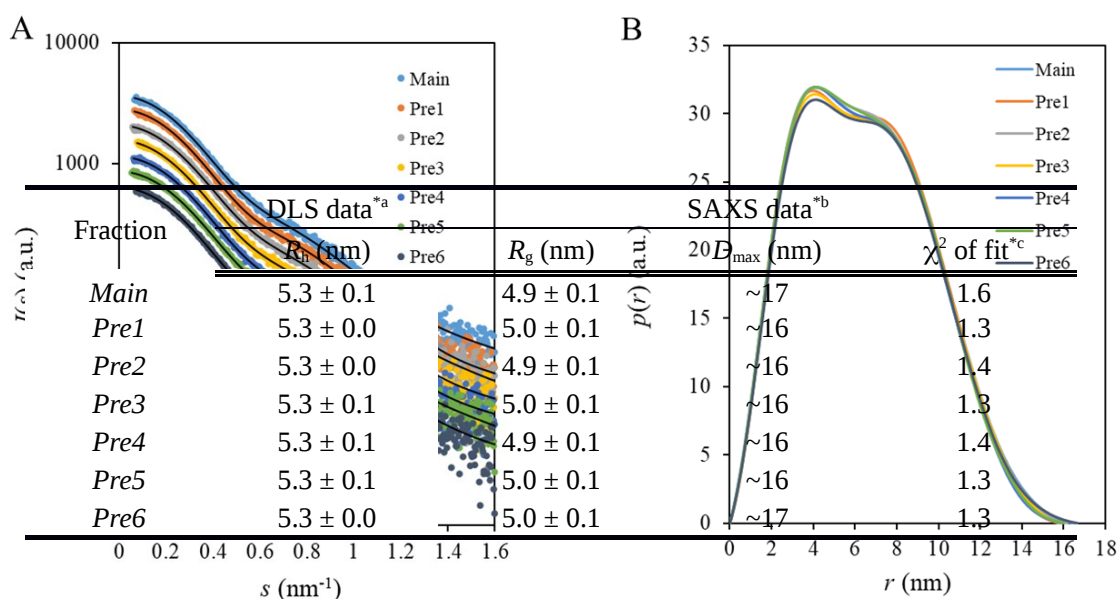


Fig. 5. (A) SAXS profiles of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* fractions (1 mg/mL) at 20°C. The scattering data of *Pre* fractions were shifted downwards in logarithmic scale to avoid overlapping. Solid lines are the result of fitting by indirect Fourier transformation. **(B)** the pair-distance distribution function [$p(r)$].

291 For the IgG1 used in this study, thermal denaturation of the CH2, CH3, and Fab domains occurs
 292 separately in order of increasing temperature [27]. To clarify which domains potentially undergo
 293 conformational changes in the *Pre* variants, the T_m of each domain of the *Main* and *Pre* fractions was
 294 determined by DSF and compared. DSF thermograms of each fraction at pH 6.1 and pH 4.1 are shown in
 295 Figure 6A and Figure 6B, respectively. At pH 6.1, the T_m of the Fab domain exceeded the upper
 296 temperature limit of the instrument, and consequently the thermal denaturation of only the two domains of
 297 CH2 and CH3 could be detected; however, because the T_m of these domains decreases with the reduction in
 298 pH, the thermal denaturation of the three domains of CH2, CH3, and Fab could be detected at pH 4.1. The
 299 thermal denaturation curves almost overlapped between the samples, but slight differences between the
 300 *Main* and *Pre* fractions were detected in the CH2 and Fab domains. The T_m of the CH2 and CH3 domains at
 301 pH 6.1 are shown in Figure 6C and 6D, and the T_m of the CH2, CH3, and Fab domains at pH 4.1 are shown
 302 in Figure 6E, 6F, and 6G. The T_m of the CH2 domain of the *Pre* fractions was slightly lower than that of the

303 *Main* fraction at both pH values, but no marked difference was detected between the *Main* and *Pre*
304 fractions in the CH3 domain. The T_m of Fab domain tended to be lower in the *Pre* fractions than in the
305 *Main* fraction at pH 4.1, but the difference was extremely small. These results suggest that the
306 conformational changes of the *Pre* variants are minor and occur at multiple sites, at least across the CH2
307 and Fab domains.

