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Transient conformational modification of immunoglobulin G during purification by protein A affinity chromatography

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ABSTRACT

Exposure of three native IgG1 monoclonal antibodies to 100 mM acetate, pH 3.5 had no significant effect on their hydrodynamic size (11.5 ± 0.5 nm), while elution from protein A with the same buffer created a conformation of 5.5 ± 1.0 nm. Formation of the reduced-size conformation was preceded by the known destabilization of the second constant domain of the heavy chain (Cγ2) by contact with protein A, then compounded by exposure to low pH, creating extended flexibility in the hinge-Cγ2 region and allowing the Fab region to fold over the Fc region. The reduced-size conformation was necessary for complete elution. It persisted unchanged for at least 7 days under elution conditions. Physiological conditions restored native size, and it was maintained on re-exposure to 100 mM acetate, pH 3.5. Protein A-mediated destabilization and subsequent restoration of native size did not create aggregates, but the reduced-size conformation was more susceptible to aggregation by secondary stress than native antibody. Protein A-mediated formation of the reduced-size conformation is probably universal during purification of human IgG1 antibodies, and may occur with other subclasses and IgG from other species, as well as Fc-fusion proteins.

1. Introduction

Potential negative consequences of exposing IgG to low pH have made protein A elution conditions an object of controversy for decades. Many investigators have expressed concern that exposure to low pH causes conformational changes leading to aggregation, and they have cited formation of turbidity upon pH neutralization of protein A elutes as proof [1–13]. Others have favored the hypothesis that IgG conformation is unaffected by such exposure [14,15], based on their observation that removal of turbidity by microfiltration had negligible effect on antibody recovery. Consistent with the latter perspective, a recent study showed that formation of turbidity was mediated through pH-dependent insolubility of chromatin-associated host cell contaminants in the eluted IgG fraction [16].

Direct characterization of domain stability with high-resolution 2-dimensional NMR has shown that the second constant domain (Cγ2) of IgG collapses entirely at pH 3.1 [17]. This makes elution conditions a legitimate concern for affinity chromatography media that are commonly eluted in the range of pH 2–3, like protein G and protein L [13], but most antibodies elute from protein A at pH 3.5 or higher, especially on current-generation recombinant protein A ligands that bind IgG exclusively at the Fc region [18]. NMR shows that domain integrity of purified IgG is virtually unperturbed at pH 3.5 [17].

These findings create an expectation that protein A affinity chromatography should be completely benign as a purification method, but the controversy surrounding elution at low pH has been screening another phenomenon that challenges that conclusion. X-ray crystallography studies have shown that the residence of protein A in its primary binding site between the Cγ2 and Cγ3 domains creates instability in the Cγ2 domain [19,20]. This suggests it must also affect IgG conformation during purification by protein A, but there is no standard for extrapolating chromatographic behavior from protein interactions constrained in a crystal lattice. The present study addresses the conformation of IgG immediately following elution from protein A and its subsequent neutralization.

2. Materials and methods

2.1. Reagents and equipment

Buffers, salts, and reagents were obtained from Sigma-Aldrich (St. Louis, MO), except allantoin, which was obtained from Merck Millipore (Darmstadt, Germany). Toyopearl AF-rProtein A-650F was obtained from Toso Bioscience (Tokyo). UNOsphere™ Q was

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obtained from Bio-Rad Laboratories (Hercules, CA, USA). Capto adhere was obtained from GE Healthcare (Uppsala, Sweden). Chromatography media were packed in XK or Tricorn™ series columns (GE Healthcare). Chromatography experiments were conducted on an ÄKTA™ Explorer 100 or Avant 25 (GE Healthcare).

2.2. Experimental methods

Three prospective biosimilar IgG1 monoclonal antibodies (Herceptin™, Avastin™, and Humira™) were expressed by mammalian cell culture in Chinese hamster ovary (CHO) cells using a tricistronic vector developed by Ho et al. [21]. They were produced in 15–30 day fed-batch cultures, harvested at 30–50% cell viability.

IgG used to support most of the study was highly purified to minimize interference with analytical methods. Cell culture harvest was clarified by adding caprylic acid to cell-free culture harvest to a final concentration of 0.4%, and allantoin to a final concentration of 2%. pH was adjusted to 5.3 with 1 M acetic acid, and the mixture stirred for 2 h. UNOsphere Q pre-equilibrated with 50 mM MES, 150 mM NaCl, pH 5.3 was added at a proportion of 5% (v/v) and mixing continued for at least 4 h. Solids were removed by centrifugation and/or microfiltration. Protein A affinity chromatography media was equilibrated with 50 mM Hepes, 120 mM NaCl, pH 7.0. Sample was loaded and the column washed with 10 column volumes (CV) of equilibration buffer, then eluted with a step to 100 mM acetic acid, pH 3.5, or the same buffer with NaCl or arginine added. Aggregates, antibody fragments, DNA and residual host cell proteins were further removed by titrating the protein A eluate to pH 8.0, adding NaCl to 1 M, loading it onto Capto adhere, and eluting with a step to 50 mM MES, 0.35 M NaCl, pH 6.0. Antibodies purified by this process contained <10 ppm host cell protein (HCP), less than 1 ppm DNA, and less than 0.1% aggregates. For the remainder of the discussion, this material is referred to as *highly purified IgG*.

