Protein–Polyelectrolyte Phase Boundaries

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The separation of proteins by polyelectrolyte coacervation or precipitation is based on electrostatically-driven complex formation. We have investigated complexation between the globular protein BSA and the polyelectrolyte poly(dimethyldiallylammonium chloride) (PDMDAAC) using light-scattering techniques to monitor solution turbidity. Turbidimetric pH titrations were used to determine the specific pH values where soluble complex formation is initiated (pH_c) and where phase separation occurs (pH_ϕ). These values, collected at different ionic strengths, can be presented as phase boundaries. The effects of macromolecular concentration, protein:polymer concentration ratio (r), and polymer molecular weight upon the phase boundary are examined. The macromolecular concentration and polymer molecular weight have little or no effect on the phase boundary. While pH_c is independent of r, pH_ϕ varies inversely with r. The use of phase boundaries in the selection of optimal pH and ionic strength for maximum yield and purity in protein separations is discussed.

Introduction

Current methods of protein purification involve an extensive series of steps and processes that increase the cost of the final product. New techniques for large-scale protein separation are therefore of interest. One of these involves the addition of polyelectrolytes, leading to selective protein phase separation (Sternberg, 1976; Bozzano et al., 1991). This phase separation is a result of the electrostatic interactions between the protein and the polyelectrolyte which, at very low ionic strength, results in tight ion-pairing and the formation of amorphous precipitates (Nguyen, 1986; Sternburg and Hershberger, 1974; Kokufuta et al., 1981). At higher ionic strengths, however, the charges on both the protein and the polyelectrolyte are shielded by counterions. Because of these shielding effects, solvent molecules are free to permeate the complex aggregates, resulting in the formation of a second liquid phase or coacervate (Burgess and Carless, 1984; Lenk and Thies, 1987; Dubin et al., 1987; Veis, 1991). Since both coacervation and precipitation concentrate the target protein, the use of polyelectrolytes for protein purification carries this additional benefit.

The theory of polyelectrolyte coacervation was first addressed by Voorn and Overbeek (Overbeek and Voorn, 1957; Voorn, 1956, 1959) and by Veis and Aranyi (1960). According to Overbeek et al., complex coacervation reflects a competition between the favorable electrical free energy, due to the attraction of oppositely charged particles, and the unfavorable entropy of mixing, which would tend to disperse the particles. Short-range electrostatic interactions are neglected, and the possibility of complexation between particles with the same charge sign is excluded. On the basis of work with gelatins of different isoionic points, Veis et al. suggested a modification to the Voorn–Overbeek theory, central to which is the formatioin of soluble complexes prior to coacervation. Phase separation is a result of the aggregation of these nearly neutral polyions. Recent electrophoretic and dynamic light-scattering studies by Dubin et al have verified the existence of such soluble complexes for protein–polyelectrolyte systems (Dubin and Murrell, 1988; Xia et al., 1993). These "primary" soluble complexes were on the same order of size as the free

polyelectrolyte and displayed a pH-dependent mobility that decreased to zero at the point of coacervation. Park et al. found that formation of soluble protein–polyelectrolyte complex was initiated at a specific pH and that this "pH_c" was a function of the ionic strength, the protein isoelectric point, and the charge density of the polyelectrolyte (Park et al., 1992). For polycations, pH_c preceded the pH of visual phase separation, designated as pH_ϕ. Since these soluble complexes can be considered a separate phase, the ionic strength dependences of both pH_c and pH_ϕ can be viewed as phase boundaries.

In applying polyelectrolytes to selective protein separations, it is important to consider both phase transitions. The phase boundaries of different proteins can be utilized for systems containing several proteins, as a means of selecting the optimal pH and ionic strength for maximum yield and purity. An example of this is shown in Figure 1, which displays hypothetical phase boundaries for a two-protein single-polyelectrolyte solution. In this figure, the broken lines represent the transition to soluble complex (pH_c), and the solid lines represent the transition to coacervate (pH_ϕ). In region A, neither protein is associated with polyelectrolyte; in region B, protein 1 has formed soluble complexes and protein 2 is nonassociated; in region C, protein 1 is coacervated while protein 2 is still unassociated; in region D, protein 2 has formed a soluble complex; in region E, both proteins are coacervated. The optimal conditions for selective protein separation are those corresponding to region C.

