Pharmaceutical Research, Vol. 26, No. 1, January 2009 (© 2008)
DOI: 10.1007/s11095-008-9722-0

Research Paper

Anion Binding Mediated Precipitation of a Peptibody

Atul Saluja,[1] Shon Crampton,[1] Eva Kras,[2] R. Matthew Fesinmeyer,[1] Richard L. Remmele, Jr.,[3] Linda O. Narhi,[3] David N. Brems,[3] and Yatin R. Gokarn[1,4]

Received June 3, 2008; accepted August 28, 2008; published online September 27, 2008

Purpose. Understand the underlying mechanism governing the salt-induced precipitation of a basic (p*I*= 8.8) protein, Peptibody A (PbA), in acidic solutions.
Methods. The rate, extent, and reversibility of PbA precipitation was monitored over 4-weeks as a function of pH (3.7–5.0), salt concentration (0–400 mM), and ion identity using a series of monovalent, Hofmeister anions (F^-, Cl^-, Br^-, I^-, ClO_4^-, SCN^-) and cations (Li^+, Na^+, K^+, Rb^+, Cs^+). The effects of salt on conformational stability and reduced valence were determined using Fourier-transform infrared spectroscopy, circular dichroism, and capillary electrophoresis/analytical ultracentrifugation.
Results. PbA precipitation occurred upon salt addition and could be modulated with solution pH, salt identity & concentration. The precipitation was sensitive to anions, but not cations, and increased with anion size. A reverse Hofmeister effect (SCN^-~ClO_4^->I^->Cl^->Br^->F^-) was observed with "salting-in" anions being the more effective precipitants. An increase in the precipitation rate below pH 4.3 indicated that protonation of aspartyl and glutamyl side-chains was also important for precipitation. The reversibility of precipitation was excellent (100%) at 4°C but decreased upon storage at 25°C and 37° C; the loss in reversibility correlated with an increase in intermolecular β-sheet content of the precipitate.
Conclusion. Salts, employed as buffering, tonicifying, and viscosity modifying agents, may adversely affect the solubility of basic proteins formulated under acidic conditions.

KEY WORDS: anion binding; Hofmeister effect; protein formulation; protein solubility; salt-induced precipitation.

INTRODUCTION

Preventing drug precipitation in parenteral solution formulations during production, storage, and upon delivery is a critical issue that needs to be addressed early in the development process. Drug precipitation can affect efficacy and pharmacokinetics if the precipitate is irreversible or slowly reversible, and safety by causing injection site reactions and/or phlebitis (intravenous injection) in patients (1,2). With protein-based drugs, there is the additional concern of the precipitated drug causing immunogenic or antigenic reactions (3,4). Thus, any drug in solution form must remain soluble (a) in the formulation through its intended shelf-life or after reconstitution in the case of lyophilized dosage forms (b) upon dilution in suitable IV diluents if employed during administration, and (c) in blood plasma upon delivery. In the case of small-molecules, drug precipitation occurs as a result of insolubility and can be effectively prevented by careful design of the formulation solvent matrix aided with equilibrium solubility measurements. The precipitation of a protein–drug, however, can occur *via* two distinctly different processes. Protein precipitation can be the result of insufficient solubility. Another mode is *via* irreversible aggregation which can lead to the formation of insoluble precipitate or particles. This mode almost always involves the formation of conformationally altered, aggregation-competent species induced by stresses such as agitation, heat and even light (5). With an increasing number of protein-based therapeutics requiring the subcutaneous delivery of very high doses (>100 mg), there is an emerging need for the development of high-concentration protein dosage forms (6,7). Ensuring adequate protein solubility in such formulations is paramount to the development of successful, solution dosage forms and requires a thorough understanding of protein solubility as a function of formulation variables and temperature.

The effects of solution pH on protein solubility are generally well-understood and it is accepted that solubility is low at or very near the isoelectric point (p*I*), and high at pH values distant (>2 pH units) from the p*I*. Protein solubility has been traditionally measured using high concentrations of 'salting out' agents such as ammonium sulfate (8,9). However, little information is available on factors governing protein solubility in solutions of moderate ionic strength

[1] Process & Product Development, 1201 Amgen Court West, MS AW2-D/3152, Seattle, Washington 98119-3105, USA.
[2] Seattle Genetics, Bothell, Washington 98021, USA.
[3] Process & Product Development, Thousand Oaks, California 91320, USA.
[4] To whom correspondence should be addressed. (e-mail: ygokarn@amgen.com)

ABBREVIATIONS: A4, 10 mM acetate buffer, pH 4.0; ADE, 10 mM acetate, aspartate and glutamate buffer; AUC, analytical ultracentrifugation; CD, circular dichroism; CE, capillary electrophoresis; FTIR, Fourier-transform infrared; PbA, Peptibody A; PBS, phosphate buffered saline; *z**, reduced valence.

(0.01–0.15 M), that are of greater pharmaceutical relevance. Understanding salt effects on protein solubility is important because isotonic amounts of sodium chloride and other neutral salts have been commonly employed in protein formulations as tonicity agents, stabilizers, and even as viscosity modifiers. For example, sodium chloride has been shown to reduce the viscosity of high-concentration monoclonal antibody formulations by inhibiting reversible self-association interactions (10). Monovalent sodium and potassium salts at concentrations up to 0.3 M have also been reported to increase the solubility of hemoglobin S (11). However, there are other reports wherein salts have been shown to deleteriously affect protein solubility and increase solution viscosity. Sodium chloride decreased the solubility of rhTFPI, and increased the viscosity of PEG-sTNFR-I solutions (12–14). These contrasting examples highlight the need for a systematic study on the effects of salt on the solubility of proteins.

This report is focused on the salt-induced precipitation of a fusion protein termed Peptibody A (PbA), an *Escherichia coli* expressed, basic (p*I*=8.8), covalent homodimer (M_w= 65,223 D), consisting of an IgG_1 Fc with a 'peptide arm' linked to each of its C-termini. Each peptide arm consists of two active, peptide sequences separated by a spacer group. The peptide arms are attached to the Fc with a flexible penta-glycine linker. PbA exhibited peculiar solution behavior. In acidic solutions of pH~4.0, the addition of an isotonic amount of sodium chloride (~0.14 M) resulted in extensive and rapid precipitation of the protein. This insolubility was unexpected given the solution pH was far from the p*I* (a condition associated with high protein solubility) and the sodium chloride concentration was lower than that normally associated with a salting out phenomenon. In this report, we attempt to elucidate the underlying mechanisms governing the salt-induced precipitation of PbA and also discuss the consequences of such phenomena for the formulation development of basic proteins.