Experimental controls were conducted in some cases with IgG purified by protein A affinity chromatography where the cell culture harvest was clarified only by centrifugation and microfiltration.

Experimental controls were also conducted in some cases with antibody purified without exposure to protein A. In brief, harvest was clarified with the caprylate-allantoin-solid phase adsorbent system described above, then fractionated by anion exchange chromatography in void exclusion mode (VEAX) [22], in 50 mM Tris, pH 8.0. NaCl was added to the IgG-containing VEAX void fraction to a final concentration of 1.0 M then applied to Capto adhere and eluted as described above.

2.3. Analytical methods

IgG purity was documented according to the methods described fully in [16]. In brief, host cell protein (HCP) content was estimated by ELISA with a Generation III CHO HCP kit from Cygnus Technologies Inc. (Southport, NC). DNA was measured using a QX100™ Droplet Digital™ PCR System (Bio-Rad Laboratories). Aggregate content was measured by analytical size exclusion chromatography (SEC) with a G3000SWxl column (Tosoh Bioscience) on a Dionex Ultimate™ 300 HPLC system (Thermo Scientific) operated at a flow rate of 0.6 mL/min, using a buffer formulation of 50 mM MES, 20 mM EDTA, 200 mM arginine, pH 6.0. Sample injection volume was 100 μL.

Solute size distributions in free solution were characterized by dynamic light scattering (DLS) using a Zetasizer ZS (Malvern Instruments, Worcestershire, UK). The sample (200 μL) was mixed gently for 10 seconds on a vortex before being placed into a quart cuvette (ZEN2112, Malvern Instruments) using a gel loading tip to avoid bubbles. Viscosity of the carrier solution was determined using a SV-10 viscometer (A&D Company, Tokyo). The backscattered light at 173° was measured and 3 measurements were averaged. Attenuation index was maintained at a value of 7–8. Analysis of the data was performed using version 7.02 of the Dispersion Technology Software provided by the manufacturer.

Circular dichroism (CD) spectroscopy was performed with a JASCO J-810 spectropolarimeter (JASCO Corp., Tokyo). Far-UV spectra (190-260 nm) were obtained with highly purified IgG at a concentration of 0.2 mg/mL using a quartz cuvette with a path length of 0.1 cm. Near-UV spectra (250–350 nm) were obtained with highly purified IgG at a concentration of 1.0 mg/mL using a quartz cuvette with a path length of 1.0 cm. For both, 32 scans were accumulated with a scan rate of 100 nm/min and time constant of 0.125 s. All Spectra were corrected by subtracting the buffer baseline and averaged 32 times. All experiments were conducted at room temperature. Relative amounts of random coil, α-helix, and β-sheet were calculated using K2D2 software [23].

Other experimental details are described or reiterated for clarity in the following section.

3. Results and discussion

3.1. Size characterization by DLS

DLS indicated hydrodynamic size of the highly purified IgG monoclonal antibodies under physiological conditions was 11.5 ± 0.5 nm. They maintained that size when titrated to pH 3.5. When highly purified native antibodies were applied to protein A and eluted with 100 mM acetic acid, pH 3.5, hydrodynamic size of the eluted IgG was 5.5 ± 1.0 nm (Fig. 1).

Addition of NaCl increased size of the protein A-eluted IgG, as did increasing pH (Fig. 2). Exposure to physiological conditions restored native size. Restored antibody maintained native size at all tested pH values from 3.5 to 8.5, and NaCl concentrations from

