Ion-specific modulation of protein interactions: Anion-induced, reversible oligomerization of a fusion protein

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Abstract: Ions can significantly modulate the solution interactions of proteins. We aim to demonstrate that the salt-dependent reversible heptamerization of a fusion protein called peptibody A or PbA is governed by anion-specific interactions with key arginyl and lysyl residues on its peptide arms. Peptibody A, an *E. coli* expressed, basic (pI = 8.8), homodimer (65.2 kDa), consisted of an IgG1-Fc with two, C-terminal peptide arms linked via penta-glycine linkers. Each peptide arm was composed of two, tandem, active sequences (SEYQGLCTRWPWMCPPQGWK) separated by a spacer (GSGSATGGSGGGASSGSGSATG). PbA was monomeric in 10 m*M* acetate, pH 5.0 but exhibited reversible self-association upon salt addition. The sedimentation coefficient (s_w) and hydrodynamic diameter (D_H) versus PbA concentration isotherms in the presence of 140 m*M* NaCl (A5N) displayed sharp increases in s_w and D_H, reaching plateau values of 9 s and 16 nm by 10 mg/mL PbA. The D_H and sedimentation equilibrium data in the plateau region (>12 mg/mL) indicated the oligomeric ensemble to be monodisperse (PdI = 0.05) with a *z*-average molecular weight (M_z) of 433 kDa (stoichiometry = 7). There was no evidence of reversible self-association for an IgG1-Fc molecule in A5N by itself or in a mixture containing fluorescently labeled IgG1-Fc and PbA, indicative of PbA self-assembly being mediated through its peptide arms. Self-association increased with pH, NaCl concentration, and anion size ($I^- > Br^- > Cl^- > F^-$) but could be inhibited using soluble Trp-, Phe-, and Leu-amide salts (Trp > Phe > Leu). We propose that in the presence of salt (i) anion binding renders PbA self-association competent by neutralizing the peptidyl arginyl and lysyl amines, (ii) self-association occurs via aromatic and hydrophobic interactions between the ..xxCTRWPWMC..xxx..CTRWPWMCxx.. motifs, and (iii) at >10 mg/mL, PbA predominantly exists as heptameric clusters.

Keywords: Hofmeister series; electroselectivity series; self-association; ion-protein interactions; anion binding; dynamic light scattering; analytical ultracentrifugation

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Abbreviations: A5, 10 m*M* acetate buffer at pH 5.0; A5N, 10 m*M* acetate buffer at pH 5.0 containing 140 m*M* sodium chloride; AUC, analytical ultracentrifugation; C_H2, constant heavy chain domain 2; C_H3, constant heavy chain domain 3; *c*(*M*), continuous molecular mass distribution function; *c*(*s*), continuous sedimentation coefficient distribution function; D_H, hydrodynamic diameter; DLS, dynamic light scattering; Fc, fraction crystallized; Fc, fragment crystallizable; FITC, fluroescein isothiocyanate; Fv, fragment variable; IAA, iodoacetic acid; IgG_1, immunoglobulin G type 1; IPA, isopropyl alcohol; M_z, *z*-average molecular weight; NaX, sodium halogen salt; PbA, peptibody A; PBS, phosphate buffered saline; PdI, polydispersity index; RPM, revolutions per minute; SE, sedimentation equilibrium; SV, sedimentation velocity; s_w, weight-average sedimentation coefficient; X, halogen ion

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Introduction

Although the differential effects of ions on protein precipitation were observed more than a century ago by Hofmeister,[1,2] the role of ions in the modulation of the solution behavior and interactions of proteins is increasingly well appreciated. Ions have been observed to impact protein stability,[3] fibril formation,[4–6] reversible self-association,[7–9] and even protein solution rheology.[10,11] An ion-specific dependence is often discussed in terms of the Hofmeister effect or the electroselectivity series;[3–5,12–14] however, the exact nature of ion-protein interactions remains unresolved. The classical view holds that such interactions are mediated indirectly through the alteration of bulk water structure,[15] although there is increasing recognition that ions interact directly with proteins.[16] Significantly, Collins has proposed[17–21] that ionic interactions in aqueous solution are governed by the relative affinities of ions for the molecular dipole of water. This qualitative description known as the "Law of Matching Water Affinities" provides a good basis for discussing interactions of dissolved salt-ions with charged protein side chains.

Therapeutic proteins often exhibit reversible self-association under different solution conditions of pH, excipients, and ionic strength. This can have a significant impact on drug development, affecting pharmacokinetics, manufacturing processes, and delivery. There are several such examples reported in literature. The hormone insulin can be stabilized as a reversible hexamer when formulated with zinc.[22,23] However, the hexameric form causes a delayed onset of glucose reduction following subcutaneous injection, which consequently has led to the development of genetically engineered, monomeric, insulin analogues such as Humalog® and Novolog®.[23] The reversible self-association of some monoclonal antibodies at high concentrations has been implicated in dramatically increasing solution viscosity, leaving significant challenges to be resolved during manufacturing and delivery of the drug product.[10,24] There also have been concerns about oligomeric forms of a drug presenting an increased immunogenic risk.[25,26] For these reasons, reversible self-association interactions have been studied in almost all classes of protein therapeutics, including peptides,[27] hormones,[28–30] growth factors,[31] cytokines,[32] antibodies,[33,34] and recently even in single-chain Fv molecules.[35]

This report is focused on characterizing the salt-dependent reversible self-association pathway of a fusion protein called peptibody A (PbA). Peptibodies form a unique class of recombinant proteins consisting of active peptide sequences attached to the Fc domain of an immunoglobulin G (IgG). In particular, PbA (see Fig. 1) is an *E. coli* expressed, basic (pI = 8.8), covalent homodimer with a sequence molecular weight of 65,223 Da and consists of an Fc domain of an IgG_1 with "peptide arms" linked to each of its C-termini. Each peptide arm consists of two active peptide sequences [SEYQGLCTRWPWMCPPQGWK] separated by a spacer group [GSGSATGGSGGGASSGSGSATG] and is attached to the Fc with a flexible, pentaglycine linker.