In this study, the ionic strength and concentration dependences of pH_c and pH_ϕ were obtained for the BSA/PDMDAAC system. These phase boundaries are discussed in terms of the nature of protein–polyelectrolyte interactions. Their utility in maximizing yield and purity in protein separation processes is considered.

Experimental Section

Materials. A commercial sample of poly(dimethyldiallylammonium chloride) (PDMDAAC), trade name "Merquat 100", was from Calgon (Pittsburgh, PA). The nominal molecular weight was 2.5×10^5, and the reported polydispersity was $M_w/M_n \approx 10$. Three PDMDAAC fractions with molecular weights of 22K, 790K, and 1250K, and polydispersities of less than 1.2
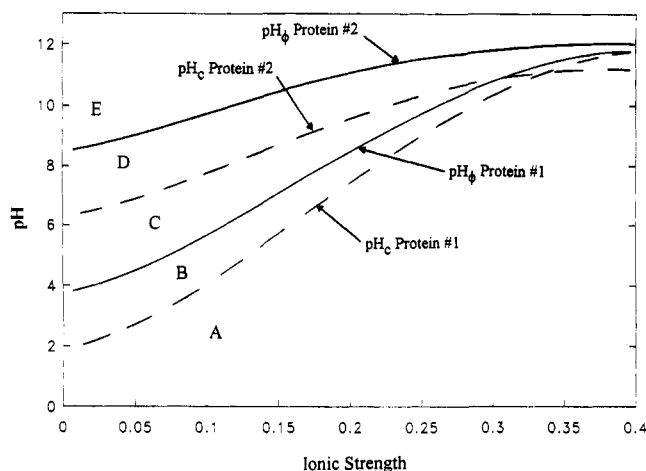
Figure 1. Schematic representation of two protein—polyelectrolyte phase boundaries and the different regions available for protein separation. Dashed lines correspond to pH_c and solid lines to pH_ϕ.

were prepared by preparative SEC and characterized by several methods (Xia et al., 1994). Bovine serum albumin (BSA), with a molecular weight of 67 000 and a pI of 4.9, was received from Sigma Chemical Co. as 95—99% pure lyophilized protein and was used without further purification.

Instruments. An Orion pH meter with a combination electrode was used to monitor the solution pH. Turbidities were measured in four ways. Transmittance was monitored with either a Brinkman PC 800 colorimeter, connected to a 2 cm path length optical probe, or a Perkin-Elmer HP8450A UV—vis spectrometer. The turbidity was reported as 100 − %T, and %T fluctuations (±0.1 %) were treated by consistently selecting the highest value. The mean intensity of 90° scattered light was measured with a 200 mW argon laser-equipped Brookhaven Instrument (Model No. BI-AT) and a Brice Phoenix Series 2000 light scattering photometer.

Turbidimetric Titrations. The dependence of solution turbidity on pH ("type 1 titration") was obtained by the addition of 0.1 M NaOH to a protein—polymer mixture at constant ionic strength (I) and at constant polymer and protein concentrations. BSA and PDMDAAC solutions were prepared independently and filtered through Gelman 0.2 μm filters prior to mixing. Upon addition of base, the solution was gently stirred until a stable %T (±0.1) reading was obtained. The stirring time was generally 2—3 min, and kinetic reversibility (with the addition of acid) was always observed. A nitrogen purge was employed during all titrations. Polymer-free blanks were used to eliminate the effect of the scattering of free protein. The possibility of gradual protein adsorption onto the probe tips was minimized by limiting the titration time, including blanks, to approximately 1 h (±10 min).

Macromolecular Charge. The net charge of BSA at various I and pH values, was determined by titrating a 25 mL polymer-free protein solution from the pI, either with 0.1 M HCl or with 0.1 M NaOH, using a 2.0 mL Gilmont microburet accurate to 0.001 mL. A solvent blank was used to determine the direct contribution of HCl or NaOH to the solution pH. By subtracting the volume of acid or base used in the blank from the volume of acid or base used in the polymer-free sample, the specific number of H$^+$ or OH$^-$ ions contributing to the net protein charge was determined. pH titration curves for BSA with and without polymer were generated by lowering the solution pH to 4.2 wth 0.1 M HCl and then
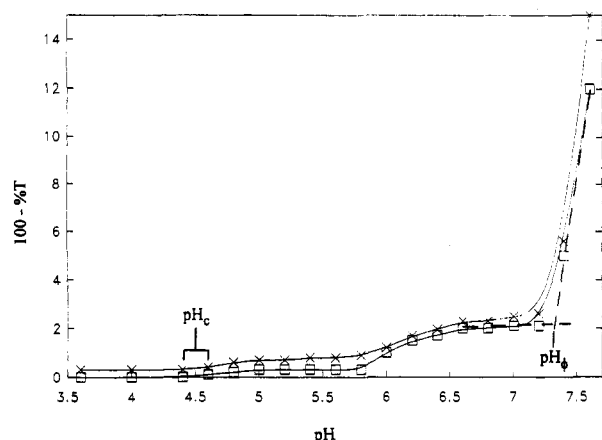