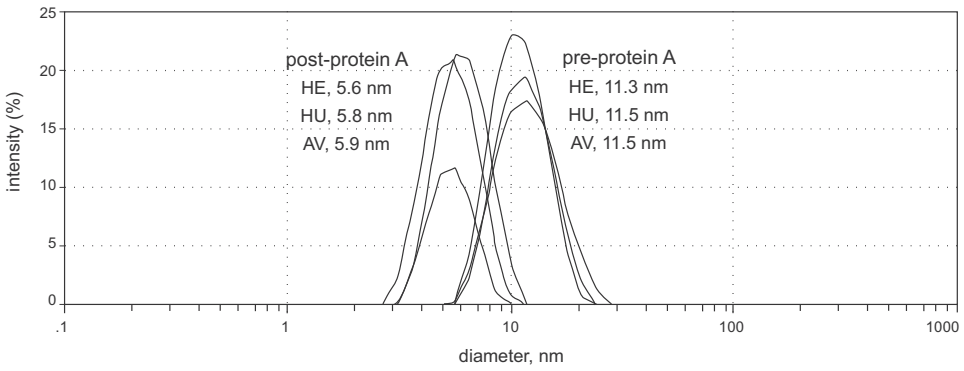

Fig. 1. DLS data showing hydrodynamic size of IgG before and after elution from protein A. Protein A-eluted samples still in 100 mM acetate, pH 3.5. HE, HU, and AV refer to biosimilar IgG1 clones for Herceptin, Humira, and Avastin.

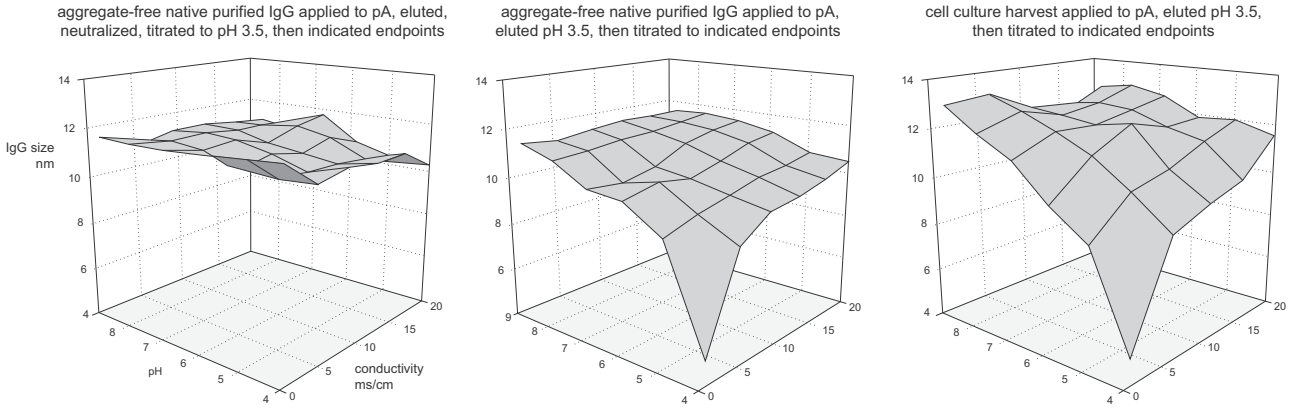

Fig. 2. Hydrodynamic size of IgG as a function of pH and NaCl concentration. Comparison of protein A-eluted IgG after neutralization, with protein A-eluted IgG still at pH 3.5.

0 to 200 mM. Aggregate content before protein A and after size restoration was ~0.1% (SEC).

IgG purified by protein A from harvests clarified only by centrifugation and microfiltration were significantly contaminated (HCP > 2000 ppm, DNA > 10 ppm, aggregates 1.5–2.5%) and caused DLS to give inflated size results (Fig. 2, panel 3). This highlights an important experimental control variable and known artifact of DLS. The intensity of scattered light is proportional to the 6th power of the solute diameter [24,25]. This causes aggregates to disproportionately dominate signal and cause overestimation of smaller solute size. This was verified experimentally in the present study with a purified IgG solution containing about ~35% aggregates (SEC). DLS produced a size estimate of 16.8 nm, versus 11.9 nm for a preparation of the same antibody containing ~0.1% aggregates.

This phenomenon has been highlighted as a beneficial feature of DLS for sensitive detection of low-level aggregates in pharmaceutical solutions, but it creates a source of uncontrolled error where the intent is to characterize native protein in aggregate-containing solutions. Except for data shown in Fig. 2, panel 3, all DLS results reported and discussed in this study were therefore based on application to protein A of highly purified IgG preparations containing not more than 0.1% aggregates.

3.2. Size characterization by SEC

SEC is widely considered to be complementary to DLS, and typically provides a valuable dimension of information that DLS cannot. It permits relative amounts of different solute size classes within a sample to be estimated with reasonable accuracy because it relies on UV absorbance instead of scatter. The one exception to the utility of SEC is when solutes interact non-specifically with the chromatography media surface, and the interaction influences their elution times [26,27]. This unfortunately proved to be the case with protein A-eluted IgG still at pH 3.5.

When protein A-eluted IgG still at pH 3.5 was applied to an SEC column equilibrated with 100 mM acetate, pH 3.5, non-specific interactions caused the applied IgG to bind. It never eluted. This documented a strong tendency for protein A-eluted IgG to associate with even a presumed inert surface under elution conditions, and emphasized the unsuitability of SEC as a corroborative assay method under such circumstances.