Peptibody A exhibited peculiar solution behavior. Although it was distinctly monomeric in a low-ionic strength pH 5.0 solution buffered with 10 m*M* acetate, it appeared to undergo rapid, reversible self-association with the addition of sodium chloride. To understand the underlying ion-protein interactions and gain insights into the assembly process, we employed a set of monovalent ions from the Hofmeister series, varied ionic strength and pH and studied their effects on PbA self-association using sedimentation and dynamic light scattering (DLS) techniques. The self-association pathway of PbA was anticipated to be complex given the symmetrical nature of the molecule (see Fig. 1) allowing for the propagation of the self-assembly process to occur via a combination of peptide–peptide, peptide-Fc, and Fc–Fc interactions (see Fig. 2). Through the systematic elucidation of the self-association pathway of PbA, our studies shed light on the importance of ion-specific protein interactions and their role in modulating the solution behavior of proteins.

Results

Solution behavior of peptibody A

Sedimentation velocity and DLS experiments with PbA in a low ionic strength, pH 5.0 solution buffered with 10 m*M* acetate (A5 buffer) revealed it to be monomeric.* There was no evidence of any concentration dependent self-association of PbA in the A5 buffer from SV and DLS experiments over a wide PbA concentration range of 0.1–35 mg/mL [Fig. 3(A–C)]. An increase in weight average sedimentation coefficient (s_w) or the hydrodynamic diameter (D_H) with increasing PbA concentration would be expected if significant self-association interactions were present. In fact, a decrease in the monomer s_w and D_H values, attributable to nonideality effects, was observed as a function of PbA concentration.

With the addition of an isotonic amount (140 m*M*) of NaCl to the A5 buffer, however, a concentration dependent increase in s_w and D_H was observed over the 1000-fold PbA concentration range examined [0.03–18 mg/mL—SV, 0.1–30 mg/mL—DLS, Fig. 3(A,B)]. This strongly indicated that PbA was undergoing reversible self-association. A key feature of the

*It should be noted that PbA itself is a covalent dimer consisting of two C_H2 and C_H3 domains of the human Fc with two peptide arms attached to the C termini of the Fc. The sequence molecular weight of the PbA monomer is 65,223 Da. The word "monomer" in this report refers to the full length PbA molecule with a molecular weight of 65,223 Da.

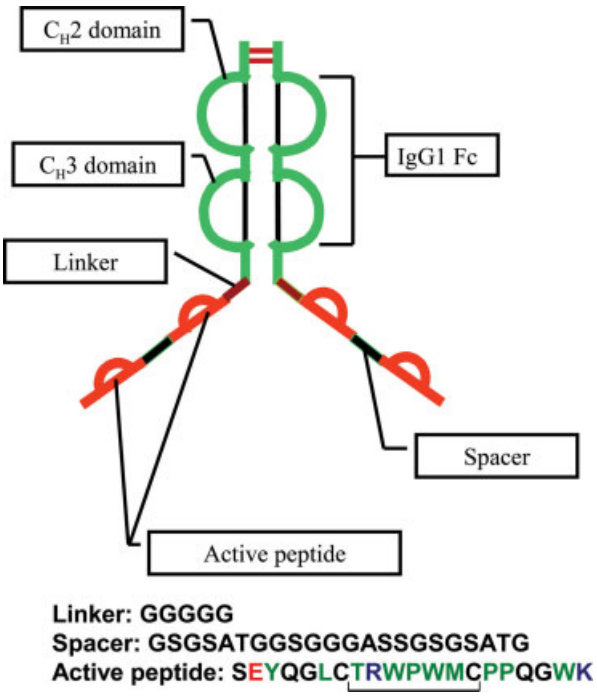

Figure 1. Structural cartoon of Peptibody A. PbA is an *E. coli* expressed, basic (pI = 8.8), covalent homodimer with a sequence molecular weight of 65,223 Da and consists of an Fc domain of an IgG_1 with "peptide arms" linked to each of its C-termini. Each peptide arm consists of two active peptide sequences separated by a spacer group and is attached to the Fc with a flexible, penta-glycine linker.

s_w and D_H versus protein concentration plots was that both isotherms appeared to asymptotically approach plateau s_w and D_H values of ~9 s and 16 nm, respectively, by 10 mg/mL PbA. In addition, the very low polydispersity indices (average = 0.05) observed in the plateau region (13–30 mg/mL) of the DLS data [Fig. 3(C)] were indicative of a monodisperse oligomeric ensemble. Generally, size distributions with polydispersity indices of less than 0.1 are considered to be monodisperse.[36,37]

Effect of ion identity, ionic strength, and pH on the self-association of peptibody A

The PbA s_w values (and hence self-association) were observed to be very sensitive to ionic strength as well as anion identity. For a fixed PbA concentration (1 mg/mL) and a given NaX (X = halogen ion) concentration, the s_w values increased with the atomic size of the monovalent anion [Fig. 4(A)]. The extent of self-association was highest in the presence of 50 m*M* sodium iodide (6.9 s) and lowest in 50 m*M* NaF (5.0 s). Monovalent cations (Li → Cs), on the other hand, did not appear to affect the self-association equilibrium appreciably. Although the observed s_w values (5.4 ± 0.2 s) were much higher than those expected for the monomer (4.2 s), indicative of self-association in the presence of all the cation-chloride salts, no significant differences in the extent of self-association between the cations could be discerned [Fig. 4(A)]. The sodium chloride concentration had a significant impact on the self-association interaction. For a given PbA concentration (1 mg/mL) and pH (5.0), the PbA s_w values increased with increasing NaCl concentration [Fig. 4(B)]. An experiment designed to study the effect of pH on PbA self-association revealed that the extent of association also increased with pH. The s_w values for 1 mg/mL PbA solutions prepared in an acetate-histidine-phosphate-Tris (5 m*M* each) buffer with 75 m*M* NaCl increased from 5.7 s at pH 4.5 to 7.4 s at pH 10.0 [Fig. 4(C)].