Figure 2. Typical type 1 titrations for [BSA] = 0.6 g/L, [PDMDAAC] = 0.12 g/L, in 0.1 M NaCl: colorimeter (□); UV (×).

titrating to pH 9.6 with 0.025 M NaOH. Care was taken to ensure that all solutions had weights that were equal to within 1 mg prior to titration. With such precautions, titration curves were reproducible to ±0.03 pH units.

Results and Discussion

Figure 2 shows the results of a "type 1 titration" monitored with a UV spectrometer and a colorimeter. A region of soluble complex formation is revealed by the increase in turbidity between the pH values of 4.6 and 7.4, which although subtle, is quite reproducible. At pH values less than 4.6 (pH_c), Coulombic repulsive forces between the positively charged protein and the positively charged polyelectrolyte prevent the formation of complexes, and the protein and polymer molecules coexist as separate entities within the solution. At pH values greater than 7.4 (pH_ϕ), a substantial increase in turbidity indicates the presence of coacervate. While pH_ϕ can be easily determined, pH_c is less exactly identified as the point at which the turbidity or scattering intensity departs from a constant value. Because of the limited sensitivity of transmittance measurements, determination of an exact pH_c value by UV absorbance is sometimes difficult. This problem can be circumvented, however, by utilizing more direct measures of particle scattering. This is demonstrated by Figure 3 which shows the same type 1 titration monitored with the Brice Phoenix and Brookhaven instruments. Because of the weak scattering of free polymer and free protein, the abrupt deviation in the total scattering intensity at pH_c can only be attributed to the presence of soluble complex. The use of these instruments entails an increase in sample preparation and run time arising from instrument sensitivity to dust and foreign particle events. Experimental time was minimized, however, by using turbidity to estimate pH_c and then total scattering intensity to determine pH_c precisely.

The effects of macromolecular concentration on pH_c and pH_ϕ can be seen in Figures 4 and 5. Figure 4 shows type 1 titrations for three different protein concentrations, with the protein to polymer concentration ratio ([BSA]/[PDMDAAC] = r) held constant. The results indicate that, within the present concentration range, the total solute concentration, at constant r, has no effect on either pH_c or pH_ϕ. Figure 5 further indicates that pH_c is independent of r as well, while pH_ϕ varies inversely with r.

The finding that pH_ϕ is inversely dependent on r can be explained if coacervation results from charge neutral-
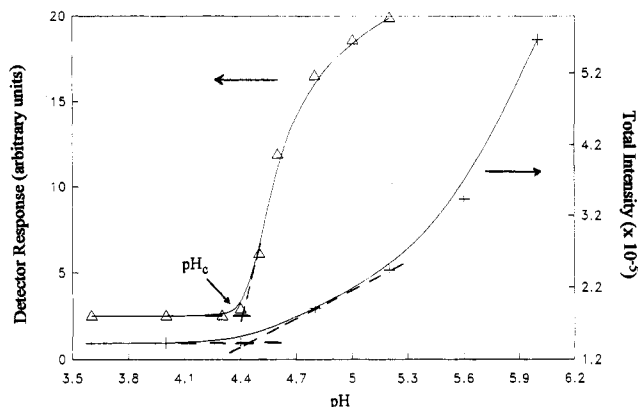
Figure 3. Verification of pH$_c$ for [BSA] = 0.6 g/L, [PDMDAAC] = 0.12 g/L, in 0.1 M NaCl: Brookhaven BI-AT (+); Brice Phoenix 2000 ($\triangle$).

