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Effects of Acid Exposure on the Conformation, Stability, and Aggregation of Monoclonal Antibodies

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ABSTRACT **Exposure of antibodies to low pH is often unavoidable for purification and viral clearance. The conformation and stability of two humanized monoclonal antibodies (hIgG4-A and -B) directed against different antigens and a mouse monoclonal antibody (mIgG1) in 0.1*M* citrate at acidic pH were studied using circular dichroism (CD), differential scanning calorimetry (DSC), and sedimentation velocity. Near- and far-UV CD spectra showed that exposure of these antibodies to pH 2.7–3.9 induced only limited conformational changes, although the changes were greater at the lower pH. However, the acid conformation is far from unfolded or so-called molten globule structure. Incubation of hIgG4-A at pH 2.7 and 3.5 at 4°C over the course of 24 h caused little change in the near-UV CD spectra, indicating that the acid conformation is stable. Sedimentation velocity showed that the hIgG4-A is largely monomeric at pH 2.7 and 3.5 as well as at pH 6.0. No time-dependent changes in sedimentation profile occurred upon incubation at these low pHs, consistent with the conformational stability observed by CD. The sedimentation coefficient of the monomer at pH 2.7 or 3.5 again suggested that no gross conformational changes occur at these pHs. DSC analysis of the antibodies showed thermal unfolding at pH 2.7–3.9 as well as at pH 6.0, but with decreased melting temperatures at the lower pH. These results are consistent with the view that the antibodies undergo limited conformational change, and that incubation at 4°C at low pH results in no time-dependent conformational changes. Titration of hIgG4-A from pH 3.5 to 6.0 resulted in recovery of native monomeric proteins whose CD and DSC profiles resembled those of the original sample. However, titration from pH 2.7 resulted in lower recovery of monomeric antibody, indicating that the greater conformational changes observed at this pH cannot be fully reversed to the native structure by a simple pH titration. Proteins 2007;66:954–962.**

Key words: antibody; acid treatment; conformation; aggregation; circular dichroism; sedimentation velocity; differential scanning calorimetry

INTRODUCTION

Antibodies are one of the most versatile reagents and are under extensive development for therapeutic uses. In both applications, the antibodies must be in the native, monomeric state to be stable and functional. In addition, aggregation of antibodies must be minimized for pharmaceutical applications. Unlike other protein reagents or therapeutic proteins, antibodies can be readily purified using affinity columns, such as Protein-A. A conventional purification approach is to bind the antibody to a Protein-A column and then elute the bound antibody at low pH. It is well known that acid induces conformational changes in antibodies, particularly the Fc domain.[1–8] Based on the premise that the eluted antibody is unstable at the pH of elution, the eluted sample is often neutralized immediately. However, it is not clear whether the acid-unfolded structures would undergo slow conformational changes if stored in acidic solution following elution.

The necessity for viral clearance from therapeutic antibodies during their manufacture creates additional opportunities for damage. The most effective means of viral inactivation is acid treatment. The lower the pH the antibody preparations are exposed to, the more effectively the viruses are inactivated. Considering the necessary involvement of low pH in antibody processing, it is important to understand the conformational changes, stability, and aggregation of antibodies at low pH and during titration from low to neutral pH values.

Here, we have examined these issues using three model antibodies: two humanized monoclonal antibodies of subtype IgG4 (hIgG4-A and -B), directed against different antigens, and a mouse monoclonal antibody, IgG1 (mIgG1).[9,10] These antibodies were exposed to low pH and characterized for conformation, stability, and aggregation using near-

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and far-UV circular dichroism (CD), sedimentation velocity, and differential scanning microcalorimetry (DSC). In addition, we examined hIgG4-A after titration from low to neutral pH to determine the impact of low pH treatment on the subsequent processing of acid-treated antibodies.

MATERIALS AND METHODS

Sample Preparation

The hIgG4-A and -B and mIgG1 antibodies used in this study were manufactured in large-scale cell culture and purified using Protein-A Sepharose, ion-exchange chromatography, and gel filtration. hIgG4-A and -B were generated against different antigens. Because of extremely low expression, only a small amount of hIgG4-B could be recovered. SDS-PAGE and size-exclusion analyses indicated that the purified antibodies were close to homogeneity.

The monoclonal antibodies were loaded onto HiTrap Protein-A columns at 4°C. After the column was washed with 20 m*M* phosphate and 0.15*M* NaCl, pH 7.0, the bound antibody was eluted with 0.1*M* citrate. The pH of the citrate ranged from 2.7 to 3.9, depending on the antibodies and experiments. The eluted protein was immediately diluted with the corresponding citrate buffer to about 1 mg/mL for CD and sedimentation experiments and to about 5 mg/mL for DSC experiments. These samples were held at 4°C for various lengths of time for stability studies. Acid-treated samples were also titrated with 1*M* Tris-base to pH 6.0 for CD and sedimentation experiments. For DSC measurements, the acid-treated samples were dialyzed against phosphate buffer at pH 6.0 to obtain an identical solvent composition for measurement of the heat capacity change of the reference cell.

In a separate experiment, mIgG1 was eluted from Protein-A columns at either pH 2.7 or pH 3.5 titrated to pH 7.6, and subjected to gel filtration using Superdex-200 and an elution solvent of 0.1*M* phosphate, pH 7.0. The monomer fraction was collected for CD analysis.

Circular Dichroism Spectroscopy

A Jasco J-715 spectropolarimeter was used with a 1-cm path-length cell for the near-UV CD and a 0.1-cm cell for the far-UV CD. Far-UV CD measurements were carried out in 25 m*M* citrate and at 0.2 mg/mL protein because the absorbance was too high when solutions of 0.1*M* citrate and 1 mg/mL protein were used. The temperature of the cells was maintained at 4°C by a Peltier cell holder and a PTC-348WL temperature control unit. The UV CD spectra were obtained using the following conditions: scan speed, 10 nm/min; 5 times accumulation; resolution, 0.1 nm; time constant, 4 s. After subtracting the solvent spectrum, the mean residue ellipticity, which normalizes the difference in the concentration of the samples, was calculated using a mean residue weight of 109.7.

