Effects of Protein Charge Heterogeneity in Protein-Polyelectrolyte Complexation

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ABSTRACT: The interaction between globular proteins and synthetic polyelectrolytes was investigated by turbidimetry and quasielastic light scattering (QELS) techniques, in 0.10 M NaCl solutions. pH titrations were used to induce the binding of three monomeric proteins of substantially different isoelectric points (bovine serum albumin, chicken egg lysozyme, and bovine pancreas ribonuclease) to cationic and anionic polyelectrolytes with a range of charge densities. The onset of association upon addition of HCl to protein/polyanion solutions, or of NaOH to protein/polycation solutions, occurs at a well-defined pH for each polymer/protein pair. This critical pH, which could be detected by either QELS or turbidimetry, corresponds to the formation of soluble polyion-protein complexes and is followed, upon progressive pH change, by phase separation (complex coacervation). Using known protein pH titration curves, the net charge on the protein at critical conditions, Z_c, could be calculated, and it was found that Z_c is frequently identical in sign to the charge on the polyelectrolyte. This finding is seen as evidence for the predominant role of "charge patches" on the protein in the formation of complexes with polyelectrolytes.

Introduction

Proteins interact strongly with both synthetic and natural polyelectrolytes. Ample evidence exists for the binding of polyanions and polycations to proteins below and above their isoelectric points, respectively. These interactions may result in soluble complexes,[1,2] complex coacervation,[3-6] or the formation of amorphous precipitates.[7-9] The practical consequences of these phase changes may include (a) the use of polyelectrolytes for protein separation,[10-12] (b) immobilization or stabilization of enzymes in polyelectrolyte complexes,[13] and (c) the modification of protein-substrate affinity.[14] Such phenomena are also undoubtedly significant in the cell, where the Coulombic association of DNA with basic histones leads to the collapse of the nucleic acid and where basic polypeptides such as polylysine are thought to profoundly influence DNA behavior. Similar electrostatic interactions between proteins and nucleic acid are likely to play a role in the transcription process.[15]

We have been investigating the interaction between the strong polycation poly(dimethyldiallylammonium chloride) (PDMDAAC) and globular proteins such as ribonuclease (RNAse), bovine serum albumin (BSA), and lysozyme.[4,11,16] Of particular interest is the preferential binding to the polycation of the lower isoelectric point proteins, which leads to selectivity in the coacervation step.[11] Polyelectrolyte coacervation thus offers the possibility of nonchromatographic protein separations that are virtually unlimited with respect to scale. In order to avoid a purely Edisonian approach to the search for polymer structures and conditions that provide highly selective protein coacervation, insight into the mechanism of phase separation is essential. Dynamic light scattering studies[16] have shown that soluble protein-polyion complexes are precursors to phase separation. A detailed knowledge of the parameters that control the binding of the protein to the polyion in solution is thus a key to the optimization of conditions for enhancing selectivity in a mixture of two or more proteins. Thus, a meaningful goal is a quantitative understanding of how polymer structural features, such as linear charge density, molecular weight, and hydrophobic character, dictate the solution binding of proteins with varying isoelectric points. Clearly, understanding the details of the interactions between synthetic polyelectrolytes and proteins may provide considerable insight into phenomena in biological systems as well.

In this study we examine the association behavior of two basic (RNAse and lysozyme) proteins and one acidic (BSA) protein with polycations and polyanions of varying linear charge densities. In a qualitative way, the linear charge density can be viewed as the inverse of the average spacing between ionophores along the polymer backbone. In the presence of any of these polycations or polyions, complex formation with protein appears to occur abruptly upon change in pH. This transition is analogous to the formation of a polyelectrolyte-micelle complex which occurs abruptly upon an increase in the surface charge density of the micelle.[17] In both cases, turbidimetric titrations may be used to observe these transitions. As will be shown, the effect of polymer charge sign and structure on these turbidimetrically observed phase changes sheds some light on the nature of the polyion-protein interaction.

Experimental Section

Materials. Structures of the synthetic cationic and anionic polymers are shown in Figure 1. Poly(dimethyldiallylammonium chloride) (PDMDAAC) was a commercial sample of "Merquat 100" from Calgon Corp. (Pittsburgh, PA), possessing a nominal molecular weight (MW) of 2×10^5 and having a reported polydispersity of $M_w/M_n \cong 10$. (Because of an earlier controversy concerning the interpretation of NMR spectra, PDMDAAC was previously thought by us and others to contain a six-membered ring as a repeating unit.) LBN52b, a homopolymer of (*N,N,N*-trimethylamino)ethyl chloride acrylate (CMA), and LBN66 (50 mol % of CMA with acrylamide) were both kindly supplied by Dr. F. Lafuma from the Laboratory of Macromolecular Physical Chemistry at University Pierre et Marie Curie, Paris.[18] The values of M_w for LBN66 and LBN52b are 5.6×10^5 and 2×10^5, respectively, and the polydispersities are estimated at ca. 2 from size-exclusion chromatography.[19] Sodium poly(styrenesulfonate) (NaPSS) was from Pressure Chemical Co. (Pittsburgh, PA) with nominal MWs of 1600 (PSS1) and 354 000 (PSS2), and a polydispersity of less than 1.1, and was used as received.[20] Sodium poly(vinyl sulfate) (PVS) with nominal MW 2000 was obtained from Polysciences Inc. (Warrington, PA) as a 5% aqueous solution. Sodium poly[[2-(acrylamido)propyl]methyl sulfate] (PAMPS) and the copolymer of AMPS with *N*-vinylpyrrolidone (1:1) (NVP-AMPS), with MWs of 2.4×10^6 and 2.0×10^6,