EXPERIMENTAL PROCEDURES

Materials

The PbA bulk drug substance was provided by the Amgen process development group. The bulk lot was received at 30 mg/mL in 10 mM acetate, 9.25% sucrose at pH 4.8. Sodium chloride, sodium bromide, sodium iodide, sodium fluoride, sodium perchlorate, sodium thiocyanate and potassium chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lithium chloride, rubidium chloride and cesium chloride were obtained from Acros Organics (Morris Plains, NJ, USA) and acetic acid, aspartic acid and glutamic acid were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). Phosphate buffered saline (PBS) was from Mediatech, Inc. (Manassas, VA, USA). All chemicals used in this work were of analytical grade or better.

Methods

Precipitation and Solubility Studies

Precipitation kinetics and solubility studies of PbA in the presence of various sodium salts were performed over a pH range of 3.7 to 5.0. Experiments were designed to study the effect of initial (starting) protein concentration, ionic strength and anion identity on the protein precipitation rate and the solubility of PbA in a given salt solution.

For studies at pH 4.0, the PbA bulk was buffer exchanged into a 10 mM acetate, pH 4.0 buffer (A4) and concentrated to 60 mg/mL using 10,000 MWCO Amicon Ultra-15® centrifugal filters (Millipore Corporation, Billerica, MA, USA). The buffer-exchanged stock was diluted appropriately with A4 and a concentrated sodium chloride solution (in 10 mM acetate, pH 3.9) to achieve final salt concentrations of 200, 300 and 400 mM and final protein concentrations of 0.5 to 30 mg/mL at each salt concentration.

For pH-solubility studies in the presence of sodium chloride, the PbA bulk was buffer exchanged into an aspartic acid, glutamic acid and acetic acid (10 mM each) buffer (ADE) at pH 4.3. This buffer was used to ensure adequate buffering capacity over the pH range of 3.7–5.0. The concentrated protein solution was diluted appropriately with pH 4.3 ADE buffer and concentrated salt solution to achieve a final protein concentration of ~30 mg/mL. At each pH, PbA solubility was measured at NaCl concentrations between 50–400 mM.

Studies to understand the effect of ion identity on PbA precipitation were conducted in solutions of sodium-anion and cation-chloride salts at pH 4.5. The six monovalent anions investigated were fluoride (F^-), chloride (Cl^-), bromide (Br^-), iodide (I^-), perchlorate (ClO_4^-), and thiocyanate (SCN^-) and the five monovalent cations investigated were lithium (Li^+), sodium (Na^+), potassium (K^+), rubidium (Rb^+), and cesium (Cs^+). As in the studies with sodium chloride, the PbA bulk was buffer exchanged into the ADE buffer at pH 4.5. The concentrated protein solution was diluted appropriately with the ADE buffer and a concentrated salt solution to achieve a final protein concentration of ~30 mg/mL and a salt concentration of 100 mM.

Before initiating these studies, the pH of the final protein solutions was checked and adjusted with 1.0 N HCl or NaOH, as appropriate, to the desired pH. For all of the precipitation and solubility experiments, 400 μL aliquots of various PbA solutions in 1.5 mL Eppendorf® tubes were placed on a rotating mixer set at 20 RPM for a period of 1–24 days. The protein concentration was determined using UV spectroscopy, in an 8453 UV–Visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) using an extinction coefficient of 2.2 mL/mg cm at 280 nm. Prior to analyzing concentration, samples were centrifuged at 13,500 RPM for 15 min in a micro-centrifuge in order to sediment the precipitate. All studies were conducted at room temperature.

Studies to Evaluate Reversibility of Precipitation

The reversibility of the salt-induced precipitation of PbA was evaluated *via* a quiescent storage study at 4°C, 25°C and 37°C over a 4-week time-period. First, precipitation was induced *in-situ*, in 3 cm^3 glass vials, by mixing a 50 mg/mL PbA solution with a 400 mM concentrated sodium chloride solution to achieve 500 μL of a solution containing 20 mg/mL PbA and 240 mM NaCl. This step was previously optimized to achieve a pH of 4.0 after final mixing. Precipitation was allowed to occur at room temperature for 24 h after which

approximately 98% of the initial amount of PbA had precipitated. Following this, vials were placed at 4°C, 25°C, and 37°C for a period of 4 weeks and analyzed at regular intervals (0, 3 days, 1, 2, 3, and 4 weeks). At each time-point, vials were pulled and the precipitate was analyzed for reversibility (resolubilization) and changes in protein secondary structure.

The reversibility of PbA precipitation over the 4-week period was assessed as follows. Twenty five micro-liters of a given precipitated sample was suspended in 975 μL of 10 mM acetate, pH 5.6 buffer and placed at 4°C on a rotating mixer at 20 RPM for 24 h. Three aliquots of 25 μL each (n=3) were withdrawn from each precipitated sample for the reversibility study. After 24 h, the suspended samples were centrifuged at 13,500 RPM for 15 min in a micro-centrifuge at 4°C to sediment any undissolved precipitate. The protein concentration in the supernatant was determined by UV spectroscopy as described earlier. The reversibility was determined as percent PbA resolubilized compared to concentration at t=0 (20 mg/mL).

Reduced Valence Measurements

Reduced valence (z^*) measurements for PbA were made at 20°C using a combination of electrophoretic mobility and sedimentation coefficient measurements (15). The z^* of a protein is related to the electrophoretic mobility (μ), Boltzmann's constant (k_B), temperature (T), elementary charge (e) and translational diffusion coefficient (D_t) as per Eq. 1.

$$z^* = \frac{\mu k_B T}{D_t e} \quad (1)$$

The translational diffusion coefficient can be related to the sedimentation coefficient (s), universal gas constant (R), molecular weight (M_w), partial specific volume of the protein ($\bar{v}$) and solvent density (ρ) as per Eq. 2.

$$D_t = \frac{sRT}{M_w(1-\bar{v}\rho)}. \quad (2)$$

The electrophoretic mobility was measured by capillary electrophoresis (CE) using a Beckman Coulter PA 800 instrument (Fullerton, CA, USA) and a 60 cm eCap amine capillary with detection at 214 nm. Samples were prepared by mixing the concentrated PbA solution (previously dialyzed in pH 4.5 ADE buffer) with appropriate volumes of ADE buffer and concentrated NaCl solution to generate 1.0 mg/mL PbA solutions with 0–50 mM NaCl. Protein samples were injected immediately after the injection of an electroosmotic flow (EOF) marker [0.02% (v/v) DMSO]. Sedimentation coefficient for PbA monomer was determined using a 1 mg/mL PbA solution, prepared in ADE buffer at pH 4.5, by sedimentation velocity. A Beckman Coulter XL-I analytical ultracentrifuge was used for this purpose and studies were conducted at 50,000 RPM.