Differential Scanning Calorimetry

Thermal unfolding was measured with a differential scanning calorimeter, nanoDSC II model 6100 (Calorimetry Sciences Corp., Utah) with platinum tubing cells. The antibody concentrations were adjusted to the following values prior to the measurements: for hIgG4-A, 5.0 mg/mL; for hIgG4-B, 1.92 mg/mL (pH 2.9) and 1.60 mg/mL (pH 6.0); for mIgG1, 2.00 mg/mL (pH 3.9) and 2.06 mg/mL (pH 6.0). The corresponding low-pH buffers and the formulation buffer (pH 6.0) were used as reference solutions for DSC measurements. These protein concentrations, except for that of hIgG4-A, are the highest achieved from sample preparation. Since our major goal was to detect endothermic unfolding and hence the presence of folded structures in acid, the different protein concentrations used in the study should not affect our conclusions, although they may affect the melting temperatures of irreversible unfolding (as occurs in our results). The heating rate employed was 1 K/min and the scanning was performed from 0 to 125°C.

Sedimentation Velocity

After loading into 2-channel charcoal-epon cells (with the corresponding buffer in the reference channel), the samples and the AN-60TI rotor were brought to 20°C in the Beckman Optima XL-A analytical ultracentrifuge. The rotor was brought to 3000 rpm to check for leaks. The rotor speed was then increased to 45,000 or 50,000 rpm and absorbance versus radius scans (at 280 nm) were recorded at 4–5 min intervals.

The distribution of species (amount of material sedimenting at different rates) was obtained using the "$c(s)$" method developed by Peter Schuck at the N.I.H. and implemented in his analysis program SEDFIT (version 6).[11] The $c(s)$ method achieves a much higher resolution of different species by computationally removing the influence of diffusion. The $c(s)$ method requires an estimate of the frictional coefficient for the major species, relative to an equivalent sphere (the f/f_0 ratio). A value of f/f_0 was selected to be consistent with the observed sedimentation coefficient of the main peak and the known mass of the hIgG4-A antibodies. In analyzing the data using SEDFIT, the size range covered was generally from ~0.6 to 20 S, and the distribution was calculated at a confidence level of 0.683 (1 σ).

RESULTS

The monoclonal antibodies used in this study (hIgG4-A, hIgG4-B, and mIgG1) were prepared from conditioned media using Protein-A, cation exchange, and size-exclusion chromatographies and were homogeneous in size (as demonstrated by sedimentation velocity). The purified antibodies were applied to a HiTrap Protein-A Sepharose column and eluted with 0.1*M* citrate, pH 2.7–3.9.

CD

The near-UV CD spectrum of native hIgG4-A, at pH 6.0 and 4°C, is shown in Figure 1(A). The spectrum is characterized by a positive peak at 292 nm (attributable to tryptophan) and several negative peaks between 260 and 290 nm

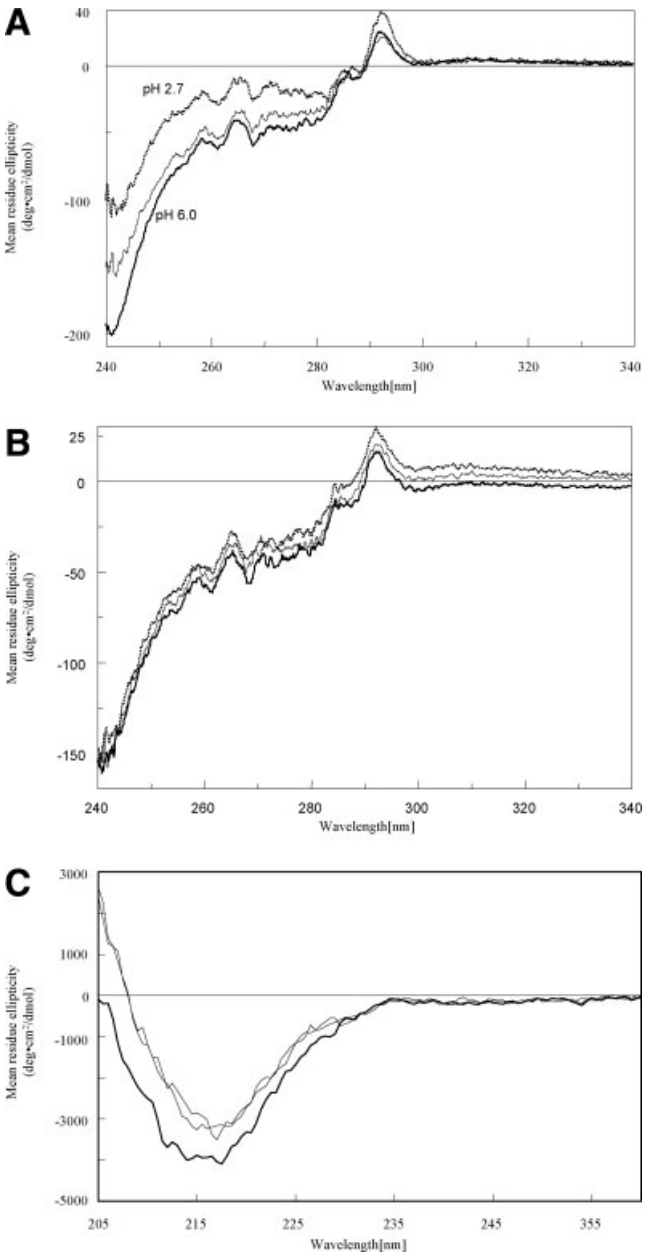

Fig. 1. The near- and far-UV CD spectra of hIgG4-A. Spectroscopic measurement conditions are shown in Materials and Methods section. (**A**) The near-UV CD spectra in 0.1*M* citrate at 4°C of hIgG4-A at pH 6.0 (thick solid line), pH 3.5 (thin solid line), and pH 2.7 (dashed line). Spectra at pH 3.5 and 2.7 were taken immediately after elution from Protein A chromatography. (**B**) The near-UV CD spectra in 0.1*M* citrate at 4°C of hIgG4-A at pH 3.5, immediately (thick solid line), 4 h (thin solid line), and 24 h (dashed line) after elution from Protein A chromatography. Thick solid and dashed lines were shifted arbitrarily downward and upward, respectively. (**C**) The far-UV CD spectra in 25 m*M* citrate at 4°C of hIgG4-A at pH 6.0 (thin solid line), pH 3.5 (dotted line), and pH 2.7 (thick solid line). Lower citrate and peptide concentrations were used to reduce absorbance.