Application of protein A-eluted IgG still at pH 3.5 to an SEC column equilibrated with 50 mM Hepes, pH 7.0 produced a 2-peak profile, but the first peak coincided with elution of the salts from the applied sample (Fig. 3). This suggested the first peak was desorbed from the column by those salts, and left it impossible to determine if the two populations were representative of underlying size differences.

Application of protein A-eluted IgG still at pH 3.5 to an SEC column equilibrated with 50 mM Hepes, 150 mM NaCl, pH 7.0 produced a 2-peak profile in which the first peak eluted in the same region as native IgG (Fig. 3), but provided no confidence that the elution time of either peak accurately reflected its size. Given that the antibody was being buffer exchanged as it passed

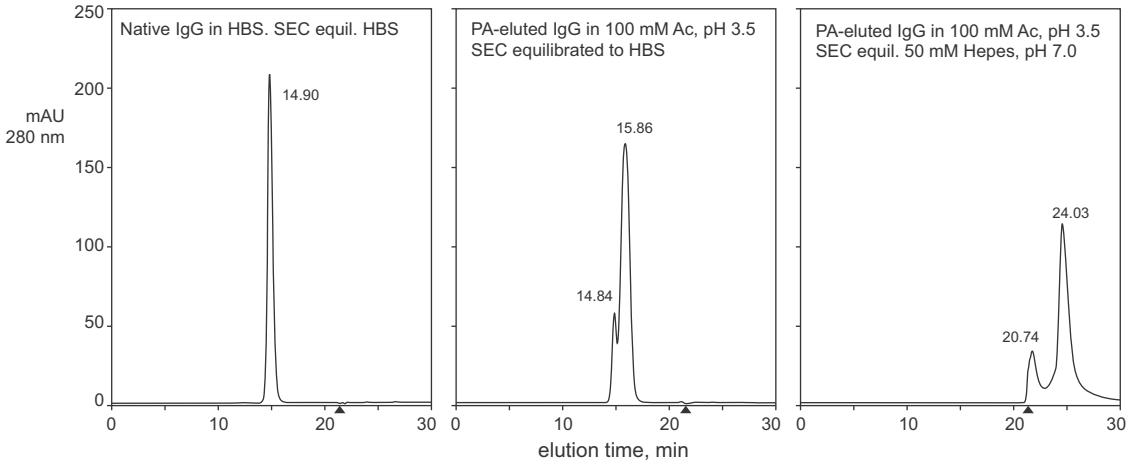

Fig. 3. Non-specific adsorption of IgG at pH 3.5 to SEC media. The black triangle at ~21 min shows where sample salts begin to elute. HBS; 50 mM Hepes, 150 mM NaCl, pH 7.0.

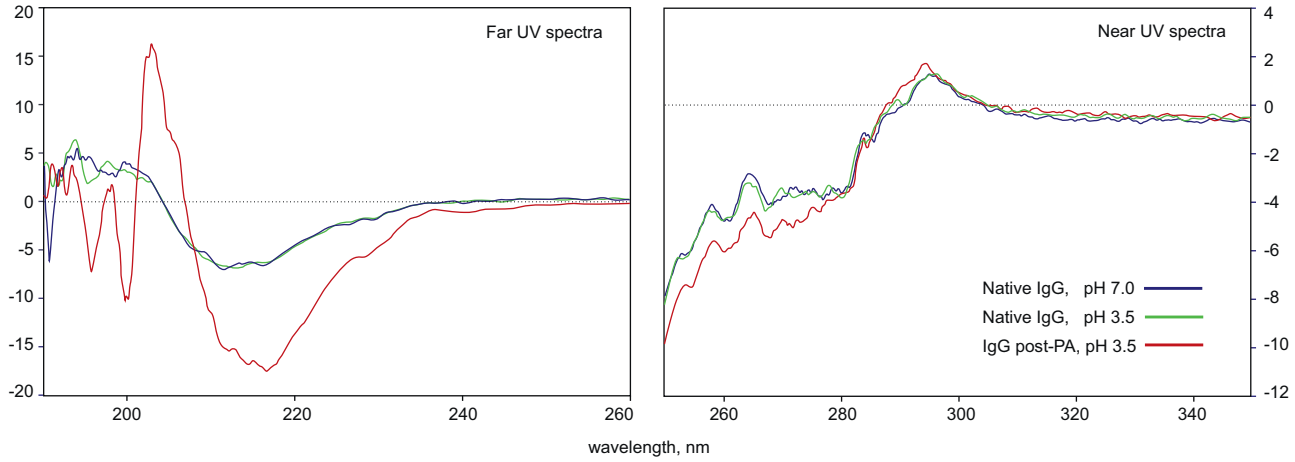

Fig. 4. Comparison of CD spectra for native IgG under physiological conditions, native IgG at pH 3.5, and the reduced-size conformation eluting from protein A at pH 3.5.

through the column, it must be understood to have been in a state of conformational-size transition that would have confounded genuine size-based fractionation. Non-specific interactions, severe immediately after injection but less after buffer exchange, would have imposed another layer of blind variability.