Fourier-Transform Infrared Spectroscopy

An FTLA Fourier-transform infrared (FTIR) spectrometer (ABB, Zurich, Switzerland) was used to study the change in secondary structure of PbA. Two calcium fluoride windows with a 6 μm spacer were used as the sample cell and FTIR spectra of native PbA in solution and precipitated samples were acquired in the transmission mode. The precipitated samples were analyzed as such without reconstitution. Sixty-four scans, with a resolution of 4 cm^{-1}, were acquired at a scan rate of 21 scans/min and averaged. Background and buffer contributions were subtracted from the spectra prior to second derivative analysis. Subtracted spectra were subjected to second derivative analysis with nine-point Savitzky–Golay smoothing.

Circular Dichroism Studies

Near-UV circular dichroism (CD) studies were employed to study the effect of temperature and sodium chloride on the tertiary structure of PbA. Experiments were conducted using a Jasco 815 spectropolarimeter (Easton, MD, USA). Solution ellipticity was measured at 273 nm as a function of pH between 2.0 and 5.0. A glycine-acetate (10 mM each) buffer was used to provide adequate buffering capacity in this range. Solutions were analyzed in the presence (25 mM) and absence (control) of NaCl at 25°C and 37°C. Samples were equilibrated at the appropriate temperature for 24 h prior to measurement. Studies were conducted at 0.5 mg/mL PbA using a 10 mm path length cell. For all samples, buffer ellipticity was subtracted prior to data analyses.

RESULTS

The initial observation regarding PbA precipitation indicated that the reaction was strongly pH and salt concentration dependent. The peptibody appeared to be adequately soluble in a neutral PBS solution (Fig. 1A) but precipitated rapidly under acidic conditions at pH 4 upon addition of sodium chloride (Fig. 1B). Immediate removal of the salt through dialysis caused the precipitation to be reversed (Fig. 1C).

To understand and elucidate the underlying mechanism governing the insolubility, we probed the effect of pH, salt concentration, starting peptibody concentration, and ion identity on the extent, rate, and reversibility of the precipitation reaction.

Effect of Solution pH, Sodium Chloride, and Protein Concentration on PbA Precipitation

The effect of solution pH and NaCl concentration on the precipitation rate of PbA was studied using a 30 mg/mL PbA solution in 10 mM ADE buffer over a pH range of 3.7 to 4.7 at NaCl concentrations of 50, 100 and 400 mM. The precipitation reaction was monitored for a period of 4-weeks. These studies revealed that both solution pH and sodium chloride played a significant role in modulating PbA precipitation (Fig. 2). The rate and extent of precipitation decreased dramatically with increasing pH. In 100 mM NaCl, the precipitation of PbA was rapid and nearly complete within 24 h at pH 3.7 (Fig. 2A), whereas precipitation at pH 4.0 was nearly complete after 10 days. At pH 4.3, the precipitation rate was even slower and the reaction appeared to plateau with ~45% of the starting PbA amount remaining in solution

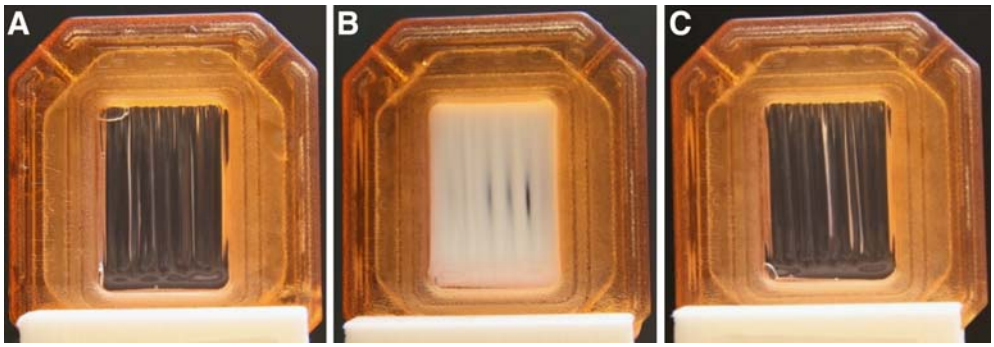

Fig. 1. Precipitation of PbA at acidic pH in the presence of salt. **A** PbA appeared to be adequately soluble in a neutral PBS solution. **B** PbA precipitated rapidly under acidic conditions at pH 4 upon addition of sodium chloride. **C** Immediate removal of the salt through dialysis caused the precipitation to be reversed.

after 4 weeks of incubation. The precipitation rate and extent further decreased at pH 4.7 with only ~5% of PbA precipitating out after 26 days.

Sodium chloride concentration also had a dramatic effect on the extent and rate of PbA precipitation; both increased with NaCl concentration. At pH 4.3, no significant precipitation was observed in the presence of 50 mM NaCl over 4 weeks; however, the precipitation was rapid and complete within 24 h at 400 mM NaCl (Fig. 2B).

The effect of PbA concentration on its precipitation rate was studied over a concentration range of 0.5–30 mg/mL PbA in 10 mM acetate buffer at pH 4.0 (A4) in the presence of NaCl (Fig. 2C). At a fixed NaCl concentration, the precipitation rate was observed to be inversely proportional to the starting PbA concentration, which decreased with increasing protein concentration. In presence of 200 mM NaCl, nearly 100% protein precipitated within 3 days from a 10 mg/mL PbA solution, whereas it took 8 days for >98% of the protein to precipitate from a 30 mg/mL PbA solution.

Effect of Ion Identity on the Precipitation of PbA

The effect of ion identity on the precipitation of PbA (30 mg/mL) was studied at pH 4.5 in the presence of 100 mM neutral salts of monovalent ions. The monovalent anions (F^-, Cl^-, Br^-, I^-, ClO_4^-, and SCN^-) were added as their sodium salts; chloride salts were used for the monovalent cations (Li^+, Na^+, K^+, Rb^+, and Cs^+). The studies revealed that the extent and rate of precipitation was particularly dependent on anion identity (Fig. 3A). The perchlorate and thiocyanate ions were the strongest precipitants and resulted in near complete precipitation within 1 day, whereas the fluoride ion was the weakest precipitant with only 2% protein precipitating in its presence over a period of 24 days. The percent protein remaining in solution after 4 weeks increased in the order $ClO4^-$, SCN^-, $I^-<Br^-<Cl^-<F^-$. There was no significant effect of cation identity on PbA precipitation. Close to 80% (±10%) of protein remained in solution for all the cations after 6 weeks of storage at room temperature (Fig. 3B).