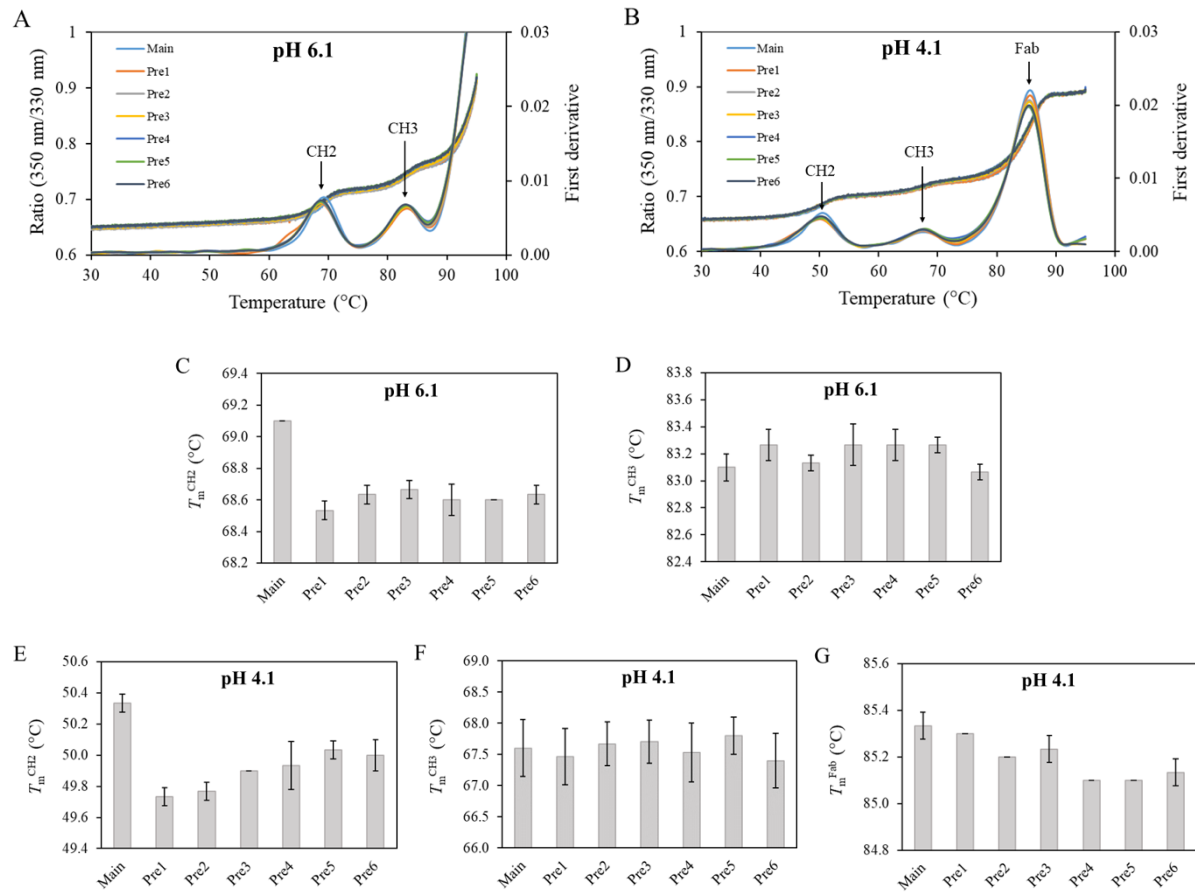
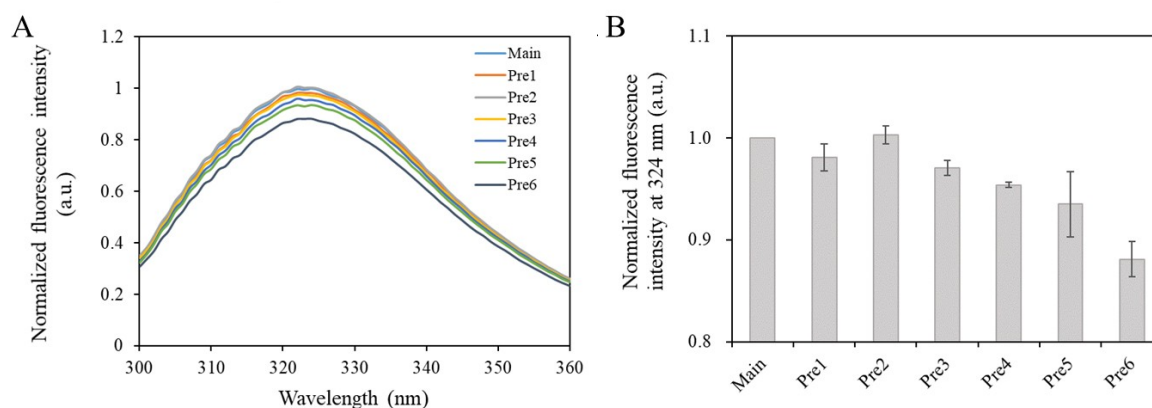