On the positive side, these results highlight a key contribution of DLS to the study. Since DLS is free of matrix adsorption effects, it is able to provide insights under conditions that confound SEC [24,25].

3.3. Conformational characterization of protein A-eluted IgG

Observation of reduced-size IgG exclusively after elution documented that protein A mediated the effect. This is consistent with X-ray crystallographic studies showing that contact between protein A and the Fc region disorders the upper third of Cγ2 to such an extent that electron densities become too weak to assign coordinates to the alpha carbon atoms [19,20]. The effect extends up through most of the hinge region, and laterally includes most of the adjacent carbohydrate chain.

This provides an important starting point, but it is evident that creation of the reduced-size conformation must involve a 2-step pathway where the low pH of elution compounds the denaturation created by initial IgG contact with protein A. Circular dichroism (CD) spectroscopy confirmed dramatic loss of structure in the reduced-size conformation (Fig. 4). β-sheet was reduced 45% compared to native molecule under physiological conditions (from 10.48% to 5.69%), accompanied by a 55% increase of α-helix (from 36.17% to 56.26%), and 28% reduction of random coil (from 53.35% to 38.05%). Native antibody titrated to pH 3.5 showed weak responses in the same wavelength ranges where the reduced-size conformation showed extreme responses, but otherwise tracked closely with native antibody under physiological conditions.

CD spectra do not define where the loss of structure occurs, but other studies highlight Cγ2 as the focal point. Cγ3 becomes disordered at pH 2.0, but not at pH 3.0 [17,28], and X-ray crystallography data show that Cγ3 is structurally unaffected by its interaction with protein A [19,20]. Differential scanning calorimetry studies have shown that rabbit polyclonal IgG folds over on itself at pH 2.0–2.5, putting the Fab region in direct contact with Fc [29,30]. It seems logical that the reduced-size conformation of IgG eluted from protein A likewise represents a folded-over configuration, where the inherent flexibility of the hinge, augmented by flexibility imposed by compound denaturation under acidic conditions, permits the Fab region to come into contact with the Cγ3 domain (Fig. 5).

3.4. Ramifications for protein A as an affinity chromatography ligand

The above findings bear on why protein A has been so successful as a ligand for affinity chromatography. The denaturation of IgG by initial contact with protein A conceptually parallels the phenomenon of induced fit in enzyme–substrate systems. IgG is forced by denaturation into conformance with the chemical topography of protein A [19,20]. This reconciles how a generally hydrophobic electronegative protein ligand (p*I* ~5.1) can offer such refined

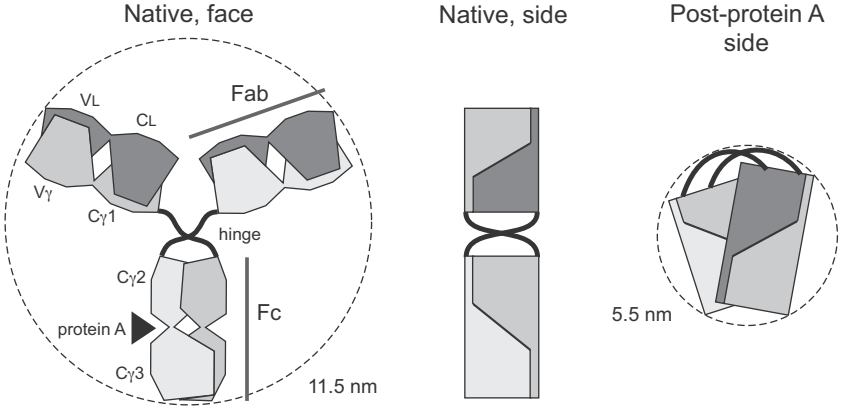

Fig. 5. Hypothetical gross-conformation of protein A-eluted IgG at pH 3.5, showing Fab folded over the Fc region.