Effect of Salt Concentration on Reduced Valence (z^*)

The z^* of PbA, measured at pH 4.5 was significantly affected by NaCl concentration (Fig. 4). The z^* decreased with salt concentration from +5.2 at 0 mM NaCl to +3.5 at 50 mM NaCl.

Reversibility of the Precipitation Reaction

While the sodium chloride induced precipitation of PbA at pH 4.0 appeared to be completely reversible upon immediate removal of the salt (Fig. 1B & C), the degree of reversibility was dependent on both storage time and temperature (Fig. 5). The amount (percent) of precipitated PbA that could be resolubilized decreased dramatically upon storage at 25°C and 37°C. Only 10% of PbA could be resolubilized after 3 days at 37°C as compared to ~37% at 25°C. By 21 days less than 5% and 1% of PbA could be resolubilized at 25°C and 37°C respectively. The reversibility of the precipitation reaction was excellent for samples stored at 4°C. Nearly 100% of the PbA precipitate could be resolubilized over the 4-week period.

FTIR Spectroscopic Analysis of the PbA Precipitate

To better understand the loss in reversibility of precipitation over time, the structure of PbA in precipitated samples was analyzed by FTIR spectroscopy (Fig. 6). Bands between 1,620–1,640 cm^{-1} were attributed to β-sheet structure and those between 1,650–1,660 cm^{-1} to α-helical structure (16). Anti-parallel β-sheet structure were identified by bands in the 1,670–1,695 cm^{-1} region. However, due to an overlap of bands from β-turn and unordered structure (1,666–1,688 cm^{-1}), accurate analysis of anti-parallel β-sheet structure in the 1,670–1,695 cm^{-1} region is often difficult (17). Formation of irreversible aggregates in proteins is usually correlated with the appearance of characteristic bands in 1,618–1,625 and 1,688–1,693 cm^{-1} regions attributed to the formation of intermolecular β-sheet structure (18,19). The second derivative FTIR spectrum of native PbA in solution showed four characteristic bands at 1,618, 1,642, 1,660, and 1,690 cm^{-1} (Fig. 6). After 4 weeks of storage at 4°C, 25°C, and 37°C, the following changes in the FTIR spectra of precipitated PbA were observed: (a) the shoulder in the major band (1,642 cm^{-1}) for native PbA developed into a separate band around 1,630 cm^{-1} in the precipitated samples, (b) with increasing storage temperature, bands at 1,630 and 1,642 cm^{-1} decreased in intensity, and (c) the band observed in

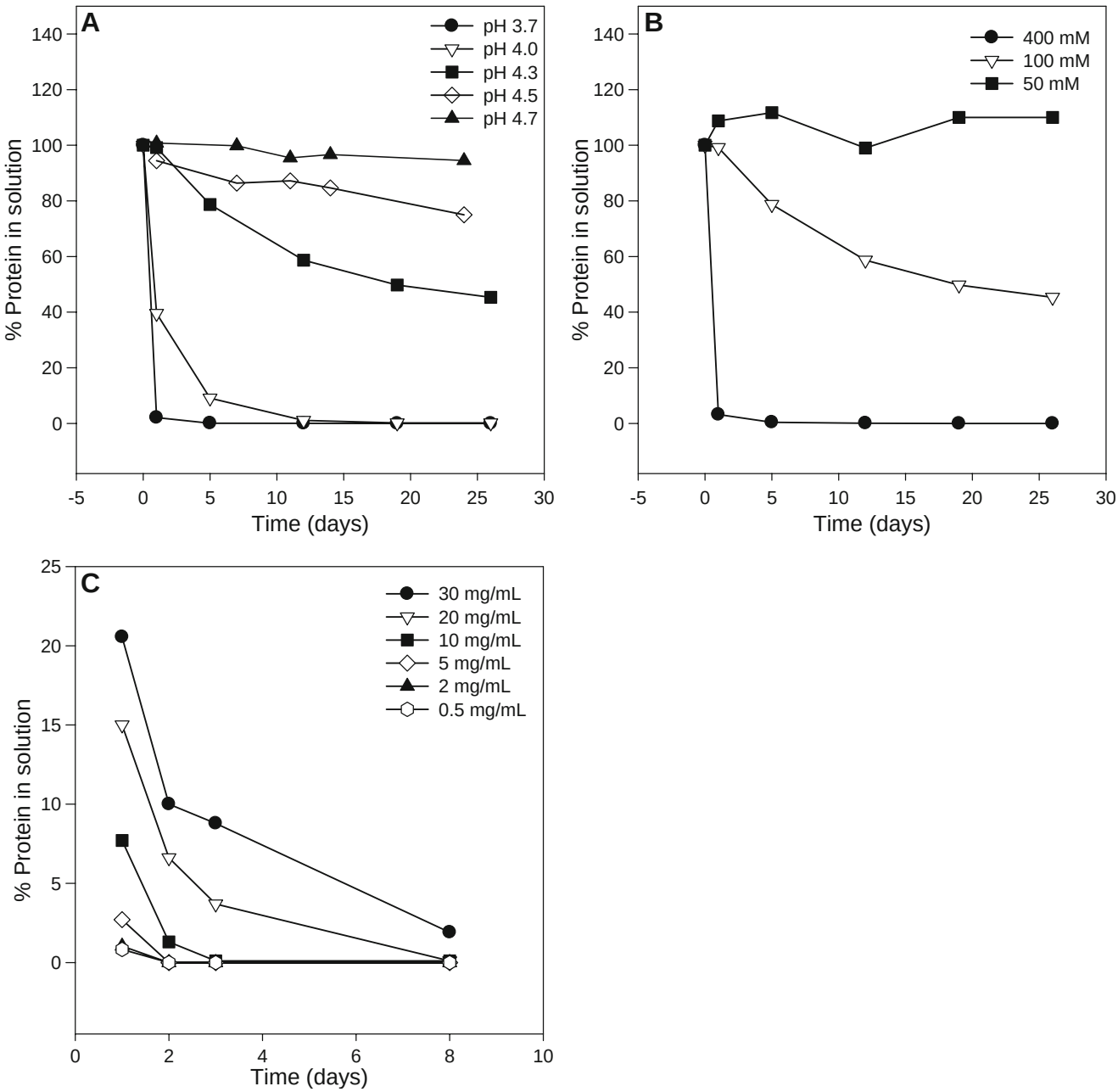

Fig. 2. Precipitation kinetics of PbA as a function of solution pH, ionic strength, and PbA concentration. **A** The rate of PbA precipitation, studied at 30 mg/mL PbA and 100 mM salt, decreased with increasing pH. While nearly 100% of the protein precipitated within 24 h at pH 3.7, only 5% protein precipitated after 26 days at pH 4.7. **B** At a fixed pH (pH 4.3), the rate of precipitation of a 30 mg/mL PbA solution increased with salt concentration. Precipitation was nearly complete in 24 h with 400 mM salt whereas the extent of precipitation appeared to saturate at 45% after 26 days at 100 mM salt. **C** At a fixed solution pH (pH 4.0) and ionic strength (200 mM NaCl) the rate of precipitation was inversely correlated to PbA concentration. The same effect of protein concentration on precipitation rate was observed at other salt concentrations (data not shown).

the FTIR spectrum of native PbA at 1,618 cm^{-1} increased in intensity. A similar trend in the band intensities was observed when spectra at a fixed temperature with increasing storage times were compared (data not shown). The results indicate that the increase in intermolecular β-sheet structure (1,618 cm^{-1}) was concomitant with a loss in native structure (1,630 and 1,642 cm^{-1}) with storage at the higher temperatures of 25°C and 37°C.