Solution behavior of Fc in the presence of salt and peptibody A

Sedimentation velocity experiments with Fc in the presence of 140 m*M* NaCl at pH 5.0 indicated that it remained monomeric over the entire Fc concentration range (0.3–10 mg/mL) examined. The monomer was observed to sediment at a rate of 3.6 s under dilute conditions (0.3 mg/mL). A linear decrease in s_w, attributable to hydrodynamic nonideality effects, was observed with increasing Fc concentration [Fig. 5(A)]. Fluorescent labeling had no effect on the association state of Fc; FITC-Fc was observed to sediment as a monomer in PBS at pH 7.5 as indicated by the sharp peak at 3.7 s in its $c(s)$ distribution [Fig. 5(B)]. In the

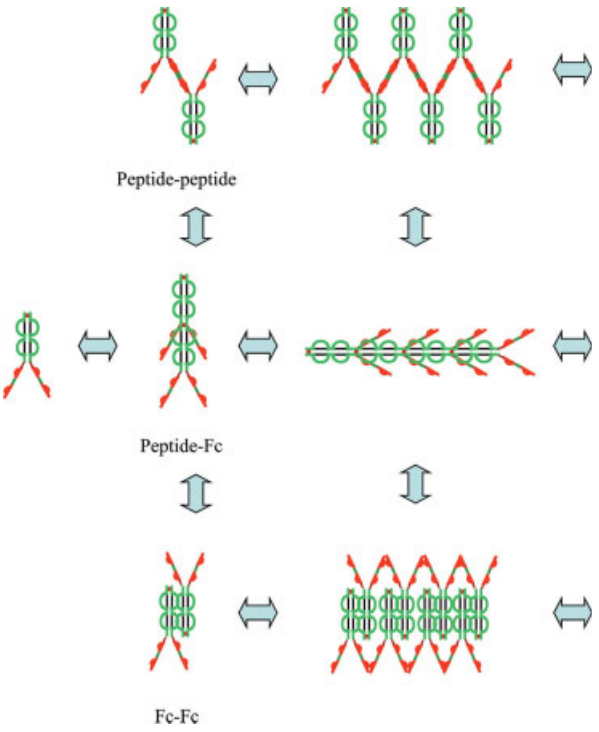

Figure 2. Generalized self-association model for PbA depicting possible association pathways mediated via peptide–peptide, peptide-Fc, or Fc–Fc interactions.

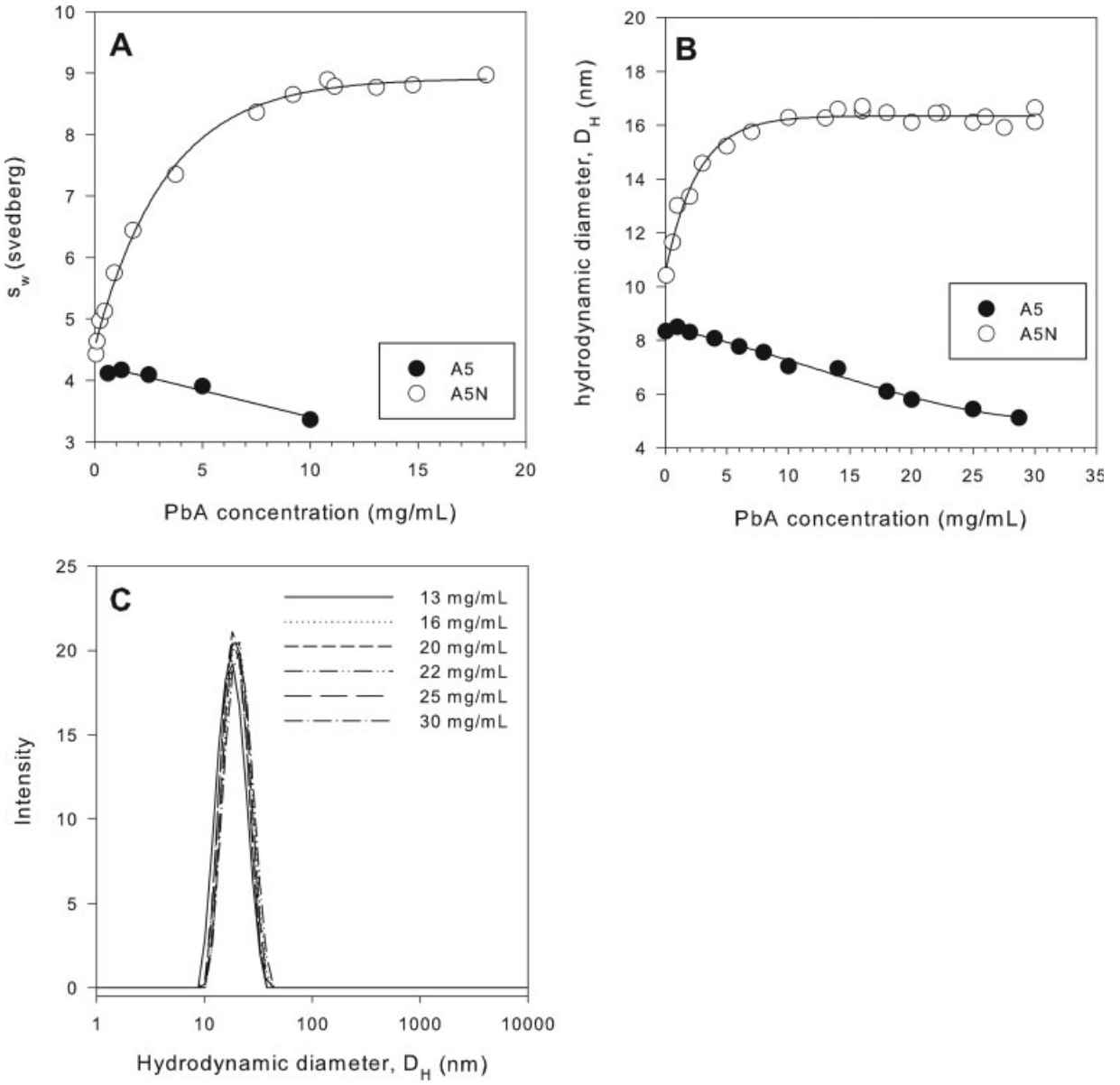

Figure 3. SV and DLS data indicate that PbA self-associates in the presence of salt. SV experiments (50,000 rpm) show the s_w of PbA to increase with PbA concentration in A5N reaching a plateau value of ~9 s above 10 mg/mL (monitored by absorbance at 230 nm for PbA concentrations <0.4 mg/mL). A decrease in s_w is observed in A5 with increasing PbA concentration (Panel **A**). The hydrodynamic diameter for PbA increases rapidly with protein concentration in A5N reaching a plateau value of 16 nm above 10 mg/mL. A decrease in hydrodynamic diameter is observed in A5 with increasing peptibody concentration (Panel **B**). The DLS data show that the PbA oligomeric ensemble in A5N from 13–30 mg/mL is monodisperse with an average pDI value of 0.052 ± 0.011 over the entire concentration range (Panel **C**).

presence of PbA, Fc displayed no self-associative behavior. The $c(s)$ distribution of FITC-Fc (monitored at 495 nm) in a mixture of FITC-Fc and PbA (0.3 mg/mL each) in PBS at pH 7.5 was super-imposable upon the control of only FITC-Fc in PBS. These experiments indicated that Fc did not participate in the PbA self-association interaction.