(attributable to tyrosine and phenylalanine). It is quite likely that multiple disulfides present in the antibody also contribute to the CD signal in this region.

The near-UV CD spectra of the antibody eluted at pH 2.7 and 3.5 are also shown in Figure 1(A). These spectra were taken at 4°C immediately after elution. Both low-pH spectra have features resembling those of the native protein at pH 6.0. Aromatic signals present in the native protein are also present in the spectra of the low-pH samples. It is evident that the spectra are shifted upward relative to the spectrum of the pH 6.0 sample. A greater upward shift was observed at pH 2.7.

The presence of aromatic signals resembling those of the native protein indicates that at pH 2.7 and 3.5 the protein retains a distinct tertiary structure and suggests that no gross conformational change has occurred. Nevertheless, the observed upward shift of the spectrum relative to that obtained at pH 6.0 does indicate that a conformational change occurs at pH 3.5 and is further enhanced at pH 2.7. This upward shift might be attributed to changes in the configuration of disulfide bonds, since disulfides in general show a broad CD spectrum in the near-UV region.

The near-UV CD spectrum for the pH 3.5 sample was also measured 4 and 24 h after elution of the antibody from the Protein-A column [Fig. 1(B)]. The spectra were, within experimental error, superimposable with the spectrum taken immediately after elution. This indicates no detectable conformational change of hIgG4-A over the course of a day when stored at 4°C at pH 3.5.

The same experiments were carried out for the pH 2.7 sample (data not shown). The spectrum after 4 or 24 h at pH 2.7 was identical, within experimental error, to that at time zero, again suggesting no conformational change upon storage at 4°C in 0.1*M* citrate, pH 2.7.

We also examined the secondary structure of hIgG4-A at low pH using far-UV CD. As the near-UV CD does not change during incubation at 4°C over 24 h, the far-UV CD measurements were done after 1-day incubation at 4°C. As shown in Figure 1(C), the far-UV CD spectrum at pH 3.5 was indistinguishable from that at pH 6.0, indicating no apparent change in the secondary structure at pH 3.5. This suggests that the observed change at pH 3.5 in the near-UV CD is not accompanied by a change in the secondary structure. The negative CD intensity peak is enhanced at pH 2.7, suggesting that the secondary structure is altered at this pH and the immunoglobulin-domain type β-sheet structure is increased, consistent with the greater observed near-UV CD change at pH 2.7. Since the CD far-UV spectral features of the native protein are retained at these low pHs, it is evident that there is no secondary structure unfolding, as is the case for the tertiary structure.

We next studied the acid conformation of mIgG1 at pH 3.9 and of hIgG4-B at pH 2.9. As shown in Figure 2(A), the near-UV CD spectrum of hIgG4-B at pH 2.9 was similar to that of the native protein, with identical peak positions, but was shifted upward relative to the pH 6.0 spectrum in a manner similar to that of hIgG4-A. The spectrum of mIgG1 at pH 3.9 was almost identical to that at pH 6.0 [Fig. 2(B)], indicating little conformational change at pH 3.9. Although the conditions have not been entirely identical, we have consistently observed that the conformation of mIgG1 in acid over 24 h incubation at 4°C resembles those seen in Figure 2(B) and also follows the trend seen for hIgG4-A, i.e., an upward shift as the pH is lowered to 3.5 or

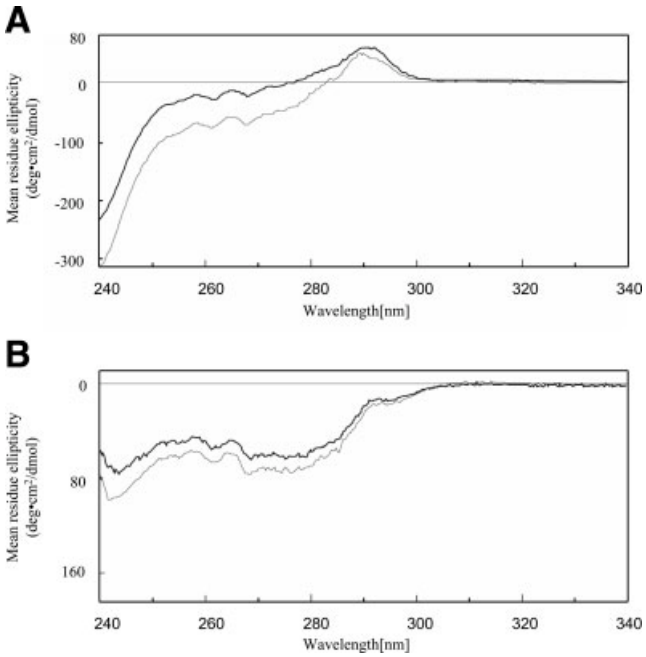

Fig. 2. The near-UV CD spectra of hIgG4-B and mIgG1 in 0.1*M* citrate at 4°C. Spectroscopic measurement conditions are shown in Materials and Methods section. (**A**) hIgG4-B at pH 6.0 (thin solid line), and pH 2.9 (thick solid line). Spectra at pH 2.9 were taken immediately after elution from Protein A chromatography. (**B**) mIgG1 at pH 6.0 (thin solid line), and pH 3.9 (thick solid line). Spectra at pH 3.9 were taken immediately after elution from Protein A chromatography.