Near-UV-CD Studies of PbA

Near-UV-CD studies were performed to monitor the conformational changes in PbA in the pH range of 3.0 to 5.0 as a function of NaCl concentration (25 mM), and temperature (25°C and 37°C) (Fig. 7). In the absence of NaCl, the onset of change in tertiary structure, as measured by an increase in the ellipticity at 273 nm (relative to pH 5.0),

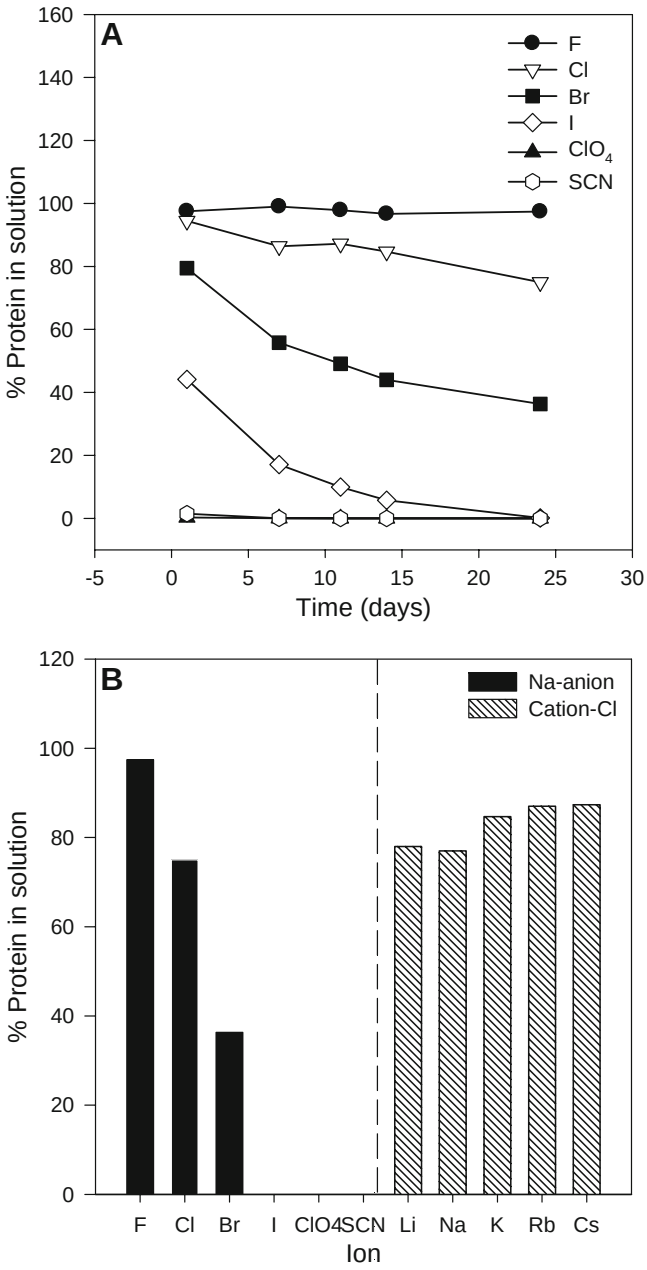

Fig. 3. Precipitation of PbA from a 30 mg/mL solution in the presence of different ions at pH 4.5. **A** At 100 mM anion concentration (Na-anion salts), PbA exhibited the fastest rate of precipitation in the presence of SCN^- and ClO_4^- with ≥99% of the PbA precipitating in 4 weeks followed by I^- (90% precipitation) >Br^-(50%)>Cl^-(13%)>F^- (2%). **B** The extent of precipitation at pH 4.5, after 26 days, followed the same order for anions. However, cation identity (100 mM cation-Cl salts) did not have a significant effect on PbA precipitation.

was observed at pH 3.4. In the presence of 25 mM NaCl, the onset of structural changes appeared to shift to pH 3.8. At 37°C the onset pH further increased to ~4.0–4.5 in the presence of salt.

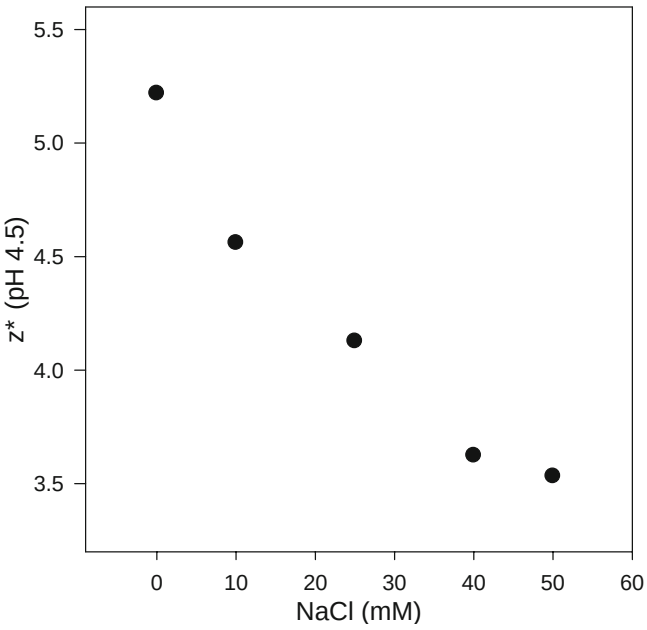

Fig. 4. Effect of salt concentration on z^* of PbA at pH 4.5: The z^* of PbA decreased from +5.2 at 0 mM NaCl to +3.5 at 50 mM NaCl.