Effect of aromatic and hydrophobic amino acids on the self-association of peptibody A

The importance of aromatic and hydrophobic interactions in mediating self-association was probed by attempting to inhibit the interaction with appropriate amino acids. Soluble amide-hydrochloride salts of tryptophan, phenylalanine, and leucine were employed for this purpose with alanine amide hydrochloride being used as a control. Inhibition of self-association was measured in terms of the decrease in s_w values of a 1 mg/mL PbA solution formulated in A5N with additional amounts (50–150 m*M*) of the hydrochloride salt of a given amino acid. It should be noted that in the absence of any inhibition, an increase in the extent of self-association with increasing amino acid-NH_2-HCl concentration was expected due to the

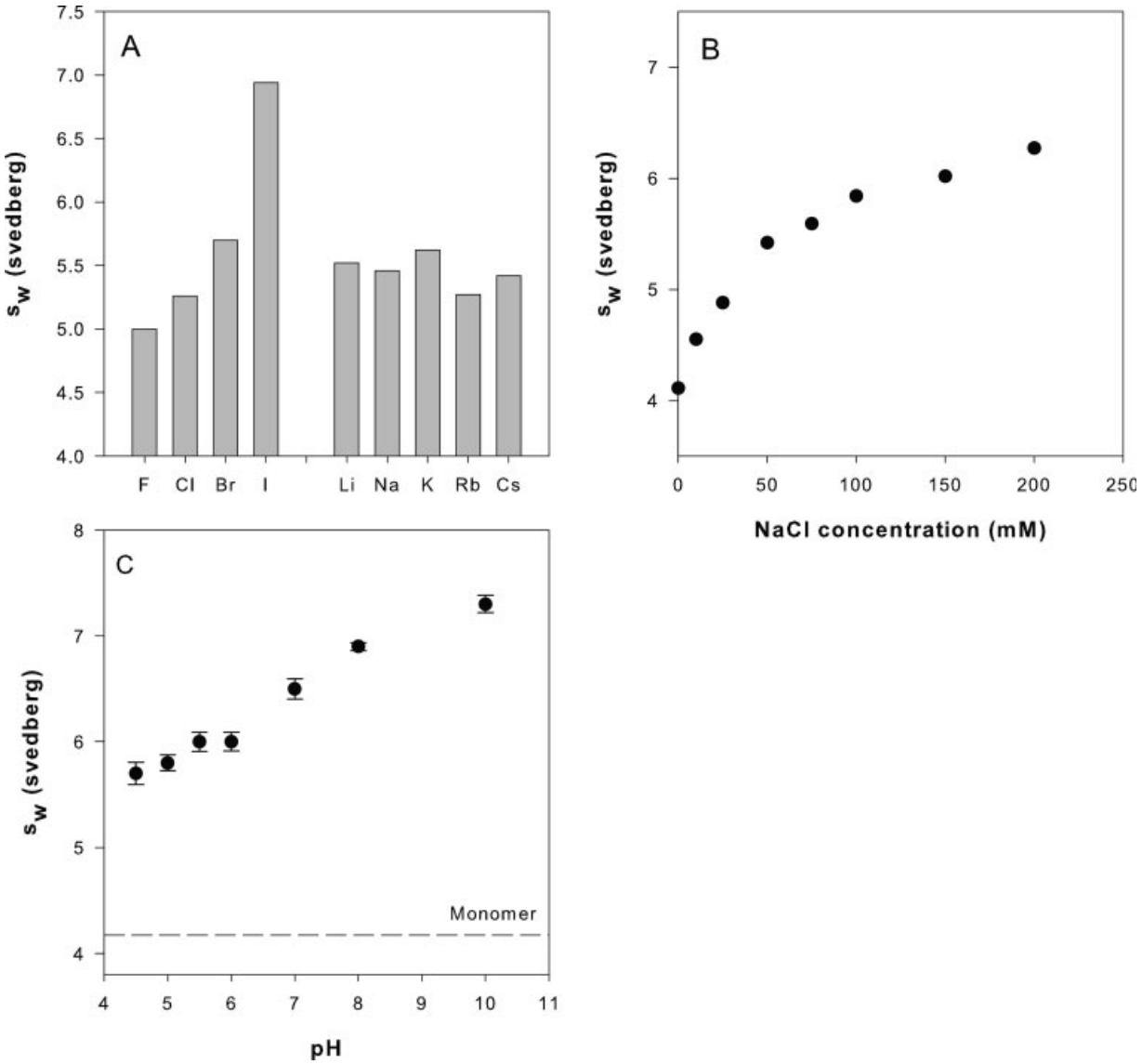

Figure 4. Factors influencing PbA self-association. Interference SV experiments (50,000 rpm) on 1 mg/mL PbA in 140 m*M* salt solutions show peptibody self-association to have a strong dependence on the identity of the halogen anion, but not the alkali cation (Panel **A**). Self-association increased with NaCl concentration (0 and 200 m*M*) (Panel **B**) and with pH (Panel **C**).

concomitant increase in chloride concentration. For example, the total chloride concentration resulting from the addition of 150 m*M* Ala-NH_2-HCl to A5N was 300 m*M*. Consistent with this expectation, a slight increase (to 6.6 s) in s_w values of PbA was observed in the case of alanine-amide. However, even with the additional chloride content, significant inhibition of the self-association interaction was observed in the presence of both aromatic compounds. With the addition of 150 m*M* phenylalanine amide or tryptophan amide, the s_w value of 6.0 s for 1 mg/mL PbA in A5N decreased to ~5.0 or 4.5 s, respectively (see Fig. 6). Inhibition of self-association was also observed in the presence of leucine amide; however, it did not appear to be as effective as the aromatic amino acid derivatives. Tryptophan amide was observed to be the most effective in inhibiting the salt induced self-association of PbA.