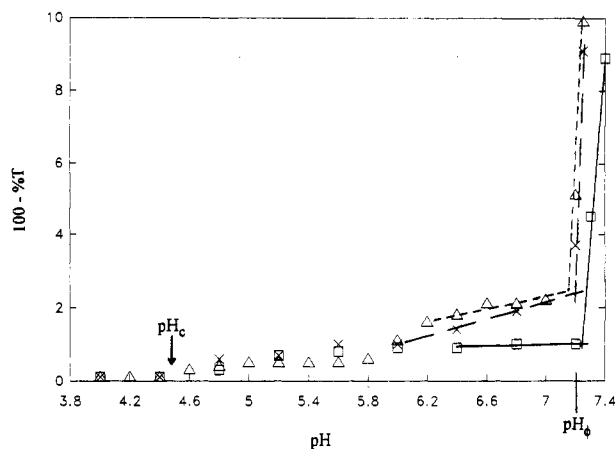**Figure 4.** Effect of total concentration on turbidimetric titrations at $r = 5$ and $I = 0.1$ M NaCl: [BSA] (g/L) = 0.12 ($\square$); 0.6 ($\triangle$); 3.0 ($\times$).
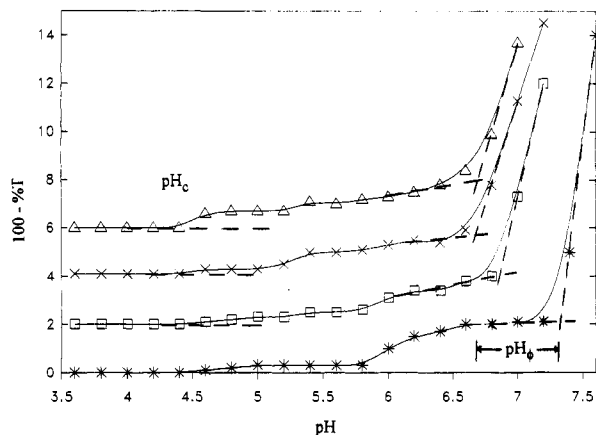
Figure 5. Effect of r on pH$_c$ and pH$_\phi$ at [BSA] = 0.6 g/L and I = 0.1 M NaCl: $r = 5$ (*); 20 ($\square$); 100 ($\times$); 200 ($\triangle$). Curves are offset for clarity. Horizontal arrow shows range of pH$_\phi$ values.

ization of the primary complex as given by eq 1, where

$$Z_T = Z_P + nZ_{pr} \qquad (Z_T)_\phi = 0 \qquad (1)$$

Z_P is the net polymer charge (independent of pH), n is the mean number of bound proteins per polymer chain, Z_T is the charge of the primary complex, and Z_{pr} is the pH-dependent protein charge (Strege *et al.*, 1990). If protein binding at pH $>$ pH$_c$ follows a mass-action equilibrium, it can be assumed that n will increase with r. The assumption that n increases with r implies that,

after complexation is initiated at pH$_c$, an equilibrium exists similar to the one schematically depicted below, where the solid ovals represent proteins and the line represents a single polymer chain.

$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad (2)$$
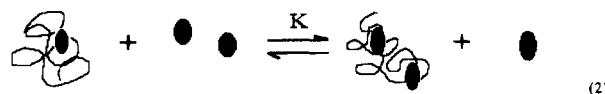
When n increases, the charge per protein molecule required to achieve complex neutralization decreases, which results in a subsequent decrease in pH$_\phi$. It should be pointed out that these simple descriptions neglect the possibility of cooperative binding, leading to a nonuniform distribution of BSA among the polymer chains. There appears to be at least circumstantial evidence for such cooperative binding (Li *et al.*, 1995; Kabanov and Mustafaev, 1981).

The absence of any effect of protein or polymer concentration on pH$_c$ is important for two reasons. First, it greately reduces the number of variables, inasmuch as pH$_c$ is then a function only of the ionic strength. Second, it suggests that *initial* complex formation is controlled by the interaction between a single protein molecule and a single sequence of polymer segments and does not follow a mass-action law. The apparent contradiction between this statement and the mass-action equilibrium of eq 2 can be explained if the binding constant (K) is a strong function of protein charge density (σ_{pr}). The electrostatic nature of the binding constant is supported by Park *et al.*, who found that both pH$_c$ and pH$_\phi$ were dependent upon the linear charge density of the polyelectrolyte (Park *et al.*, 1992). These findings indicate that the binding constant is a function of protein surface charge density and polyelectrolyte linear charge density. Prior to pH$_c$, σ_{pr} is too small to initiate complexation. At pH $>$ pH$_c$, however, σ_{pr} has increased enough for the mass action effects to be observed, i.e. $n = f(r)$.