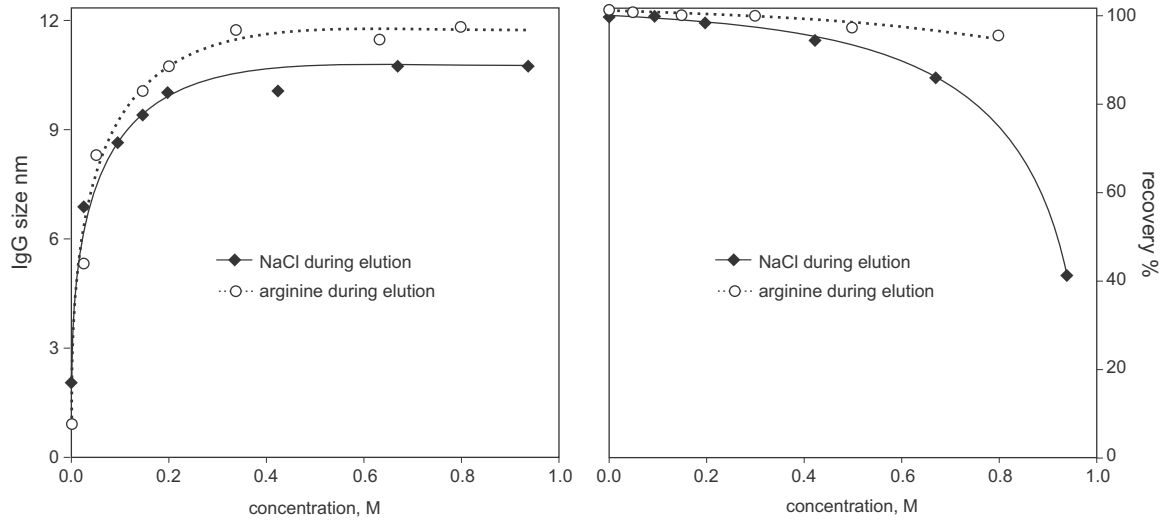

Fig. 6. Relative effects of NaCl and arginine during pH 3.5 elution on hydrodynamic size and IgG recovery. NaCl and arginine added to a foundation buffer of 100 mM acetate, pH 3.5.

specificity, and why its affinity for IgG is so high [31–34]. Another chromatography-enabling feature of initial contact denaturation is that it increases sensitivity of Cγ2 to compound denaturation upon exposure to pH 3.5. This eliminates the need for the more extreme elution conditions imposed by other biological affinity ligands, like protein L, protein G, and immunoaffinity. It also highlights an additional parallel with enzyme-substrate systems, in this case representing the phenomenon whereby enzymes reduce the activation energy required to convert one substrate conformation to another.

3.5. The effect of eluted IgG size on elution efficiency

Other lines of evidence suggest that IgG must be in the reduced-size conformation to achieve complete elution. Having shown that IgG size increased as a function of NaCl concentration even at low pH, a series of experiments was conducted in which NaCl was included at increasing concentrations during elution at pH 3.5. IgG recovery decreased dramatically (Fig. 6). IgG that failed to elute was recovered in a subsequent NaOH wash. Addition of arginine to elution buffers had the same molar influence on size as NaCl. Arginine's impact on IgG recovery was less negative than NaCl, but still supported the conclusion that the reduced-size conformation is necessary for complete elution.

The difference in recovery between NaCl and arginine is consistent with known features of the interaction between IgG and protein A. Most of the binding energy between IgG and protein A is hydrophobic, and the remainder from four hydrogen bonds [19,20]. NaCl is often used to promote protein binding on hydrophobic interaction and hydrophobic multimodal chromatography media, and would be understood to disfavor dissociation of IgG from protein A. Arginine has been recommended to enhance elution of IgG from protein A, specifically because it relaxes hydrophobic interactions [1]. Its guanido residue — a guanidine analog — is also known to be a strong hydrogen donor-acceptor. This suggests arginine permitted better recovery than NaCl because its suppression of hydrogen bonding and hydrophobic interactions partially compensated for the increase in IgG size, versus NaCl, which compounded the effects of IgG size.

We note a superficial conflict between the present data and a published conclusion that arginine enhances elution of IgG from protein A through relaxation of hydrophobic interactions [1]. The authors relied on peak width as an indicator. We also observed very substantial narrowing of the elution peak with increasing arginine content, but elution profiles indicated it was a function of higher buffer capacity producing a steeper transition to the endpoint pH. Most discussions of arginine center on the properties of its guanido residue, but it also carries a carboxyl. At acidic pH, only the carboxyl groups contribute to buffer capacity. 500 mM arginine-carboxyls would be understood to increase buffer capacity 6-fold over 100 mM acetate by itself.

Fig. 7 illustrates these effects. Note especially the relative slopes of the pH curves. Columns were loaded with 25 mg/mL highly purified IgG, then eluted as discussed, and cleaned with 50 mM NaOH. The large IgG load caused the elution peaks to saturate the UV monitor at 280 nm, causing the peaks to appear broad at the top. Profiles at 254 also suffered from saturation, but to a much lesser degree that revealed conventionally shaped elution peaks for acetate and acetate-arginine. The wavelength ratio of 254–280 nm in the acetate-NaCl elution is typical for IgG and provides a reference to appreciate the degree of optical distortion by monitor saturation in the other profiles.