Sedimentation equilibrium experiments with peptibody A

Sedimentation equilibrium (SE) studies were performed to determine the molecular weight of the PbA oligomeric ensemble in the plateau region of the DLS isotherm [Fig. 3(B)]. Experiments were conducted at 12–20 mg/mL PbA in A5N. The resulting equilibrium concentration distributions could be fit to the single, ideal-species model and yielded a *z*-average molecular weight (M_z) value of 433 kDa (see Fig. 7) with an RMSD of 0.012.

Discussion

Self-association of peptibody A occurs via its peptide arms

Peptibody A is a basic (pI = 8.8), symmetric molecule composed of two peptide arms attached to the C

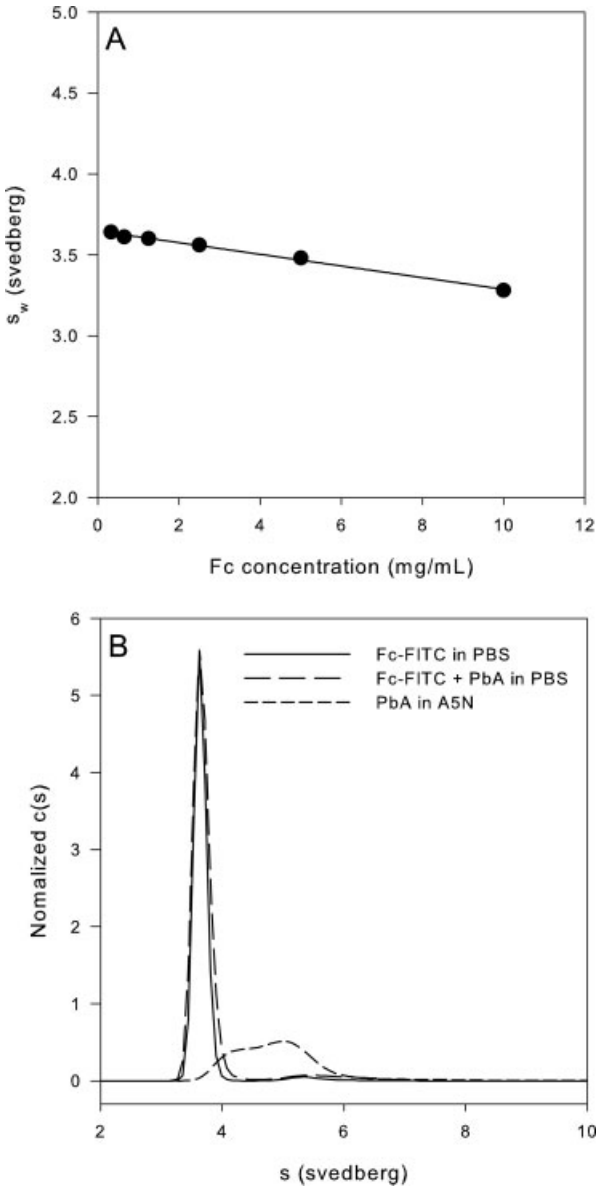

Figure 5. AUC experiments with Fc-FITC indicated PbA does not self-associate through Fc–Fc or Fc-peptide interactions. The s_w of 0.3 mg/mL FITC-Fc obtained from SV absorbance (495 nm) experiments at 60,000 rpm matched those of the unlabeled Fc (Panel **A**); the *c*(*s*) distribution of FITC-Fc was unchanged by the addition of PbA to 0.3 mg/mL (Panel **B**).

termini of a human IgG_1 Fc. The Fc itself is a symmetric, covalent dimer composed of two C_H2 and C_H3 domains. The structural symmetry of PbA led to construction of a generalized assembly model in which reversible self-association of the peptibody was proposed to occur via a combination of intermolecular peptide-peptide, peptide-Fc, and/or Fc–Fc interactions (see Fig. 2). The symmetry allowed for the possibility that self-association could propagate indefinitely.

The sedimentation velocity experiments with Fc and FITC-Fc were useful in demonstrating that self-association of PbA was likely to be mediated through its peptide arms. Over the wide Fc concentration range of 0.3–10 mg/mL examined, there was no indication of any rapid, reversible self-association of Fc under the A5N conditions [Fig. 5(A)]. The observed monotonic decrease in s_w with increasing Fc concentration was a result of hydrodynamic nonideality, which becomes significant at higher protein concentration.[38] Based on this, the possibility of PbA self-association being mediated by Fc–Fc interactions could be eliminated. Because PbA is expressed in *E. coli*, the Fc C_H2 domain is not glycosylated. It was important to ensure that the lack of glycosylation was not causing the reversible self-association observed in PbA.

With no evidence of Fc–Fc mediated self-association, we were able to simplify the PbA reversible self-association model, leaving peptide-Fc and peptide–peptide interactions as the only two possible pathways. The FITC labeling of Fc enabled us to selectively follow its sedimentation in the presence of PbA by setting the detection wavelength to 495 nm. It is important to note here that the SV experiment with Fc + PbA mixture was conducted at pH 7.5 in PBS (which contained 140 m*M* NaCl), conditions in which the extent of self-association was expected to be even greater than at pH 5.0 [Fig. 4(C)]. The *c*(*s*) analysis of the 495-nm signal indicated that the sedimentation coefficient of FITC-Fc was unaffected by the presence of PbA and that it