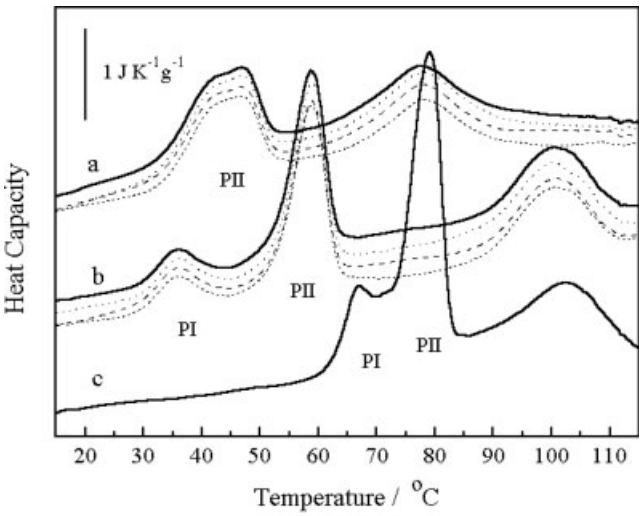

Fig. 3. pH and time dependence of hIgG4-A heat capacity in 0.1*M* citrate. hIgG4-A adjusted to each pH by Protein A chromatography was kept at 4°C for up to 10 days and measured by nanoDSC. Calorimetry conditions are described in Materials and Methods section. (**a**) At pH 2.7: immediately after elution (solid line, top), 3 days (dotted line), 6 days (dashed line), 10 days (thin dashed line, bottom). (**b**) At pH 3.5: immediately after elution (solid line, top), 1 day (dotted line), 3 days (dashed line), 10 days (thin dashed line, bottom). (**c**) At pH 6.0.

2.7, suggesting that the acid conformation is stable over time at 4°C. There was not enough material for hIgG4-B.

DSC

We used DSC to evaluate the thermal stability of native hIgG4-A and the hIgG4-A samples eluted at low pH. The native hIgG4-A showed a major endothermic peak at 78°C (P_{II}) and a minor peak (P_I) at 67°C (Fig. 3). The observed biphasic thermal transition is typical for antibodies.[12] At pH 3.5, the thermal unfolding also consisted of two transitions, although the peaks were somewhat broader. In addition, at pH 3.5, the T_m of both peaks shifted to lower temperatures, to 58°C for PII and to 35°C for P_I. The decreased melting temperature can simply be attributed to general pH-induced destabilization. This is consistent with the CD data indicating that no gross conformational change occurred at pH 3.5. At pH 2.7, the minor peak observed at pH 6.0 and pH 3.5 (P_I) disappeared. This suggests further destabilization of the protein structure and possibly further conformational changes, in particular for the domain responsible for the P_I transition. At pH 2.7, the major peak (P_{II}) was split into two peaks with T_ms of 41 and 46°C, indicating further destabilization of the more stable domain. The samples recovered from the DSC cell were turbid, consistent with the irreversibility of thermal unfolding at both the pH 6.0 and 3.5. Unfolding at pH 2.7 was partially reversible, as indicated by the small but significant peaks in the reheating of the pH 2.7 sample (data not shown), and the solution recovered from the DSC cell was transparent.

DSC was also carried out after storing the pH 3.5 sample at 4°C for 1, 3, and 10 days and after storing the pH 2.7 sample at 4°C for 3, 6, and 10 days (Fig. 3). DSC profiles were identical at each pH to the scan obtained immediately after elution, consistent with the CD results that no conformational change occurred over time when the antibodies were stored at pH 3.5 or 2.7.

The assignment of the broad peak observed above 90°C in each DSC curve remains unclear. This type of peak can often be observed in DSC of proteins at acid pH, suggesting that there may be some secondary unfolding of residual conformations in the molecules shown in P_I or P_{II}, or dissociation of antibody aggregates.

We also carried out DSC analyses for hIgG4-B and mIgG1. Figure 4(A) shows the DSC scan of hIgG4-B at pH 6.0 and 2.9. There is only one endothermic peak with a T_m of 73°C for the pH 6.0 sample, corresponding to unfolding of the native protein. This peak is shifted to 44°C at pH 2.9. As shown in Figure 4(B), mIgG1 shows a doublet endotherm with T_ms of 68 and 72°C for the native protein. At pH 3.9, there is an endothermic peak at 51°C. These results clearly show endothermic unfolding of the antibodies at low pH and hence indicate that they are distinctly folded at acidic pH, although their stability is substantially reduced.

Sedimentation Velocity

The 1 mg/mL hIgG4-A sample was diluted to 0.5 mg/mL immediately before sedimentation velocity experiments. Figure 5 shows the sedimentation coefficient distribution for a control sample at pH 6.0. This distribution has been

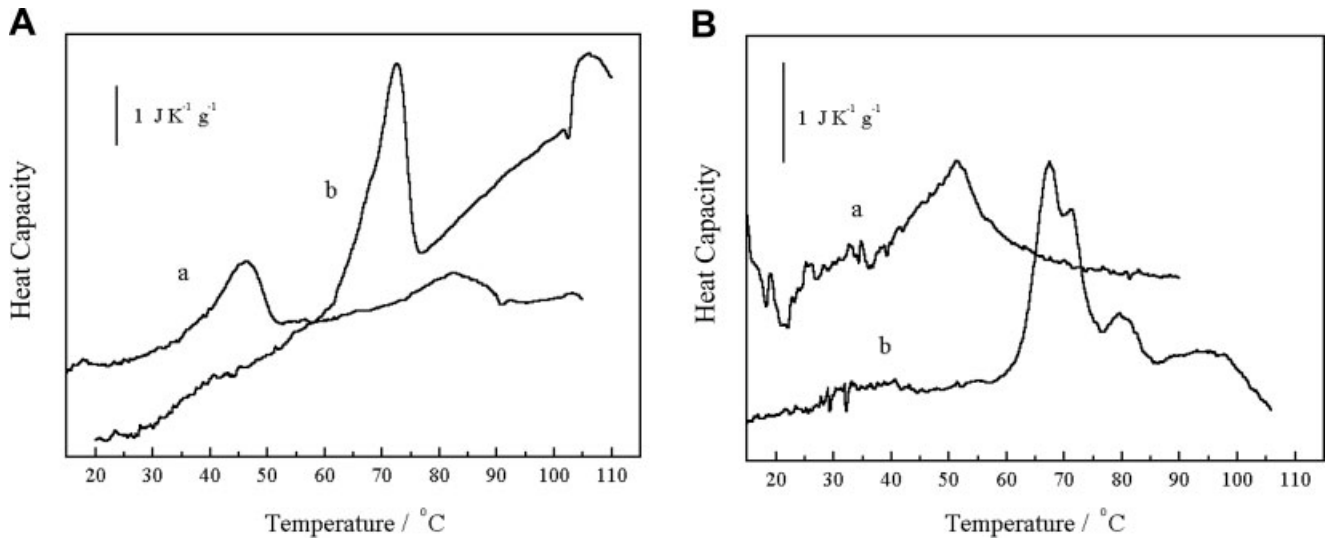

Fig. 4. pH dependence of heat capacities of human IgG4-B and mouse IgG1 in 0.1*M* citrate. Calorimetry conditions are described in the Materials and Methods section. (**A**) Humanized IgG4-B: a, pH 2.9 and 1.92 mg/mL; b, pH 6.0 and 1.60 mg/mL. (**B**), mouse IgG1: a, pH 3.9 and 2.00 mg/mL; b, pH 6.0 and 2.06 mg/mL.