On the basis of electrostatic shielding effects, one would expect pH$_c$ and pH$_\phi$ to increase with increasing ionic strength. The expected qualitative dependence of these values on I is seen in Figure 6, which clearly indicates that, as the ionic strength is increased, pH$_c$ and pH$_\phi$ also increase.

The effect of polyelectrolyte molecular weight on pH$_c$ and pH$_\phi$ is shown in Figure 7, which contains type 1 titrations for PDMDAAC fractions with molecular weights 22K, 780K, and 1250K at I = 0.10 M NaCl. The constant pH$_c$ and pH$_\phi$ values shown, 4.4 and 6.8, respectively, are in good agreement with the values obtained for the polydisperse Merquat sample (4.4 and 6.9). These results show that neither the polymer MW nor its polydispersity have any effect on pH$_c$ or pH$_\phi$. This result is consistent with the preceding description of the interaction in terms of a relatively short sequence of polymer segments.

The dependence of pH$_\phi$ on polymer molecular weight was also examined by Sheih and Glatz (1994) for PAA−lysozyme and PAA−ovalbumin complexes. Within the weight range of 5000−500 000, pH$_\phi$ was reported to be independent of polymer molecular weight, although some deviation in pH$_\phi$ was observed at MW = 4 000 000. This effect might result from self-aggregation of larger colloids. However, differences between Glatz's system and ours preclude more direct comparisons.

The dependence of pH$_c$ and pH$_\phi$ on ionic strength constitutes a phase boundary which, as shown in Figure 8, separates the nonassociative, soluble complex, and coacervate regions. The results for titrations carried out at different r values confirm the insensitivity of pH$_c$ to r at different I.
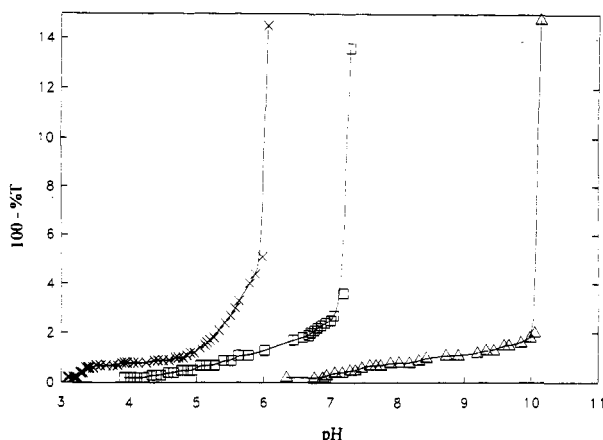
Figure 6. Dependence of pH_c and pH_ϕ on ionic strength (I) at $r = 5$ and [BSA] = 0.6 g/L: $I = 0.02$ (×); 0.08 (□); 0.2 (△) M NaCl.
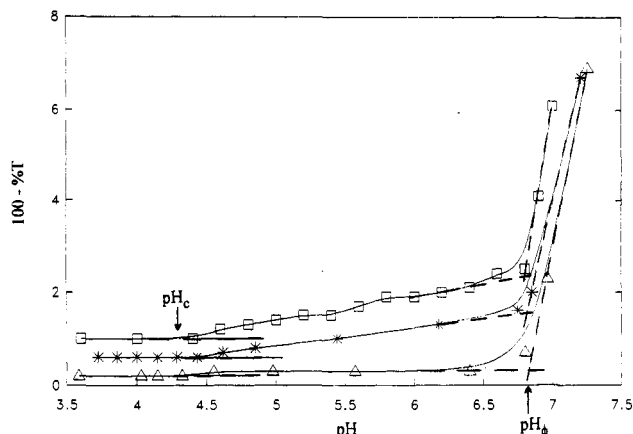

Figure 7. Effect of polymer molecular weight on pH_c and pH_ϕ at [BSA] = 0.6 g/L, $r = 5$, and $I = 0.1$ M NaCl: molecular weight = 22K (△); 790K (*); 1250K (□).