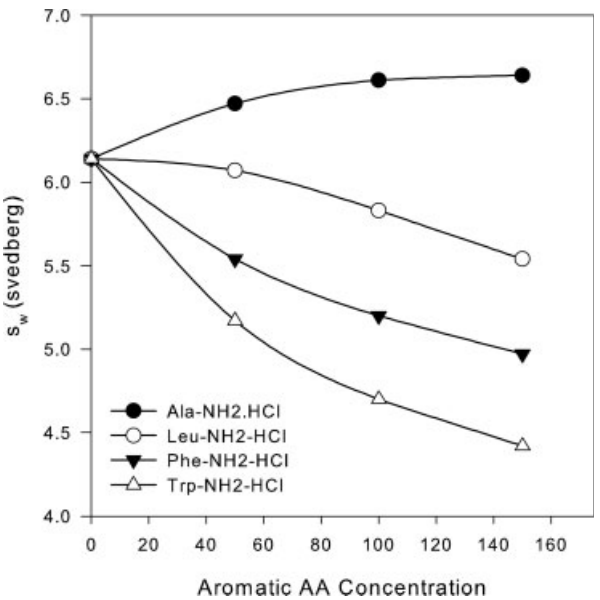

Figure 6. Aromatic and hydrophobic amino acids inhibit self-association of PbA. The addition of chloride salt of tryptophan-, phenylalanine-, or leucine-amide decreases the degree of self-association of 1 mg/mL PbA in A5N, as measured by interference, SV experiments (50,000 rpm). The degree of self-association increases in the presence of alanine amide hydrochloride due to the concomitant increase in chloride concentration.

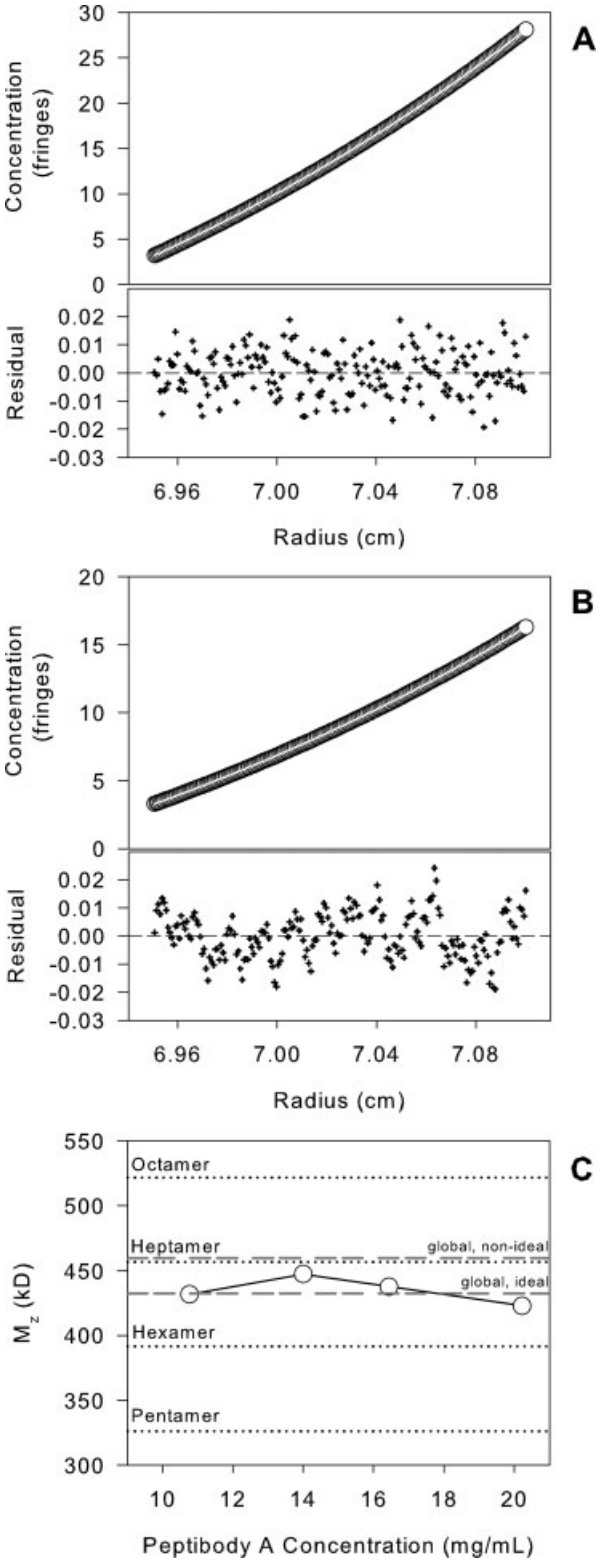

Figure 7. Sedimentation equilibrium studies of PbA (12–20 mg/mL) in A5N. Experiments were performed at 3000 rpm and 20°C using interference detection. The data could be described well by the single ideal species model for the entire PbA concentration examined (20 mg/mL—Panel **A**, 12 mg/mL—Panel **B**). The molecular weight from individual fits (single, ideal) and from the global single ideal and nonideal species fits are presented in Panel C.

migrated as a monomer (from its corresponding $c(M)$ analysis, data not shown). Further, the $c(s)$ distribution of FITC-Fc from the mixture experiment was super-imposable with that of the control ($c(s)$ distribution of FITC-Fc alone). If peptide-Fc interactions were significant in mediating the reversible self-association of PbA, then the c(s) distribution should be broad, similar to that observed for PbA in A5N [Fig. 5(B)]. Given that Fc did not appear to self-associate in the presence of PbA, the peptibody was most likely to self-associate via its peptide arms.

Anion binding is essential and self-association is mediated via a combination of aromatic and hydrophobic interactions

The salt dependence of the reversible self-association of PbA indicated that charge interactions were important in the mediation of the interaction. Combined with the evidence that self-association of PbA occurred only through its peptide arms, a model which included ion binding to specific residues in the peptide arms as a necessary step for self-association could be constructed. Upon inspection of the amino acid sequence of the peptide arm, the possible roles of anion binding and tryptophan (aromatic and hydrophobic) interactions in mediating PbA self-association became apparent. Each active sequence in a single peptide arm of PbA (see Fig. 1) contains two basic amino acids, lysine and arginine, with the latter near two tryptophan residues in a disulfide-closed loop (-CTRWPWMC-). We tested our hypothesis that charge neutralization of the arginyl and lysyl side chains rendered the peptibody self-association competent and that self-association was mediated through the peptide loops via hydrophobic and aromatic interactions. In the absence of a significant anion concentration, repulsion between intermolecular, positively charged arginine and lysine residues prevented self-association.