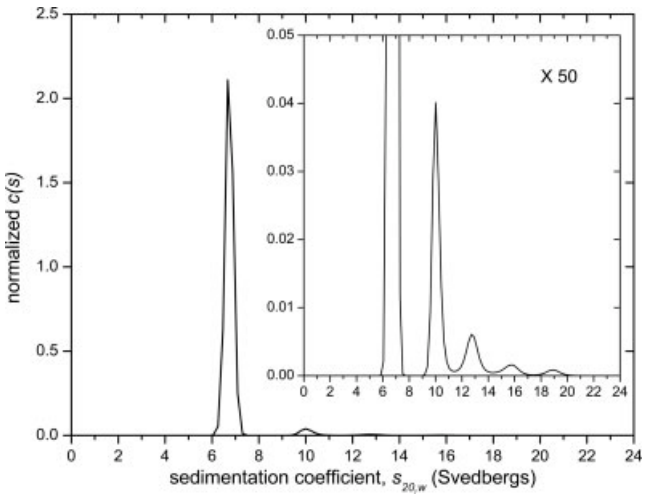

Fig. 5. Normalized sedimentation coefficient distribution for native hIgG4-A at pH 6.0, 20°C. The inset shows the same data at an expanded vertical scale so the minor components can be seen. Experimental conditions are described in the Materials and Methods section.

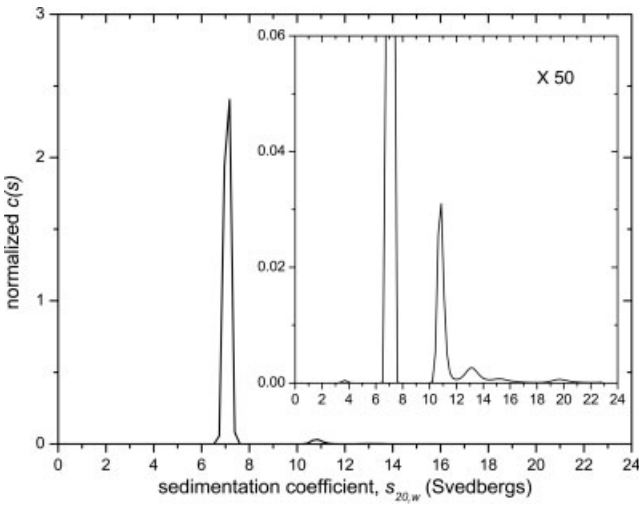

Fig. 6. Normalized sedimentation coefficient distribution for hIgG4-A at pH 3.5, 20°C immediately after elution from a Protein-A column.

normalized so that the total area under the curve is 1.0. The main peak, corresponding to the monomer, has a sedimentation coefficient, $s_{20,w}$, of 6.74 S and represents 96.2% of the sample. There are also some minor aggregate peaks present, more clearly seen in a 50-fold expanded version (inset to Fig. 5). The species at 10.0, 12.7, 15.8, and 18.9 S represent 2.7, 0.7, 0.2, and 0.1% of the total, respectively. Based on the ratio of their sedimentation coefficients to that of the monomer, these aggregate peaks presumably correspond to dimer, trimer, tetramer, and hexamer. We have not studied this material by sedimentation velocity at other protein concentrations to definitively establish whether these aggregates represent reversible or irreversible oligomers, but these are probably irreversible species.

Figure 6 shows the size distribution of the pH 3.5 sample immediately after elution, with the vertical scale expanded a 50-fold expanded vertical scale in the inset to show the minor components. In addition to the main peak at 7.08 S (which is 97.5% of the total protein), there are minor peaks at 10.9 S (presumably the dimer), 13.1 S (trimer), and traces (0.1% or less) of material at ~15.2 and 19.8 S (possibly tetramer and hexamer). In total, there is somewhat less aggregate than was seen in the control sample, suggesting either that some of the aggregates were not eluted from the Protein-A column or that the aggregates dissociated at pH 3.5. There is also possibly a peak sedimenting more slowly than the monomer at ~3.5 S, which would represent some sort of lower mass fragment (but species <0.1% of the total cannot be reliably de-

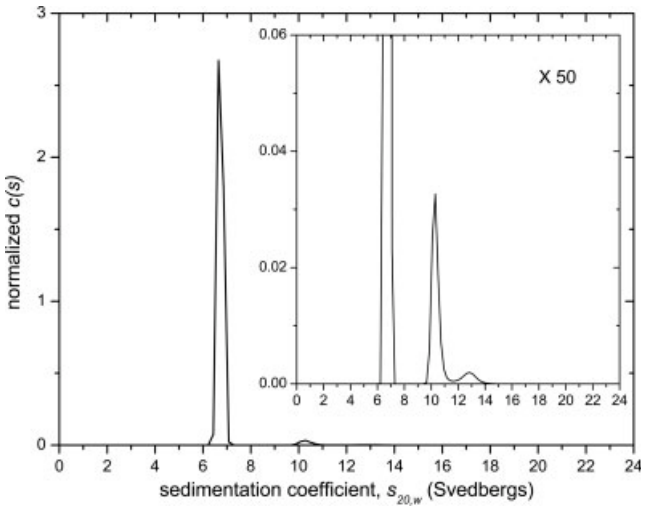

Fig. 7. Normalized sedimentation coefficient distribution for hIgG4-A at pH 2.7, 20°C immediately after elution from a Protein-A column.