Figure 8. BSA/PDMDAAC phase boundaries, pH_c (lower); pH_ϕ (upper) at [BSA] = 0.6 g/L: $r = 5$ (+); 10 (*); 20 (□); 40 (×). Since pH_c is independent of r, only one symbol is used for pH_c.

Figure 9. Data of Figure 8 presented as $(Z_{pr})_c$ (upper curve) and $(Z_{pr})_\phi$ (lower curve) vs $I^{1/2}$. Since pH_c is independent of r, only one symbol is used for $(Z_{pr})_c$.

In Figure 9, the data of Figure 8 are plotted as Z_{pr} (net protein charge) versus $I^{1/2}$, corresponding to both pH_c (upper curve) and pH_ϕ (lower curve). We first note the linearity of the upper $(Z_{pr})_c$ boundary. $I^{1/2}$ dependence is characteristic of systems controlled by Coulombic forces, and this observation confirms the supposition that binding is driven by electrostatic interactions between the protein and the polyelectrolyte. Thus, it can be proposed that the degree of shielding or the thickness of the ionic atmosphere has an important effect on the value of $(Z_{pr})_c$.

The linear dependence of $(Z_{pr})_c$ on $I^{1/2}$ resembles the results of McQuigg et al. (1992), who studied polyelectrolytes and oppositely charged micelles and observed a linear dependence of micelle surface charge density (σ) on $I^{1/2}$ at the point of incipient polyelectrolyte binding. Theoretical justifications for the linearity of σ_{crit} with $I^{1/2}$ were first reported by Evers et al. for the binding of polyions to planar charged surfaces (Evers et al., 1986). For the same systems, Muthukumar (1987) found a more complex relation between σ_{crit} and I. On the other hand, Odijk (1980) also reached the conclusion that σ_{crit} was proportional to $I^{1/2}$ from a completely different approach. While the treatments of Evers, Muthukumar, and Odijk are for polyelectrolytes and oppositely charged planar surfaces, Manning et al. recently considered polyelectrolytes and spherical colloids (Manning, 1994). They defined the critical binding energy as the sum of a favorable ionic attraction energy and an unfavorable elastic stress arising from the bending of the polymer around the colloid. Combining the ionic attraction energy (from the Debye–Huckel approximation, with no coun-
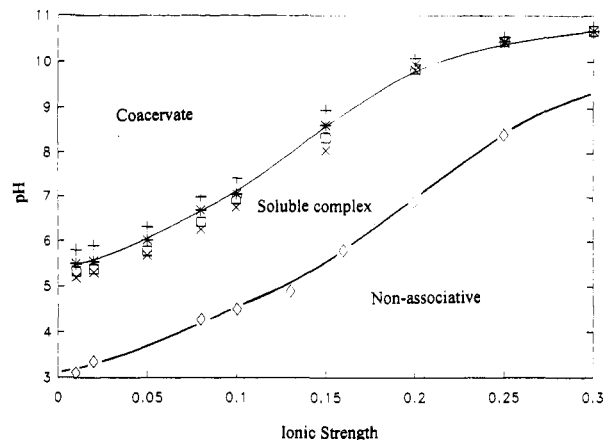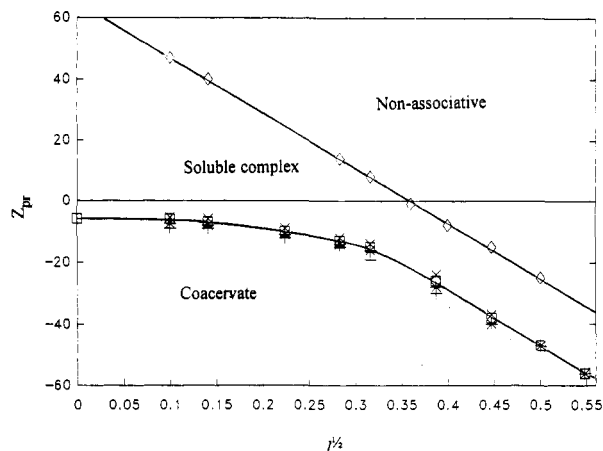
terion condensation), with the elastic stress (from a Hookean bending constant, obtained from the persistence length), they calculated the critical binding colloid charge density and observed that σ_{crit} was proportional to $I^{1/2}$.