3.6. Susceptibility of the reduced-size conformation to aggregation

Guo et al. [35,36] recently traced the presence of IgG aggregates to formation of an unstable intermediate conformation created by contact with a cation exchanger. This invites an expectation that conformational modification of IgG by its interaction with protein A might also induce aggregation, especially considering that variations in Cγ2 conformation have been found to correlate with formation of aggregates in pharmaceutical formulations [17]. Surprisingly, aggregate content of reduced-size IgG neutralized after 7 days at pH 3.5 (and all lesser intervals) was identical to its aggregate content prior to loading the already highly purified antibody onto protein A (0.1%).

This provides some comfort that prolonged exposure to low pH during virus inactivation is unlikely to promote excessive aggregate formation by itself, but it should not be understood to indicate that the reduced-size conformation is inherently stable. A series of experiments was performed in which the pH of freshly protein A-eluted IgG and native IgG were reduced in parallel to pH 3.0, held for 1 h, then titrated to physiological pH. No aggregate formation was induced in the native IgG, but extensive aggregation was observed in the protein A-eluted antibody (Fig. 8). Note also

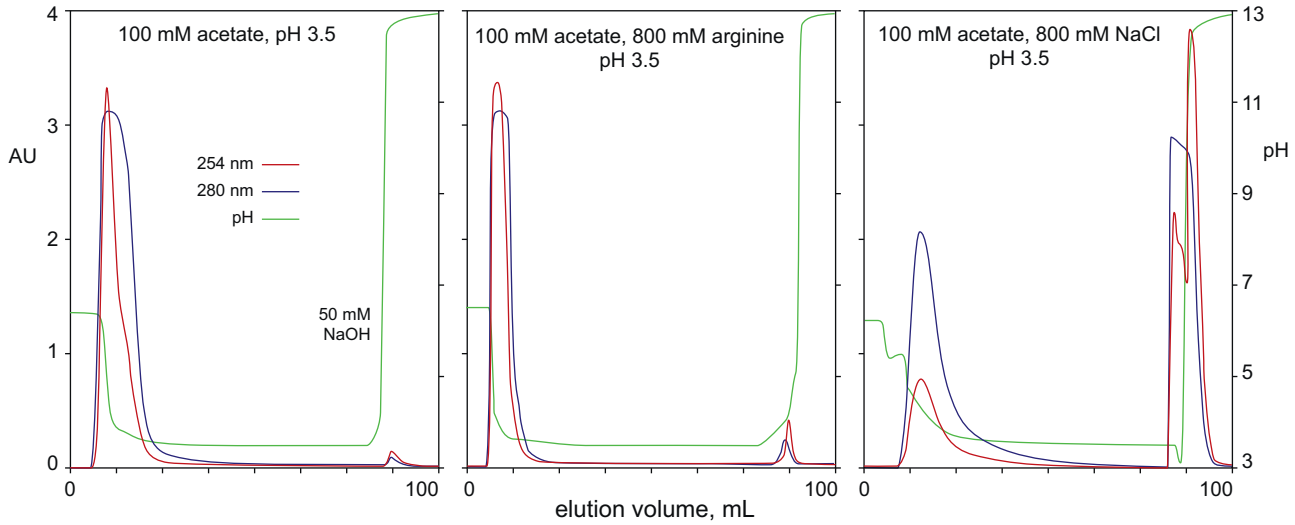

Fig. 7. Comparison of protein A elution profiles showing the effects of arginine and NaCl in the elution buffer.

the greater IgG peak width that partly obscured the trailing H2L impurity (2 heavy chains, 1 light chain). This suggested that even the non-aggregated IgG population had an elevated tendency to participate in non-specific interactions, which implies a tendency toward further aggregation over time.

These results underline an important caution about eluting protein A at pH values below pH 3.5, but their greater significance is their confirmation of the general phenomenon whereby protein A-mediated denaturation elevates vulnerability of IgG to aggregation by secondary stress.

3.7. IgG conformation as a purification process variable

Overall results of the study highlight a final point, that IgG conformation following protein A chromatography is a process control variable in its own right. It has an impact on recovery of IgG from protein A columns, which makes it an economic issue. It has an impact on aggregate formation, which makes it a process development and product quality issue. IgG size can be modulated at any stage of protein A chromatography by simple modifications of buffer content and pH, and its effects measured to guide development of the most favorable conditions for any given antibody.