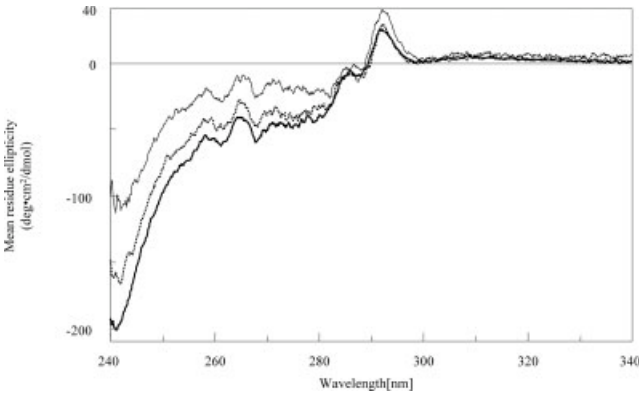

Fig. 8. The near-UV CD spectra of hIgG4-A after low pH treatment. Spectroscopic measurement conditions are shown in Materials and Methods section. hIgG4-A at pH 6.0 in 0.1*M* citrate (thick solid line), titrated from pH 2.7 (dashed line, contains 0.1*M* citrate and Tris), and at pH 2.7 in 0.1*M* citrate (thin solid line).

tected). This 3.5 S peak slowly increased in area to 1.6% after incubation at 4°C and pH 3.5 for 24 h (data not shown). The nature of this species is not entirely clear. The low sedimentation coefficient suggests that it represents an antibody fragment. One likely possibility is that this species is a half-molecule, which could arise from slow dissociation of antibodies in which the disulfide bond linking the two heavy chains has not been formed. The amount of the main peak (monomer) was essentially unchanged with time after 24 h incubation, accounting for 96.5% of the total. More significantly, the sedimentation coefficient of the main peak also remained unchanged. This again indicates that the conformation of hIgG4-A at pH 3.5 is stable, consistent with the CD and DSC analyses.

The sedimentation coefficient distribution acquired immediately after elution at pH 2.7 was not significantly different from that obtained after elution at pH 3.5 (Fig. 7). The main peak at 6.73 S was 97.7% of the total. Aliquots of the sample were examined 4 and 24 h after elution at pH 2.7. The pH 2.7 sample showed only slight time dependence. After 4 h, 97.1% of the sample was in the main peak, with 0.9% in the fragment peak at 3.6 S and 2.0% in the dimer peak at 9.2 S; after 24 h, the main peak was 96.9%, with 2.8% dimers and 0.3% trimers (data not shown).

The sedimentation coefficient for the monomer at low pH provides further evidence for the retention of a unique folded structure, but one slightly different from the native state at neutral pH. We have not studied the concentration dependence of these values, and certainly at low pH, the electrostatic effects will depress the measured values more, but at 0.5 mg/mL and fairly high ionic strength, the concentration effects should be small. At pH 3.5, the monomer actually sedimented faster than at neutral pH (it is more hydrodynamically compact). At pH 2.7, the sedimentation coefficient dropped back to equal that at neutral pH, supporting the difference in conformation between pH 3.5 and 2.7 detected by CD.

pH Titration

When hIgG4-A was titrated with 1*M* Tris-base from pH 3.5 to 6.0, the majority of the protein returned to the native state. Sedimentation analysis showed a monomer content of 97.5%, nearly identical to that of both the original sample and the pH 3.5 sample (data not shown). There is little effect of incubation at pH 3.5 on the monomer content of pH-titrated samples. The near-UV CD of the sample titrated to pH 6.0 after pH 3.5 treatment also resembled that of the original sample and there is little effect on the spectra of incubation after pH titration or incubation at pH 3.5 prior to titration (data not shown).

The behavior of material titrated from pH 2.7 was quite different. As shown in Figure 8, the CD spectrum of the hIgG4-A titrated to pH 6.0 immediately after elution at pH 2.7 was halfway between the original spectrum and that at pH 2.7. There was no effect of incubation at 4°C after pH titration (data not shown). When hIgG4-A eluted with 0.1 *M* citrate at pH 2.7 was immediately titrated to pH 6.0 and subjected to sedimentation analysis (Fig. 9), the monomer peak accounted for only 67% of the sample, with the balance forming a broad range of soluble oligomers. A similar degree of aggregation was observed for the same antibody incubated at 4°C for 4 and 26 h in pH 2.7 citrate buffer prior to titration, reflecting the fact that incubation at pH 2.7 does not cause time-dependent changes in the acid-induced conformation. However, incubation at 4°C over 24 h after pH titration resulted in a slight decrease in monomer content and hence a slight increase in aggregate content (data not shown), indicating that the aggregates formed after titration from pH 2.7 do not dissociate, at least during this period.

The aggregation did decrease at lower protein concentration. The hIgG4-A eluted at pH 2.7 was first diluted 10-fold with water or 0.1*M* citrate, pH 2.7, and then brought to pH 6.0 with 1*M* Tris-base. The monomer peak increased to ~80% for both dilutions (data not shown). Since the viscosity and density of the sample buffer after titration are not known, no comparison can be made

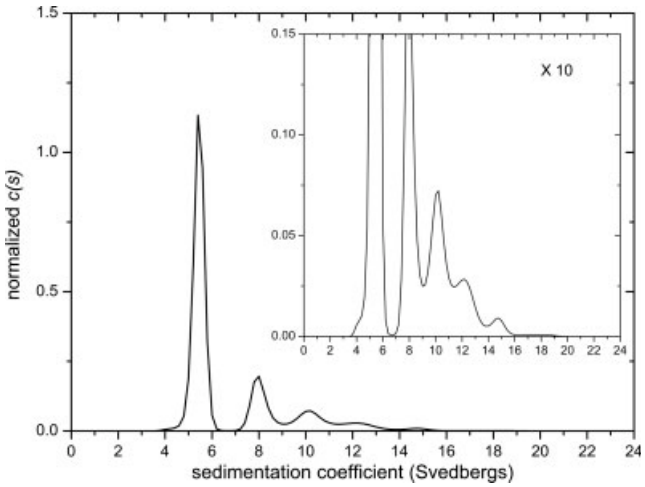

Fig. 9. Normalized sedimentation coefficient distribution for hIgG4-A eluted at pH 2.7 and immediately titrated to pH 6.0, as in Figure 8. Note that the *x*-axis for this figure is raw sedimentation coefficient rather than standardized values because we were unable to calculate the viscosity and density of the buffer after the titration.