Inspection of the low ionic strength region of Figure 9 reveals the initial formation of soluble complex at pH values such that the net charge on the protein is positive (same sign as that of the polyelectrolyte). The formation of complexes between proteins and polyelectrolytes under conditions at which the protein net charge is of the same sign as the polymer's has been observed by others (Tsung and Thompson, 1965; Hoffe, 1964; Mustafaev et al., 1975). One interpretation of this behavior depends on the presence of a negatively charged "patch", such that complexation at pH_c is a result of electrostatic interactions between the polyelectrolyte and some local protein region, as opposed to the global protein surface. Evidence for the presence of protein charge patches was reported by Lesins and Ruckenstein (1989), who found significant retention of positively charged proteins on positively charged anion exchange columns. Further evidence of a protein charge patch was reported by Kopaciewicz et al. (1983), who also showed that proteins could be retained on an ion exchange column, even when the protein charge had the same sign as the column.

A paradox emerges from the linearity of $(Z_{pr})_c$ in both $Z_{pr} < 0$ and $Z_{pr} > 0$ regimes. The simplicity of the relationship observed when the total protein charge is used suggests that the interaction is controlled by the global charge. However, the observation of $Z_{pr} > 0$ at
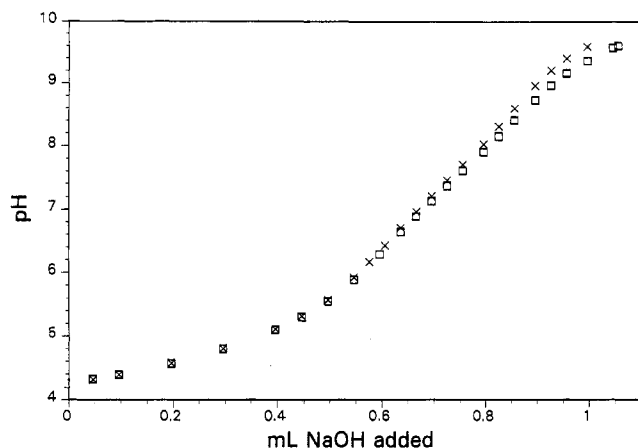
Figure 10. pH titration curves for BSA (×) and BSA in solution with PDMDAAC (□) in I = 0.16 M NaCl, [BSA] = 3.0 g/L, and r = 5.

Figure 11. (A) Change in BSA charge due to complexation with PDMDAAC vs pH; from data in Figure 10 after conversion of moles of OH^- to protein charge. (B) Type 1 titration for [BSA] = 3.0 g/L and [PDMDAAC] = 0.6 g/L in 0.16 M NaCl. Broken lines corresponding to constant ΔZ or constant $100 - \%T$ shown to guide the eye.

low I indicates the presence of negatively charged protein patches which act as polyelectrolyte binding sites. One possible explanation for this contradiction is that the global charge of BSA is proportional to the patch surface charge density at pH < pH$_c$.

An alternate explanation for initial complexation at pH < pI was offered by a reviewer of this paper, who suggested that the charge on complexed protein might be more negative than the charge on free protein at the same pH because binding to the polymer produces a pK_a shift in the direction of higher protein acidity. Therefore potentiometric titrations were carried out for BSA, (3.0 g/L), in 0.16 M NaCl, in the presence and absence of PDMDAAC (0.6 g/L). As shown in Figure 10, the pH titration curve of BSA in the presence of PDMDAAC does indeed diverge from the polymer-free control in the expected direction. The shift, although subtle, is nevertheless reproducible. No particular influence of phase separation on the titration curve is observed. From the horizontal difference between the two curves, corresponding to the difference in OH^- uptake, we can calculate the difference in net charge for protein in the presence and absence of PDMDAAC, at any pH, ΔZ_{pr}, shown in Figure 11A.

It is not clear whether the effect of PDMDAAC on protein dissociation can account for pH$_c$ < pI; in the vicinity of pH$_c$, ΔZ_{pr} is quite small, less than one charge unit. However, it is possible that larger absolute values of ΔZ_{pr} might be observed at lower I where pH$_c$ < pI is found. Furthermore, it is important to note that the measured value of ΔZ_{pr} represents an average between the free and bound proteins that are likely to coexist near pH$_c$: in other words, experiments carried out in the presence of excess polymer might reveal larger pK shifts.