Fig. 6. DSF thermograms of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* fractions (0.5 mg/mL) at **(A)** pH 6.1 and **(B)** pH 4.1. The y-axes represent the ratio of intrinsic fluorescence at emission wavelengths of 330 and 350 nm (350 nm/330 nm) and the first derivative. Each curve represents the mean of three experiments. The peaks of the first derivative correspond to the thermal denaturation of CH2, CH3, and Fab domains in order of increasing temperature. The T_m (°C) of **(C)** CH2 and **(D)** CH3 domains at pH 6.1. The T_m of **(E)** CH2, **(F)** CH3, and **(G)** Fab domains at pH 4.1. Each column represents the mean $\pm$ SD of three experiments.

3083.6 ANS and intrinsic fluorescence analysis

309 To further investigate the conformational changes occurring in the *Pre* variants, the hydrophobicity of
 310 each fraction was evaluated by ANS, a fluorescent probe that binds to hydrophobic regions. The ANS
 311 fluor^{Fig. 7.} **(A)** ANS fluorescence spectra of isolated *Main*, *Pre1*, *Pre2*, *Pre3*, *Pre4*, *Pre5*, and *Pre6* *Pre*
 312 fract^{fractions} at 25°C. The concentrations of ANS and IgG1 were 200 μ M and 2.7 mg/mL, respectively.
 313 inten^{The} The excitation wavelength was 385 nm. The fluorescence intensity was normalized to the intensity I_m
 of the *Main* sample at 494 nm. Each curve represents the mean of three experiments. **(B)**
 Normalized fluorescence intensity at 494 nm and **(C)** λ_{max} (nm). Each column represents the mean
 $\pm$ SD of three experiments.



B

fractions at 25°C. The concentration of IgG1 was 3 mg/mL. The excitation wavelength was 285 nm. The fluorescence intensity was normalized by the maximum intensity of the *Main* sample. Each curve represents the mean of three experiments. **(B)** Normalized fluorescence intensity at 324 nm. Each column represents the mean $\pm$ SD of three experiments.

Main *Pre1* *Pre2* *Pre3* *Pre4* *Pre5* *Pre6*

emission wavelength (λ_{max}) of ANS in the *Pre* fractions was slightly blue-shifted compared to that of the *Main* fraction. Subsequently, environmental changes around aromatic residues were investigated by intrinsic fluorescence measurements. The intrinsic fluorescence spectra under excitation at 285 nm are shown in Figure 8A. The fluorescence intensity tended to decrease slightly as the *Pre*-fraction number increased (Fig. 8B).

4. Discussion

As previous characterization studies on charge variants of therapeutic mAbs have shown, the full characterization of post-translational modifications in acidic variants of mAbs is challenging and remains an open area of investigation [9, 14–16]. It is usually found that quantitative measurements of identified acidic species leave some gaps, and that the sum of the identified acidic species does not fully account for the total acidic regions in the elution profile. Neill et al. reported that the basic variants of a mAb detected by CEX could be all fully accounted for by identified post-translational modifications, whereas the acidic variants could only be partially explained by deamidation and isomerization [9]. Dada et al. characterized both the acidic and basic regions of imaged capillary isoelectric focusing profile of an IgG1 mAb by using

328preparative immobilized pH gradient isoelectric focusing fractionation and reported that unlike the basic
329variants where the detected modifications are discrete and exclusive, all acidic peaks to a varying degree
330shared similar modifications [16].