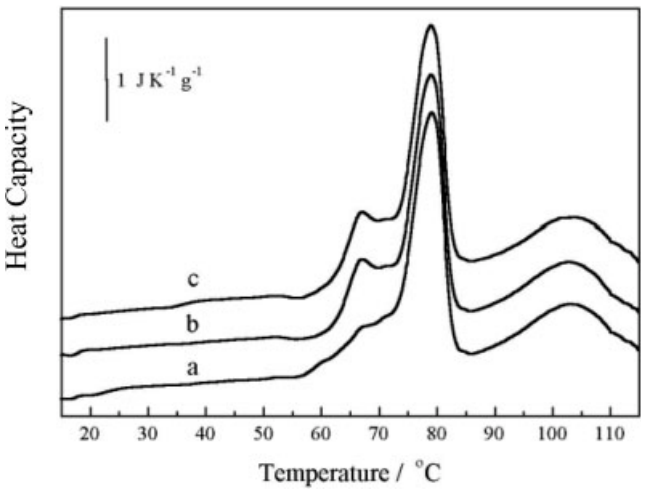

Fig. 10. DSC profile of the pH-titrated sample of hIgG4-A in 0.1*M* citrate. Experimental conditions for calorimetry are described in the Materials and Methods section. The concentrations of the samples were adjusted to 5 mg/mL. (**a**) Titrated from pH 2.7 to 6.0; (**b**) titrated from pH 3.5 to 6.0; (**c**), original sample (pH 6.0).

between the sedimentation coefficient of the monomer in the original and pH-titrated samples.

Somewhat surprisingly, the DSC of the pH-titrated sample was similar whether titrated from pH 2.7 or 3.5. Figure 10 shows the DSC profiles of hIgG4-A titrated from three different pH values. They are similar to the profile of the original sample, particularly in the PII transition, indicating that the folded structure resembled that of the native state. The DSC profile of the sample titrated from pH 2.7 thus differs from the sedimentation and CD data, which showed only ~67% monomers and a different CD profile than the original structures. The DSC data clearly indicate that the core structure involved in endothermic unfolding of PII is nearly identical between the pH-titrated and the original samples.

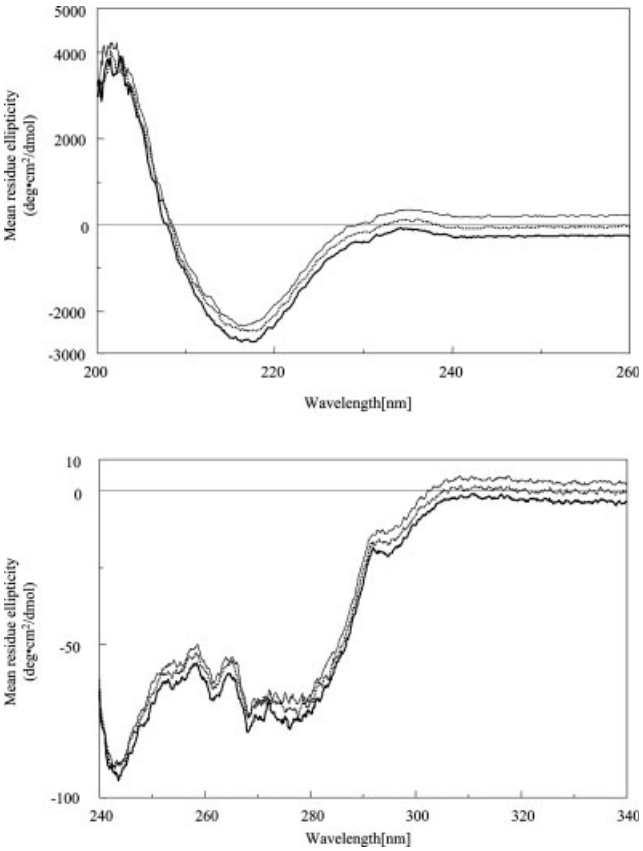

Fig. 11. The near- and far-UV CD spectra of the purified monomers of mIgG1 in 0.1*M* phosphate, pH 7.0. Native, thin solid line; monomer from pH 3.5, dashed line; and monomer from pH 2.7, thick solid line were shown. Thin and thick solid lines were shifted arbitrarily upward and downward, respectively. The monomers were purified by gel filtration as described in Materials and Methods section. In this case, phosphate buffer was chosen so that the far UV CD spectra can be determined. The monomers were purified by gel filtration as described in the Materials and Methods section. In this case, phosphate buffer was chosen so that the far-UV CD spectra could be determined.

Both CD and DSC analyses after pH titration were done without first purifying the hIgG4-A, but we did purify the monomer fractions after pH titration. Figure 11 shows the near- and far-UV CD of the purified monomers of mIgG1, indicating that the structure of the monomers obtained at pH 2.7 or 3.5 have structures identical to those of the original monomers. Therefore, the slightly altered CD and DSC profiles seen after pH titration (in particular from pH 2.7) and before purification are due to the presence of protein aggregates. An identical result was observed for hIgG4-A (data not shown).

DISCUSSION

Protein-A efficiently purifies antibodies, but often causes the antibodies to lose potency and form aggregates.[13-19] To avoid these problems, antibodies eluted at low pH from

Protein-A columns are typically neutralized immediately, based on the premise that a prolonged exposure to low pH exacerbates these problems. Immediate neutralization is not possible for therapeutic antibodies because they must be stored at low pH for viral inactivation and because it is generally not practical because of the large production scale. It has been unclear, however, whether it is the exposure to the acidic condition or the changes in pH (from low pH to neutral, from neutral to low pH, or both) that cause aggregation. In one previous case, acidic pH was reported to dissociate antibody dimers instead of inducing aggregation,[19] and a similar observation has been made in the current study.