The fact that 3 different human IgG1 monoclonal antibodies behaved similarly suggests that most antibodies of this species and subclass will do the same. This conclusion is fortified by the fact that the X-ray crystallographic studies originally describing protein A-mediated denaturation of IgG were performed with Fc fragments from human polyclonal IgG1. IgG from other species and subclasses, and the Fc component from fusion proteins, may undergo similar changes, though perhaps to different degrees. NMR studies demonstrate that secondary factors such as differential glycosylation particularly affect Cγ2-mediated aggregation [17]. It seems reasonable to anticipate that they may also modulate the degree of conformational change induced by contact with protein A.

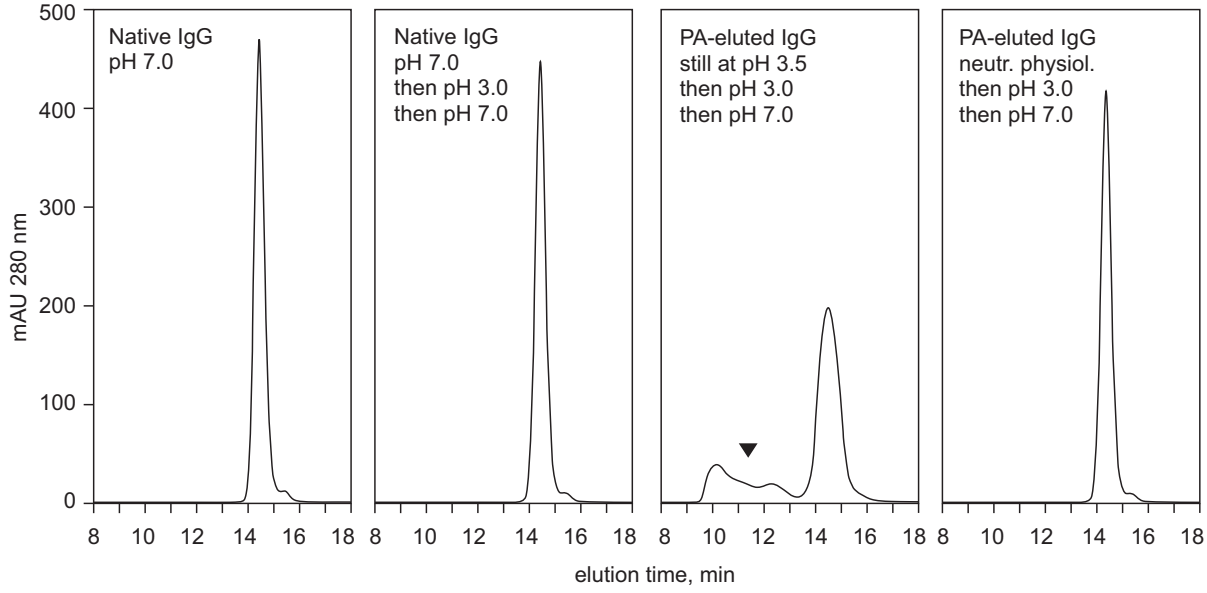

Fig. 8. SEC profiles illustrating increased vulnerability of protein A-purified IgG to aggregation by exposure to pH 3.0. The black triangle emphasizes aggregate content.

4. Conclusions

Purification of IgG1 monoclonal antibodies by protein A affinity chromatography induced a conformational variant with about half the hydrodynamic size of the native IgG; ~5.5 nm versus ~11.5 nm. CD spectroscopy revealed ~45% loss of β-sheet, with a 55% increase of α-helix and 28% reduction of random coil. Formation of the reduced-size conformation occurred in two steps, where the first was denaturation of the upper third of the Cγ2 domain under physiological conditions by its initial contact with protein A, and the second where low pH elution compounded the denaturation created by initial contact with protein A. The small size of eluted IgG was consistent with a configuration where the Fab region was folded over in contact with the Cγ3 region. The reduced-size conformation persisted as long as the IgG remained resident in elution conditions. IgG size increased towards native dimensions with elevation of pH and/or elevation of NaCl concentration, and native size was fully restored under physiological conditions. Restored IgG maintained native size upon re-exposure to pH 3.5.

Elution efficiency was highest when IgG was in its most-reduced-size conformation, but fair elution efficiency was obtained with larger conformations in the presence of additives that weaken hydrogen bonds and hydrophobic interactions between IgG and protein A, such as arginine. Formation of the reduced-size conformation and its subsequent restoration to native size did not produce aggregates, but aggregation of the reduced-size conformation was induced by secondary stress factors, and those aggregates persisted after neutralization. Observation of the reduced-size conformation with 3 different IgG1 monoclonal antibodies suggest the phenomenon is probably universal for this human subclass, and may occur with other subclasses and species, as well as with Fc fusion proteins.

Acknowledgements

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