The positively charged guanidino group of arginines and the ε-amine of lysines are weakly hydrated and expected to preferentially bind weakly hydrated anions.[17–21,39] The responsiveness of PbA self-association to halogen anion identity and its relative unresponsiveness to alkali cation identity [Fig. 4(A)] supported anion binding as being critical for self-association. The binding affinity of halogen ions to the arginyl and lysyl side-chains is expected to increase with increasing anion size.[18] Indeed, the greatest extent of self-association was observed in the presence of iodide, and it progressively decreased with decreasing anion size, from $I^- \rightarrow Br^- \rightarrow Cl^- \rightarrow F^-$ [Fig. 4(A)].

The observed pH dependence of the PbA s_w value (at a fixed NaCl concentration of 75 mM) also supported anion binding being an important step in the reversible self-association process [Fig. 4(C)]. With increasing pH, the effective charge of PbA (pI = 8.8) was expected to decrease progressively, requiring a

proportionately lower anion concentration to achieve equivalent charge reduction. For a given ionic strength, this would free-up an increasing concentration of anions (with increasing pH) for binding to the arginyl side chains in the peptide loops (which were expected to be always positively charged, $pK_a = 12$). Consequently, an increase in the extent of self-association would be expected with increasing pH. This expectation was consistent with our data, in which the s_w values of PbA steadily increased from 5.7 s at pH 4.5–7.4 s at pH 10.0.

Anion binding has been reported to be important in several biological systems, for the modulation of ion transport across membranes,[40] enzymatic activity, and even self-association.[7] Anion binding to arginine and lysine residues on the interaction interface of hemoglobin (from Archid Mollusks) has been reported to be critical for its ionic strength dependent reversible polymerization.[7] Although there was valid evidence to suggest that anion binding enabled self-association of PbA, the specific residue-level interactions through which self-association was mediated remained to be investigated. It was recognized that the peptide loop -CTRWPWMC- was fairly hydrophobic (except for R) and that self-association via this hydrophobic patch was likely. Further, because there were two such patches per peptide arm (see Fig. 1), a stable interaction interface involving both peptide loops [...xxxCTRWPWMCxxx...xxxCTRWPWMCxxx] was likely to be favored. However, at least five types of interactions between two intermolecular peptide arms were theoretically possible and considered: (i) R→W, cation-π interactions,[41] (ii) K ←→ E ion-pair interactions, (iii) W←→W aromatic interactions, (iv) W-X-W type interactions, coordinated by anions, especially halides,[42] and (v) intermolecular hydrophobic interactions.

Both R→W and K←→ E are ionic interactions and expected to be screened with increasing ionic strength. Contrary to this expectation, PbA self-association was enhanced with increasing ionic strength suggesting R→W, and K←→ E interactions were unlikely to be responsible for PbA self-association. Evidence in support of W←→W aromatic interactions and/or hydrophobic interactions came from the ability of tryptophan-amide, phenylalanine-amide, and leucine-amide to inhibit the self-association interaction (see Fig. 6). And, because both phenylalanine and leucine could also inhibit the self-association (albeit to a lesser extent), the fourth possibility of W-X-W interactions was considered unlikely. The ability of the weakly charged indole nitrogen of tryptophan to interact with halides has been reported in literature. The haloalkane dehalogenase enzyme is known to employ this type of interaction to coordinate a halide ion between two adjacent tryptophan residues in its active site.[42] The tryptophan coordination "strength" of halides is expected to increase with ionic size (I^- better than Cl^-), which in the case of PbA could potentially have helped explain the observation of increased self-association with increasing halide ion size. However, if W-X-W were the only interaction involved in mediating PbA self-association, then inhibition of the interaction by phenylalanine and leucine (with no expectation to coordinate halides) would not be observed. On the other hand, the inhibition of W ←→ W aromatic interactions by free phenylalanine was possible, as formation of similar W ←→ F aromatic interactions is well known. Given the abundance of hydrophobic residues in the peptide loops (-CTRWPWMC-), self-association could also simply be mediated by hydrophobic interactions upon neutralization of arginines and lysines. In this event, greater inhibition of self-association by phenylalanine and leucine, being more hydrophobic than tryptophan, would be expected. In contrast, tryptophan was observed to be most effective at inhibiting PbA self-association. Although W ←→W interactions appear to be significant, the self-association of PbA probably occurs through a combination of aromatic and hydrophobic interactions. The active peptide sequence is largely hydrophobic, with 20% of the amino acids having aromatic side chains. From our studies with tryptophan- and phenylalanine-amide, we have strong evidence that tryptophan-aromatic interactions play a significant role. Furthermore, the sensitivity of the self-association interaction to salt concentration is consistent with side-chain charge neutralization of the arginyl and lysyl residues within the active peptides being essential. Neutralization would reduce the energetic cost of bringing the residues into close proximity with like-charges and/or burying them in a hydrophobic/aromatic interaction. Although there is no evidence for the residue-level interactions to be highly specific or structured, our proposition of cluster formation (next section) is more consistent with nonspecific, aromatic/hydrophobic interactions.

Oligomer geometry, stoichiometry, and a model for peptibody A self-association

The plateau in the DLS and SV isotherms in A5N [Fig. 3(A,B)] at high PbA concentrations (>10 mg/mL) was suggestive of a termination step in the PbA oligomerization pathway. With increasing protein concentration, PbA self-association may propagate via its peptide arms and terminate with the formation of cyclic oligomers. Alternatively, PbA may form heptameric clusters in which peptide arms are buried in a central core. The monodispersity of the PbA oligomers in the plateau region [Fig. 3(C)] is consistent with both models.