As a first step to understand the effects of acid on the structure and aggregation of antibodies, we examined the structure of hIgG4-A at low pH after elution from a Protein-A column using sedimentation velocity, near- and far-UV CD, and DSC. At pH 2.7 and 3.5, native hIgG4-A was primarily a monomer, with a small amount of several different oligomers present. Two conclusions can be made about the structure of this antibody at low pH after elution from a Protein-A column. First, it has a distinct folded structure. CD analysis showed aromatic CD features at wavelengths apparently unchanged in low pH solution, proving that a distinct tertiary structure is present. The immunoglobulin-domain type secondary structure is also retained at low pH. DSC analysis showed endothermic unfolding at both the pH 3.5 and 2.7. The sedimentation velocity experiments show that the samples at pH 3.5 or 2.7 mainly consist of monomers. All of these results are consistent with a unique folded structure for the antibody at low pH. The pH 3.5 or 2.7 samples also showed no increase in aggregation. The same conclusion can be made for two other antibodies, mIgG1 and hIgG4-B, which also retained distinct folded conformations at low pH, as demonstrated by both their near-UV CD and the presence of endothermic unfolding peaks in DSC.

The second conclusion is that the structure obtained upon elution is stable at the elution pH when stored at 4°C. No significant time-dependent changes were observed in the near-UV CD spectra, DSC profiles, or sedimentation coefficient of the main peak, indicating that no additional conformational changes occurred at low pH nor was there any increase in aggregation. This suggests that, at least for the hIgG4-A used here, it is not necessary to neutralize the antibody immediately after elution at low pH, nor should there be any concern about prolonged incubation at low pH for viral clearance. This was further confirmed by the pH titration experiments, which showed no effect of incubation at low pH on the structure, stability, and aggregation of the pH-titrated samples.

The one time-dependent effect of exposure to low pH we did observe was dissociation of a small amount of the antibody into smaller fragments (the ~3.5 S species). This likely represents formation of a half-antibody because of incomplete disulfide formation,[20] which appears to be particularly common for the IgG4 subtype.[21] It is unlikely that the small amount of fragment detected by sedimentation velocity would produce a significant change in either the CD or DSC data.

These conclusions, then, raise a further question: why do we see aggregation after elution at low pH? We propose that aggregation occurs during the course of neutralization of low pH samples and have undertaken a study to clarify this proposal. We observed significant aggregation of the hIgG4-A when the material at low pH was directly subjected to gel filtration analysis using a neutral pH elution solvent (data not shown). This strongly suggests that aggregation must occur during the pH change in the gel filtration column, since the present study showed that the low pH treatment alone induces no aggregation. When the hIgG4 was titrated from pH 2.7 to 6.0, the near-UV CD did not return to the profile of the original sample, and sedimentation analysis revealed formation of soluble oligomers (total ~30%). Such aggregates contributed to an altered CD profile, since the purified monomers showed an identical structure to that of the original sample after titration from pH 2.7. It is clear from the altered CD profile of the aggregates that the protein has retained a long-term "memory" of its exposure to pH 2.7, including decreased stability against further aggregation. The failure to fully return to the native conformation at neutral pH implies that it has become kinetically trapped in an altered conformation, and must overcome a substantial energy barrier in order to return to the normal conformation. This type of kinetic trapping may be one important mechanism of damage during antibody processing.

The DSC data of the pH-titrated hIgG4-A from pH 2.7 led to an interesting observation. Despite the above conformational differences of the pH-titrated sample, the DSC profile was similar to the original or the sample titrated from pH 3.5. This may be explained if the samples titrated to pH 6.0, even from pH 2.7, have the native-like core structure (corresponding to the PII transition), although they form some soluble oligomers and have altered near-UV CD signals. However, subtle conformational changes remaining from the exposure to pH 2.7 persist after titration and play a key role in aggregation. An alternative hypothesis is that the conformational "memory" of the pH 2.7 exposure is due to kinetic trapping in an altered conformation that is separated from the native structure by a high energy barrier. As the temperature is raised during the DSC experiments, the titrated sample is able to revert to the native conformation at a rate sufficiently high that the differences in conformation and association are erased prior to the onset of the PII unfolding transition. Thus, the structural differences between the sample titrated from pH 2.7 and the others are subtle, yet sufficient to alter the environment of aromatic residues and to cause formation of soluble oligomers.

These data indicate that the properties of hIgG4-A used here are significantly different from those of some other antibodies. In fact, in this study, mIgG1 was prone to aggregation at pH 3.9 at or above room temperature and hence yielded a large number of aggregates when the process temperature was not maintained well below room temperature (data not shown). Is the difference due to different spe-

cies (human vs. mouse) or different subclass? Naturally, we wished to extend the observations made for hIgG4-A to other human monoclonal antibodies. Unfortunately, we had only hIgG4-A in sufficient quantity for extensive characterization. Surveying the literature, we found that antibody CB4-1 and its Fab fragment show extensive pH-dependent conformational changes in CD and fluorescence analyses.[7] CD and DSC showed that a human hemoglobin-β chain antibody undergoes extensive acid-induced unfolding, with only some secondary structure retained.[1] A MAK33 antibody showed a peculiar acid-induced conformation, designated as an alternatively folded state because of its unique thermal stability; this conformation is different from the native state and has a tendency to aggregate,[5] a type of acid conformation is known as the A-state or molten globule for globular proteins.[22,23] Similar behavior was observed with the CH3 domain of MAK33.[6] Mouse IgG2a (MN12) possesses particular acid stability, showing a nonrandom conformation even at pH 2.5.[24] However, MN12 gradually changes its conformation to yield precipitates at low pH. In contrast, the hIgG4-A antibody studied here retained the conformation specific to each pH for up to 10 days. It is not clear whether the differences between the reports are due to the different properties of the antibodies or the different experimental conditions. We have observed that pH, but not ionic strength, plays a major role in the acid conformation of hIgG4-A, but the temperature and the ionic strength or type of buffer, greatly, affect the stability. As described above, different results reported for acid conformation could be in part due to process temperatures. We are now collecting several human monoclonal antibodies via collaboration and plan to compare them systematically. It is also possible that the way the sample is neutralized from low pH will determine the structure and aggregation of acid-exposed samples. We suggest here that aggregation associated with low pH treatment during purification or viral clearance could be suppressed by more appropriate pH titration protocols in the case of acid-stable antibodies like hIgG4-A.

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