DISCUSSION

PbA Precipitation is Mediated by Anion Binding to Basic Side-Chains and Protonation of Acidic Side-Chains

PbA, a basic protein with a p*I* of 8.8, has a net positive charge in acidic solutions (pH 4–5) through its arginyl, lysyl

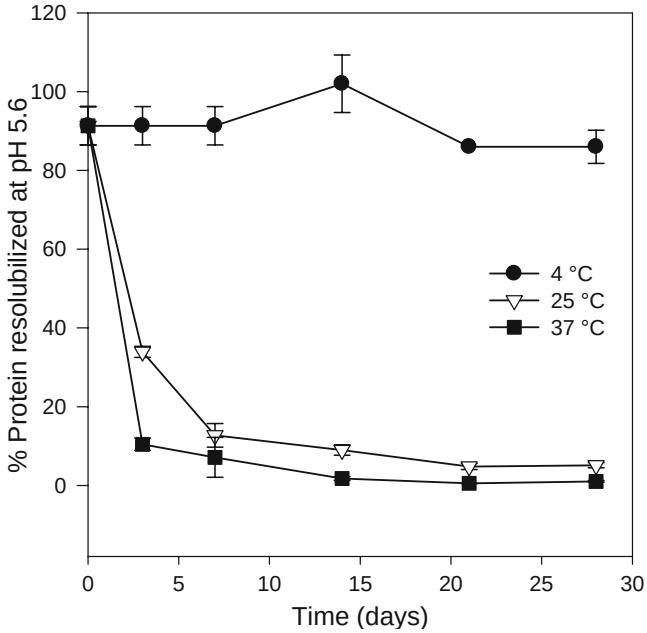

Fig. 5. Reversibility of PbA precipitate generated in 20 mg/mL PbA solution at pH 4.0 with 240 mM sodium chloride. Nearly all the precipitated protein could be resolubilized in 10 mM acetate buffer at pH 5.6 after storage at 4°C for 4 weeks. However, irreversibility was observed for samples stored at 25°C and 37°C for the same time period.

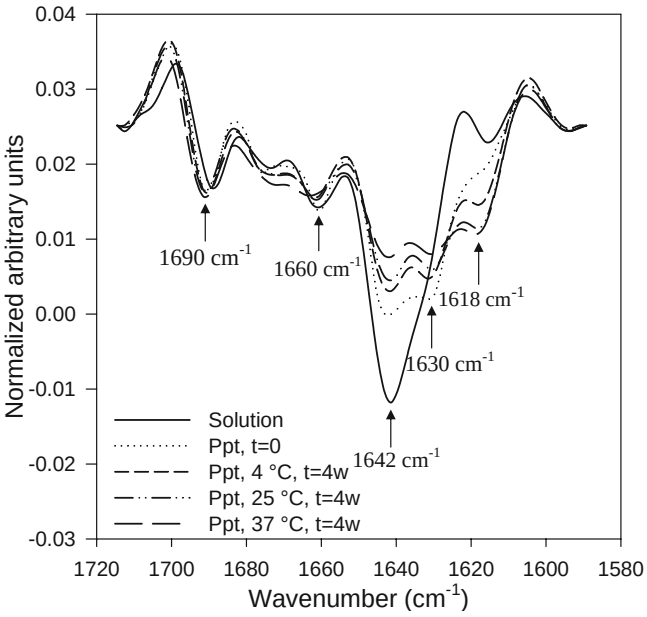

Fig. 6. Effect of temperature on secondary structure of precipitated PbA as studied by FTIR spectroscopy. A 20 mg/mL solution of PbA was precipitated with 240 mM NaCl and stored at 4°C, 25°C and 37°C for 4 weeks. The main band observed at 1,642 cm^{-1} for the native PbA in solution split into two bands in the precipitated samples (t=0) and decreased in intensity with temperature (t=4w samples). The band at 1,618 cm^{-1} increased in intensity with temperature indicating the growth of intermolecular β-sheet structure in the precipitated samples.

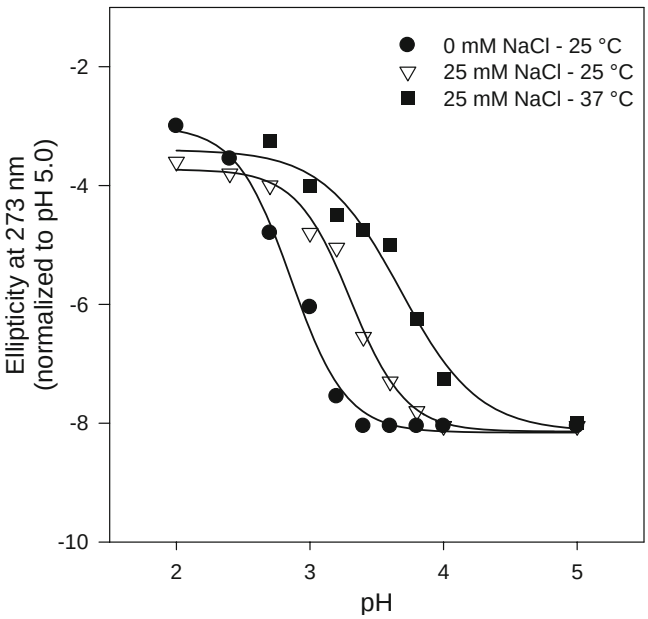

Fig. 7. Effect of salt on the tertiary structure change for PbA monitored in the solution state by CD spectroscopy. Salt destabilized the conformation of PbA in a temperature dependent manner. In the absence of NaCl, the onset of tertiary structure transition was observed at pH 3.4. In the presence of 25 mM NaCl, transition onset shifted to pH 3.8 at 25°C and to pH~4.0–4.5 at 37°C.

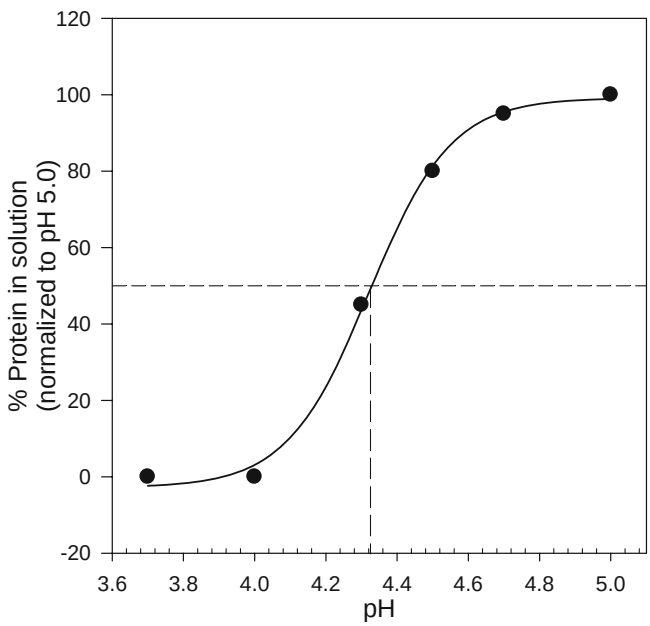

Fig. 8. Effect of solution pH on the extent of PbA precipitation (30 mg/mL initial PbA concentration) in100 mM NaCl solutions. Percent PbA remaining in solution after 26 days decreased with decreasing solution pH and paralleled the pH-titration of a weak carboxylic acid with a pK_a value ~4.3.

and histidinyl residues, and thus is expected to possess high aqueous solubility under these conditions. The precipitation of PbA, at pH 4.0, occurred only upon salt addition. Further, ion identity, ionic strength, and pH had profound effects on the precipitation reaction suggesting charge neutralization was important in mediating precipitation. We propose that PbA precipitation is modulated by the coupling of two effects: (a) anion binding to basic side-chains, and (b) protonation of acidic side-chains with decreasing solution pH. In combination both effects result in the formation of net-neutral PbA molecules, which can aggregate and lead to precipitation.