331 As described in Introduction section, the acidic fractions of the IgG1 mAb used in this study have also
332been extensively characterized previously by various techniques. However, interestingly, most of them
333showed no differences from the *Main* fraction (unpublished results). Since few post-translational
334modifications were detected, we speculated that the main attributes characterizing these unidentified acidic
335variants would be alterations in their higher order structure. Interestingly, Gervais et al. report that, on the
336basis of capillary isoelectric focusing, liquid chromatography-mass spectrometry, and liquid
337chromatography-tandem mass spectrometry data, the acidic variants of L-asparaginase do not include
338primary sequence modifications (such as deamidation), and they concluded on the basis of SAXS and ion
339mobility-mass spectrometry data showing slight conformational differences between the acidic and main
340peaks that the most prevalent acidic peak is a conformational variant [17]. That consideration was further
341supported by the results of refolding experiments demonstrating that the acidic peak could be converted
342into the main peak after unfolding using 8M urea and 2.2 M N-ethylurea and refolding by dialysis against
343water [17]. Even such a small conformational change may alter the overall surface charge distribution,
344owing to changes in the local distribution of charged residues. With reference to the study by Gervais et al.,
345in this study we investigated whether conformational changes occur in the unidentified acidic variants of
346IgG1, and we analyzed the conformational changes occurring in the acidic fractions in more detail.

347 First, we examined whether refolding without a reducing agent could generate the acidic variants of
348IgG1. By applying stepwise dialysis, the use of arginine hydrochloride during refolding [23, 24], and a low
349concentration of the IgG1, we found conditions under which the IgG1 could be refolded without
350aggregation formation and under which the acidic variants could be generated. The results of the refolding
351studies revealed that the unidentified acidic variants of IgG1 would be conformational variants.
352Interestingly, *Pre1* was generated from *Main*, *Pre2* was generated from *Pre1*, and *Pre3* was generated from
353*Pre2* by the refolding process, suggesting that the acidity of the IgG1 molecular surface increased in a
354stepwise pattern as certain conformational changes accumulated in the *Main* molecule and these variants
355were eluted as multiple *Pre* peaks depending on the degree of accumulation of the conformational changes.
356The fact that only a small amount of *Pre2* and no *Pre3* were generated from *Main* is likely due to the low

357 probability of multiple conformational changes occurring simultaneously within a single molecule. With L-
358 asparaginase, the refolding process generated the main peak from the acidic peak [17], but this was not the
359 case for the IgG1 used in this study, presumably because the activation energy barrier for changing from
360 *Pre* variants to the *Main* variant is very high in IgG1. The *Pre1* peak generated from *Main* by refolding
361 process was symmetric. On the other hand, the *Pre1* peak originally contained in the IgG1 DS was
362 asymmetric, which is likely due to the presence of sialylated IgG1 as described in Introduction section in
363 addition to the conformational variant.

364 SAXS experiments were performed to investigate the conformational changes occurring in the *Pre*
365 variants, but no marked differences in SAXS profiles were detected between the *Main* and *Pre* fractions,
366 unlike in the case of L-asparaginase [17]. As well, no difference was detected between the *Main* and *Pre*
367 fraction's R_h measured by DLS. These results indicate that the conformational changes occurring in the *Pre*
368 variants are likely localized/minor and do not greatly affect the overall global disposition of the IgG1 in
369 solution. Since IgG1 is a multi-domain/modular protein that is present in solution as a population-state
370 ensemble, the minor conformational changes occurring in the *Pre* variants may be difficult to detect by
371 SAXS due to the net averaging of conformational fluctuations. By isolating the Fc and Fab domains after
372 fragmentation by enzyme treatment (such as papain digestion) and comparing the SAXS data between the
373 *Main* and *Pre* fractions, it may be possible to identify those domains in which the conformational changes
374 occur. However, isolating the Fc and Fab domains of the *Pre* fractions for SAXS experiments is time-
375 consuming and labor-intensive; therefore, in this study, the SAXS experiments were limited to the
376 measurement of the whole IgG1. Online CEX-SAXS, which can obtain SAXS data while isolating and
377 purifying each domain using enzyme-treated IgG1, may be a suitable tool for detecting conformational
378 changes occurring in the *Pre* variants [28–30]. Further studies will be required in order to detect the
379 conformational changes in the *Pre* variants using SAXS.