The preceding argument suggests consideration of a two-state model, in which bound proteins constitute a single, well-defined species, so that the gradual divergence of the two curves in Figure 10 represents a transition from the titration curve of the unbound protein (upper curve) to the titration curve of the fully bound protein (currently unknown, but perhaps approached by the lower curve at high pH). Comparison between ΔZ_{pr} vs pH and turbidity vs pH in Figure 11A,B may be relevant. ΔZ_{pr} deviates from zero at the turbidimetric pH$_c$, but also, the curves continue to be very similar at higher pH. If the region 5 < pH < 9 corresponds to the progressive formation of a well-defined soluble complex, the scattering intensity and ΔZ_{pr} might both vary with the relative concentration of this species, giving rise to the behavior seen in Figure 11. While these interpreta-
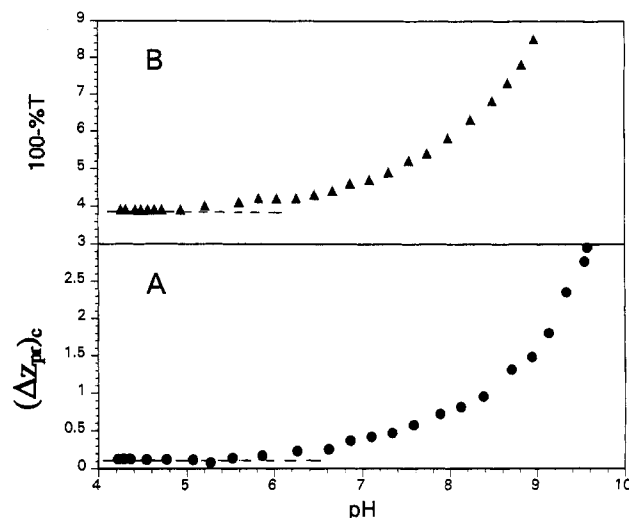
tions are at present highly speculative, they point the way toward further analysis of pH titration curves obtained at a variety of ionic strengths and protein: polymer stoichiometries.

A second interesting feature of Figure 9 is the asymptotic behavior of $(Z_{pr})_\phi$ at lower ionic strengths. This behavior is important for two reasons. First, it lends support to the electroneutrality condition (eq 1): since the polymer charge is always positive, zero net charge can only be obtained with Z_{pr} < 0, so that coacervation requires $(Z_{pr})_\phi$ < 0. Second, it presents a qualitative approach to selecting the best ionic strength for maximum protein yield. For optimal protein separation in multi-protein solutions, one would prefer to minimize the pH range of the soluble complex, i.e., pH$_c$-pH$_\phi$. At lower I, pH$_c$-pH$_\phi$ widens, leading to the likelihood of a larger pH regime over which several proteins may complex simultaneously, hence poorer separation. At higher I, however, is seems probable that the number of proteins bound per polymer chain will diminish, along with the yield. For this system then, the optimum ionic strength would correspond to the location of the slope change of the $(Z_{pr})_\phi$ curve, i.e. I = 0.09 M or $I^{1/2}$ = 0.30. Similar conclusions were offered by other groups (Clark and Glatz, 1990; Niederauer *et al.*, 1994; Suominen *et al.*, 1993) who have found that better protein selectivity could be attained at moderate ionic strength.

According to eq 1, it is possible to determine the number of bound proteins at the coacervation point. On the basis of the average polymer molecular weight and the charge per formula unit of PDMDAAC, the average total charge of the polyelectrolyte chain is 1.2×10^3. When this value is divided by the charge on the protein at pH$_\phi$ [$(Z_{pr})_\phi$ = -12 for BSA at I = 0.1, r = 40], the number of bound proteins can be estimated at *ca.* 100. This value is consistent with the results obtained by Ahmed, Kokufuta, Xia, and Dubin (1994), who reported that for the BSA/PDMDAAC system at an ionic strength of 0.1 M NaCl, and in the limit of $r \rightarrow \infty$, the average number of bound proteins per polymer chain is approximately 120.

Conclusions

The use of phase boundaries for optimizing selective protein separations is so far untried. However, the

presence of the primary soluble complex, originally predicted by the Veis–Aranyi theory, appears to be an important characteristic of these systems. These soluble complexes are initiated at a specific ionic strength-dependent pH_c, and visible phase separation (pH_ϕ) is achieved when their charge approaches zero. The phase boundaries arising from the ionic strength dependence of pH_c and pH_ϕ lead to insight into the binding mechanism which could help optimize purity and yield in future protein separations.

Acknowledgment

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