The responsiveness of PbA precipitation to anion identity relative to cation identity (Fig. 3) suggested anion binding was critical for precipitation. Larger anions, with lower charge densities, are expected to interact more favorably with the positively charged ammonium, guanidinium, and imidazolium groups on the protein surface as compared to smaller anions of higher charge density (20). This is because the degree of hydration decreases with ion charge density or increasing ion size in the order $F^->Cl^-> Br^->I^->SCN^-\sim ClO_4^-$. The fluoride ion, the smallest and consequently the most hydrated anion in the series, is expected to interact favorably with water molecules as compared to the positively charged groups on the protein surface. Consistent with the ion size and anion binding hypothesis, the rate and extent of precipitation at pH 4.5 and 100 mM sodium salt concentration could be ranked in the order $SCN^-\sim ClO_4^->I^->Br^->Cl^->F^-$ (Fig. 3). A direct reflection of anion binding to PbA can be observed in the reduction of its z^* (measured at pH 4.5) with increased sodium chloride concentration (Fig. 4). By 50 mM NaCl, the reduced valence of PbA decreased to +3.5 from +5.2 at pH 4.5 and was expected to reduce further at higher salt concentration.

The rate and extent of PbA precipitation increased dramatically with decreasing solution pH (from pH 5.0 to 3.7) suggesting protonation (or neutralization) of glutamyl and aspartyl side-chains may be important for precipitation. This is supported by the precipitation experiments conducted with 100 mM NaCl (Fig. 2A). While no significant precipitation was observed at pH 5.0 (data not shown) in 100 mM NaCl, it was measurable below pH 4.7 with a sharp increase in both the extent and rate observed below pH 4.3. The composite acidic-residue pK_a of PbA (pK_{Na}), calculated using all its constituent aspartic (n=48, pK_a=3.9) and glutamic acids (64, 4.3) and the C-termini (2, 3.7) is ~4.2. It then stands to reason that the *average* protonation of the acidic residues of PbA can be modeled by a monoprotic weak acid with a pK_a of ~4.2. The effect of solution pH on the extent of PbA precipitation can be better appreciated by plotting the 26 day values for percent PbA remaining in solution from Fig. 2A against solution pH (Fig. 8). Interestingly, the extent of precipitation *vs.* pH curve parallels the pH-titration of a weak carboxylic acid with a pK_a value ~4.3 indicating that the extent of precipitation correlates well with the protonation of the PbA acidic side-chains with decreasing pH. It is important to note that protonation of acidic residues alone is insufficient to induce PbA precipitation. In the absence of salt, the PbA molecules have a significant net positive charge that prevents aggregation. With the addition of sufficient salt, anion binding leads to a significant reduction in the reduced valence of PbA, and under these conditions, PbA can aggregate and precipitate.

An important point that deserves discussion is the effectiveness of anions in causing PbA precipitation in relation to their position in the Hofmeister series. The anions can be arranged in the order $F^- < Cl^- < Br^- < I^- < ClO_4^- \sim SCN^-$, with F^- being kosmotropic (or the least chaotropic) to SCN^- and ClO_4^-, which possess a strong chaotropic character. The chaotropic ions are considered to be 'salting-in' ions based on their ability to increase protein solubility while the kosmotropic ions are considered to be 'salting-out' from their ability to precipitate proteins. PbA precipitation displayed a reverse Hofmeister effect; the salting-in anions (SCN^-, ClO_4^-) were the most effective in precipitating PbA while the salting-out ions appeared to be the least effective. The salt-induced insolubility observed in PbA is similar to that observed in the case of lysozyme, another basic (pI=9.4) protein, wherein precipitation in acidic solutions with salt addition was attributed to be a result of anion binding (21).

Reversibility & Kinetics of PbA Precipitation

Reversibility of the salt-induced PbA precipitation at pH 4.0 was dependent on the storage temperature of the suspended PbA precipitate. While precipitation could be completely reversed in samples stored at 4°C over a 4-week period, the percent reversibility dropped rapidly for samples stored at the higher temperatures of 25°C and 37°C (Fig. 5). The loss in the reversibility of PbA precipitation indicated that irreversible structural changes in PbA were being induced by the combination of salt and high storage temperature. The FTIR and CD studies provided supporting evidence to this hypothesis. In FTIR studies (Fig. 6), the maximal decrease in native structure content (splitting and decrease in band at 1,642 cm^{-1}) along with a concomitant increase in intermolecular β-sheet structure (increase in band at 1,618 cm^{-1}), attributable to irreversible aggregates, was observed for samples stored at 25°C and 37°C. After 4 weeks, the 4°C samples also exhibited a loss in native structure, indicating that loss in reversibility of PbA precipitation at 4°C could be expected at longer storage times (greater than 4-weeks). The CD results indicated that in addition to altering the solubility of PbA, salt destabilized the protein below pH 4.0 as evident from the pH-unfolding isotherms at 25°C and 37°C (Fig. 7). The destabilizing effect of salt on PbA conformation may result in the loss of reversibility of the precipitation reaction.

An interesting aspect of the precipitation reaction was the strong dependence on ion identity and its inverse dependence on protein concentration. The rate dependence on ion identity can be rationalized on the basis of anion binding. The larger anions (of lower charge density) which preferentially pair with the positive residues on the protein surface can form intermolecular linkages and increase protein aggregation (22). The apparent inverse dependence of the precipitation rate on starting (t=0) PbA concentration may suggest that the ion concentration & the surface area for particle growth may be rate limiting at higher PbA concentrations.