380 In the SEC, HIC, DSF, and fluorescence analyses, slight differences were observed between the *Main*
381 fraction and the *Pre* fractions, however, the differences detected were extremely small. The results of HIC
382 and ANS fluorescence showed that the *Pre* variants were slightly more hydrophobic than the *Main* variant.
383 In DSF, the T_m of the CH2 and Fab domains were slightly decreased in the *Pre* fractions, suggesting that the
384 conformational changes in the *Pre* variants occur at multiple sites, at least across these two domains. The
385 intrinsic fluorescence intensity of the *Pre* fractions decreased in a CEX retention time-dependent manner,

386 suggesting that the minor conformational changes of the *Pre* variants occur around aromatic residue
387 regions. Wen et al. reported that microconformational changes within aromatic hydrophobic regions of
388 several representative proteins could be induced by incubation with amine or guanidine additives [31].
389 Taking into account the results of that study, it could be assumed that, in the *Pre* variants, the side chains of
390 aromatic residues are exposed to water due to local denaturation around the aromatic side chains, resulting
391 in a decrease in the intrinsic fluorescence intensity and an increase in the hydrophobicity of the molecular
392 surface. In SEC, the elution time of the monomer peak was slightly shorter for the *Pre* fractions;
393 considering the results of HIC, fluorometry, and DSF, this was presumably due to the slightly expanded
394 conformation of the *Pre* variants compared to that of the *Main* variant due to local denaturation. This
395 hypothesis is consistent with the results of a previous study, in which SAXS measurements detected a
396 slightly expanded state in the acidic peak of L-asparaginase [17].

397 For cytochrome C, acidic peaks were also generated by the refolding process. It is interesting to note that
398 conformational variants resulting from the refolding process were detected as acidic peaks rather than basic
399 peaks both in IgG1 and in cytochrome C. One possible explanation is that the exposure of aromatic amino
400 acid side chains to water is induced by local denaturation, resulting in altered interactions with the column
401 resin and the subsequent detection as acidic peaks. However, further studies are needed to clarify the
402 mechanism of this phenomenon. On the other hand, refolding of ribonuclease A did not change the CEX
403 profile, and no acidic peaks were generated. The stability of ribonuclease A is well known. The classical
404 method for purification of ribonuclease A from bovine pancreas relies on its high stability that can maintain
405 its integrity and solubility under severe conditions [32]. Conformational changes such as those seen in this
406 study may be less likely to occur for highly stable proteins such as ribonuclease A.

4075. Conclusion

408 Charge variation is one of the most common forms of heterogeneity in therapeutic mAbs, and thorough
409 characterization of charge variants is important to identify critical quality attributes. However, it is not
410 unusual that the sum of the quantitative values of the identified acidic species does not fully account for the
411 total acidic regions in the elution profile. In this study, acidic variants of IgG1, which had been rarely
412 identified in previous characterizations, were found to be generated by the process of refolding without a

413reducing agent, indicating that these acidic variants are conformational variants. On the other hand, no
414conformational changes were detected by SAXS experiments for the intact IgG1, indicating that the
415conformational changes are minor. The results of HIC and ANS fluorescence showed that the *Pre* variants
416were slightly more hydrophobic than the *Main* variant. In DSF, the T_m of the CH2 and Fab domains were
417slightly decreased in the *Pre* fractions, suggesting that the minor conformational changes in the *Pre* variants
418occur at multiple sites, at least across these two domains. The intrinsic fluorescence intensity of the *Pre*
419fractions decreased in a CEX retention time–dependent manner, suggesting that the minor conformational
420changes of the *Pre* variants occur around aromatic residue regions. Taken together, these results suggest
421that, in the *Pre* variants, the side chains of aromatic residues are exposed to water due to local denaturation
422around the aromatic side chains, resulting in a decrease in the intrinsic fluorescence intensity and an
423increase in the hydrophobicity of the molecular surface. For cytochrome C, acidic peaks were also
424generated by the refolding process. One possible explanation is that the exposure of aromatic amino acid
425side chains to water is induced by local denaturation, resulting in altered interactions with the column resin
426and the subsequent detection as acidic peaks. Further studies are needed to elucidate in more detail the
427conformational changes, their formation mechanisms, and the mechanisms by which they are detected as
428acidic variants. This study provides new insights into the characterization of acidic variants of therapeutic
429mAbs that are not currently fully understood.

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