The determination of the molecular weight of PbA oligomers (and hence stoichiometry) was complicated by potential thermodynamic nonideality effects, which can be significant at high protein concentrations. Self-association and nonideality have opposite effects on the measured molecular weight by SE in a reversibly interacting system. While self-association leads to an

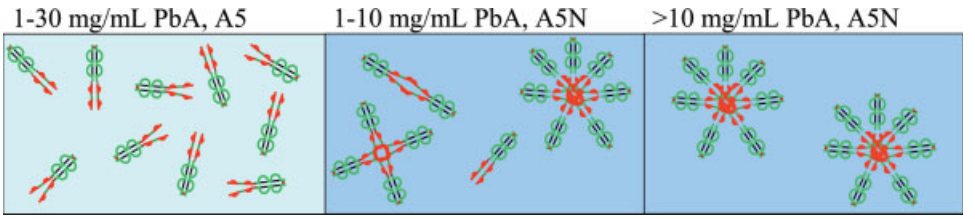

Figure 8. Cartoon depicting the proposed self-association model for PbA in A5N. In 10 m*M* acetate, PbA remains monomeric. Upon addition of an isotonic amount of NaCl, at low to intermediate concentrations PbA (< 10 mg/mL), it exists as a mixture of dimeric to heptameric oligomers. At higher concentrations, PbA self-associates to form heptameric clusters.

increase in buoyant molecular weight, nonideality effects will lower the value. Still, the sedimentation equilibrium data between 12 and 20 mg/mL PbA in A5N could be described well using the single-ideal species model as supported by the randomness of the residuals to the fits and yielded a *z*-average molecular weight of 433 kDa (Fig. 7, Panel C global ideal fit), consistent with a molecular stoichiometry of ~7.

It is indeed tempting to interpret the sharp increases and plateauing of s_w and D_H in their respective isotherms in terms of a cooperative, monomer ← → heptamer, self-association interaction. However, the propagation of PbA self-association via a sequential oligomerization pathway terminating at a heptamer is also likely and thus cannot be ruled out. On the basis of the evidence presented, and employing parsimony, we propose the following self-association model for PbA in A5N (see Fig. 8):

1 Upon salt addition, anion binding renders PbA self-association competent by neutralizing the peptidyl arginyl and lysyl amines
2 Self-association propagates via a combination of intermolecular, aromatic, and hydrophobic interactions between . . .xx**CTRWPWMC**..xxx.. **CTRWPWMC**xx.. motis on the peptide arms
3 At low to intermediate concentrations (<10 mg/mL), PbA exists as a mixture of dimeric to heptameric oligomers. At higher concentrations, PbA self-associates to form heptameric clusters with a D_H of 16 nm.

Materials and Methods

Peptibody A

The bulk drug substance lots of PbA were supplied by Amgen Process Sciences & Protein Sciences groups. The lot from Protein Sciences was formulated at 10 mg/mL in 10 m*M* acetate, 5% sorbitol at pH 5.0 (A5S), whereas the lot from Process Sciences was received at 30 mg/mL in 10 m*M* acetate, 9.25% sucrose at pH 4.8. The *E. coli* expressed human IgG_1 Fc (FC) was received at a concentration of 25 mg/mL in A5S from Amgen's Protein Sciences group. The L-tryptophan amide and L-phenylalanine amide hydrochloride salts were purchased from CalBiochem (San Diego, CA). All chemicals were of analytical grade or better.

Preparation of solutions

For all experiments, the PbA bulk was first dialyzed into 10 m*M* acetate, pH 5.0 (A5) buffer and concentrated to 30 mg/mL. When conducting experiments in the 10 m*M* acetate, 140 m*M* NaCl, pH 5.0 (A5N) buffer, appropriate volumes of a 3*M* sodium chloride stock solution prepared in 10 m*M* acetate, A5 buffer, and the PbA bulk in A5 were mixed proportionately to achieve the desired protein and NaCl concentrations. All other solutions were prepared similarly by dilution and/or addition of the stocks of the desired chemicals to the bulk in A5 buffer.

Analytical ultracentrifugation: sedimentation velocity and sedimentation equilibrium

Sedimentation experiments were conducted in a Beckman Coulter XL-I analytical ultracentrifuge at 20°C, using either the absorbance or interference optical system. Experiments were performed using cells fitted with sapphire (specifically used in interference experiments) or quartz windows, and 12-mm path-length, charcoal-epon centerpieces. Samples were centrifuged at 3000 rpm for equilibrium experiments and 50,000 rpm or 60,000 rpm for velocity experiments. With the absorbance optical system, sedimentation was monitored using a detection wavelength of 280 nm in all experiments except for those involving the FITC labeled Fc (FITC-Fc), in which case a wavelength of 495 nm was used to selectively detect the sedimentation of FITC-Fc. When employing the absorbance optical system for SV experiments, scans were gathered using a radial step size of 0.003 in continuous mode, with no replicates.

The software program SEDFIT (kindly provided by Dr. Peter Schuck, NIH, Bethesda, USA) were employed to analyze the SV data (using the *c*(*s*) and *c*(*M*) distribution analysis).[43,44] The program HETEROANALYSIS (kindly provided by Prof. James Cole, University of Connecticut, Storrs, USA) was employed to determine the apparent z average molecular weights

from the SE data by applying the single ideal and non-ideal species models.

Dynamic light scattering experiments

DLS studies were conducted using the Zetasizer Nano ZS® instrument (Malvern Instruments, Worcestershire, UK) at 20°C to determine the hydrodynamic diameter (D_H) of PbA. One hundred microliters of a given solution was placed in a disposable DTS1060, folded capillary cuvette (also from Malvern Instruments) and analyzed in the DLS mode. Prior to any DLS measurements, the PbA bulk was desalted and eluted into a given buffer (A5N or A5) using NAP-5® columns (GE Healthcare, Piscataway, NJ) and then concentrated to ~30 mg/mL using 10,000 MWCO Amicon Ultra-15® centrifugal filters (Millipore Corporation, Billerica, MA). The D_H was measured as a function of PbA concentration from 0.1 to 30 mg/mL.

FITC labeling of FC

Fluorescent labeling of the FC with fluroescein isothiocyanate (FITC) was accomplished using the FLUOROTAG™ FITC Conjugation Kit (Sigma Aldrich # FITC-1) and following the protocol (FITC-1) provided with the kit. The reaction conditions were optimized (as prescribed in the protocol) to achieve a FITC/protein (F/P) conjugation ratio of 1. The actual F/P ratio in the case FITC-Fc was 1.1. After the removal of the unreacted FITC, the purified FITC-Fc was transferred into Dulbecco's PBS and used immediately in sedimentation experiments.

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