Implications for the Formulation Development of Basic Proteins

Proteins are subject to a variety of covalent degradation reactions (*viz.* deamidation, isomerization, hydrolysis, covalent aggregation), and non-covalent degradation processes that include surface adsorption and irreversible aggregation. The pH dependence of covalent degradation reactions in proteins has been well-documented in the literature, and in general, tend to be minimal in the pH range of 4.0–6.5 (23, 24). The non-covalent stability of a protein has a more complex, protein specific, pH dependence that can be correlated to the effect of pH on the conformational and colloidal stability of the protein (25,26). Thus, proteins displaying adequate physical stability in the pH 4.0–6.5 range present a greater chance to be formulated as liquid dosage forms. A significant number of therapeutic proteins currently under development in the pharmaceutical industry, such as monoclonal antibodies, possess basic pIs and are often formulated under mildly acidic (pH 4.0–6.5) conditions to minimize covalent degradation rates. In the case of such proteins, the addition of even small quantities of salts as buffering, tonicifying, or viscosity modifying agents under acidic conditions may play a significant role in modulating solubility. Interestingly, the use of sodium chloride and in particular chaotropic salts has been proposed to reduce the viscosity of high-concentration mAb formulations (27). However, the PbA solubility results reported here along with those of lysozyme (21) suggest that both ionic strength and the chaotropic nature of anions may adversely impact the solubility of basic proteins in acidic solutions.

REFERENCES

1. E. Koren, L. A. Zuckerman, and A. R. Mire-Sluis. Immune responses to therapeutic proteins in humans—clinical signifi-

cance, assessment and prediction. *Curr. Pharm. Biotechnol.* **3**:349–360 (2002).
2. E. D. Lobo, R. J. Hansen, and J. P. Balthasar. Antibody pharmacokinetics and pharmacodynamics. *J. Pharm. Sci.* **93**:2645–2668 (2004).
3. A. Braun, L. Kwee, M. A. Labow, and J. Alsenz. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-a) in normal and transhenic mice. *Pharm. Res.* **14**:1472–1478 (1997).
4. A. S. Rosenberg. Effects of protein aggregates: An immunologic perspective. *AAPS Journal.* **8**:Article 59: (http://www.aaps.org) (2006).
5. H. C. Mahler, R. Muller, W. Friess, A. Delille, and S. Matheus. Induction and analysis of aggregates in a liquid IgG1-antibody formulation. *Eur. J. Pharm. Biopharm.* **59**:407–417 (2005).
6. B. Dani, R. Platz, and S. T. Tzannis. High concentration formulation feasibility of human immunoglubulin G for subcutaneous administration. *J. Pharm. Sci.* **96**:1504–1517 (2007).
7. R. J. Harris, S. J. Shire, and C. Winter. Commercial manufacturing scale formulation and analytical characterization of therapeutic recombinant antibodies. *Drug Dev. Res.* **61**:137–154 (2004).
8. K. L. Shaw, G. R. Grimsley, G. I. Yakovlev, A. A. Makarov, and C. N. Pace. The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. *Protein Sci.* **10**:1206–1215 (2001).
9. S. R. Trevino, J. M. Scholtz, and C. N. Pace. Amino acid contribution to protein solubility: Asp, Glu, and Ser contribute more favorably than the other hydrophilic amino acids in RNase Sa. *J. Mol. Biol.* **366**:449–460 (2007).
10. J. Liu, M. D. H. Nguyen, J. D. Andya, and S. J. Shire. Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. *J. Pharm. Sci.* **94**:1928–1940 (2005).
11. W. A. Eaton, and J. Hofrichter. Sickle cell hemoglobin polymerization. *Adv. Protein Chem.* **40**:63–279 (1990).
12. B. L. Chen, X. Wu, S. J. Babuka, and M. Hora. Solubility of recombinant human tissue factor pathway inhibitor. *J. Pharm. Sci.* **88**:881–888 (1999).
13. A. C. Herman, T. C. Boone, and H. S. Lu. Characterization, formulation, and stability of Neupogen (Filgrastim), a recombinant human granulocyte-colony stimulating factor. *Pharm. Biotechnol.* **9**:303–328 (1996).
14. B. A. Kerwin, B. S. Chang, C. V. Gegg, M. Gonnelli, T. Li, and G. B. Strambini. Interactions between PEG and type I soluble tumor necrosis factor receptor: modulation by pH and by PEGylation at the N terminus. *Protein Sci.* **11**:1825–1833 (2002).
15. J. A. Durant, C. Chen, T. M. Laue, T. P. Moody, and S. A. Allison. Use of T4 lysozyme charge mutants to examine electrophoretic models. *Biophys. Chem.* **101–102**:593–609 (2002).
16. A. Dong, P. Huang, and W. S. Caughey. Protein secondary structures in water from second-derivative amide I infrared spectra. *Biochemistry.* **29**:3303–3308 (1990).
17. P. I. Haris, and F. Severcan. FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *J. Mol. Catal., B Enzym.* **7**:207–221 (1999).
18. A. Dong, B. Kendrick, L. Kreilgard, J. Matsuura, M. C. Manning, and J. F. Carpenter. Spectroscopic study of secondary structure and thermal denaturation of recombinant human factor XIII in aqueous solution. *Arch. Biochem. Biophys.* **347**:213–220 (1997).
19. M. van de Weert, P. I. Haris, W. E. Hennink, and D. J. Crommelin. Fourier transform infrared spectrometric analysis of protein conformation: effect of sampling method and stress factors. *Anal. Biochem.* **297**:160–169 (2001).
20. K. D. Collins. Charge density-dependent strength of hydration and biological structure. *Biophys. J.* **72**:65–76 (1997).
21. P. Retailleau, M. Ries-Kautt, and A. Ducruix. No salting-in of lysozyme chloride observed at low ionic strength over a large range of pH. *Biophys. J.* **73**:2156–2163 (1997).
22. K. D. Collins. Ions from the Hofmeister series and osmolytes: effects on proteins in solution and in the crystallization process. *Methods.* **34**:300–311 (2004).
23. N. P. Bhatt, K. Patel, and R. T. Borchardt. Chemical pathways of peptide degradation. I. Deamidation of adrenocorticotropic hormone. *Pharm. Res.* **7**:593–599 (1990).
24. C. Oliyai, and R. T. Borchardt. Chemical pathways of peptide degradation. VI. Effect of the primary sequence on the pathways of degradation of aspartyl residues in model hexapeptides. *Pharm. Res.* **11**:751–758 (1994).
25. E. Y. Chi, S. Krishnan, T. W. Randolph, and J. F. Carpenter. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. *Pharm. Res.* **20**:1325–1336 (2003).
26. W. Wang. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* **185**:129–188 (1999).
27. S. Kanai, J. Liu, T. W. Patapoff, and S. J. Shire. Reversible self-association of a concentrated monoclonal antibody solution mediated by Fab–Fab interaction that impacts solution viscosity. *J. Pharm. Sci.* **94**:1928–1940 (2008).