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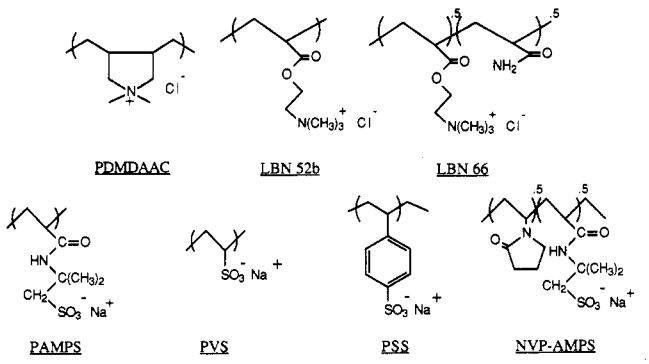

Figure 1. Structures of polyelectrolytes studied.

respectively, were prepared by free-radical polymerization by D. W. McQuigg of Reilly Industries (Indianapolis, IN).[21] The polydispersities of PVS, PAMPS, and NVP-AMPS were not determined. Bovine serum albumin (BSA), bovine pancreas ribonuclease (RNAse), and chicken egg lysozyme were obtained from Sigma Chemical Co. as 95–99% pure lyophilized proteins, with isoelectric points of 4.9, 9.0, and 11.0, respectively.

Selection of Conditions. In previous work[4,11] we have shown that the pH at which phase separation occurs in solutions containing PDMDAAC and one of several globular proteins depends strongly on both the protein/polymer weight ratio, r, and the ionic strength, I. In this work, where the intent is to focus primarily on the influence of polyion charge sign and charge density, we choose to fix these parameters at $r = 5$ and $I = 0.10$. The former is chosen because it corresponds to a bulk ratio of ca. 20–50 protein molecules per polymer chain, which is close to previous estimates[11] of the microscopic stoichiometry near the point of polymer saturation. This protein concentration is sufficient to ensure complex formation but below the range corresponding to excess protein. The salt concentration was selected so as to maintain constant ionic strength irrespective of pH adjustment, without being large enough to reduce the intrinsic protein solubility.

Turbidimetric Titrations. Solutions of 1.0 g/L of protein and 0.2 g/L of polymer, corresponding to a protein/polymer weight ratio (r) of 5.0, were prepared in 0.10 M NaCl at a pH chosen to ensure comiscibility (e.g., acidic in the case of polycations). The optical probe (2-cm path length) of a Brinkmann PC800 fiber-optics probe colorimeter and a combination pH electrode connected to a Beckman Φ34 pH meter were both placed in the solution. The pH was adjusted to near the isoelectric point (IEP) by addition of NaOH or HCl. Titrant (0.05–0.50 M NaOH or HCl) was delivered from a 2.0-mL Gilmont microburet (Great Neck, NY) with gentle stirring at 23 ± 1 °C. Changes in turbidity were monitored at 420 nm and reported as $100 - \%\ T$ which is linearly proportional to the true turbidity for $T > 0.9$.

Quasielastic Light Scattering (QELS). Preliminary measurements of apparent Stokes radii of proteins were made by quasielastic light scattering with an Oros Model 801 (Cambridge, MA) at 24.0 ± 0.5 °C on 5.0 g/L solutions of protein in 0.1 M NaCl. The sample was introduced by syringe into a flow cell of 7-μL scattering volume through a 0.20-μm Anotec in-line disposable filter. A 35-mW solid-state laser irradiated the cell with 780-nm polarized light, and scattered photons were accumulated at 90° for 30 s with an actively quenched solid-state avalanche photodiode. The autocorrelation function was analyzed using the method of exponential sampling to yield a mean diffusion coefficient and a corresponding apparent Stokes radius. The size polydispersity was calculated from the second cumulant. The goodness of fit was monitored by the sum-of-squares deviation of the data from the theoretical curve and the deviation of the baseline from the expected value. More detailed studies of the apparent size distributions of polyelectrolyte–protein mixtures were carried out by measurement of 90° scattering with a Brookhaven system equipped with a 72-channel digital autocorrelator (BI-2030 AT) and a Jodon 20-mW He–Ne laser (Ann Arbor, MI). After pH adjustment, solutions of protein and polyelectrolyte in 0.10 M NaCl were filtered through 0.20-μm filters into an optically clean, dust-free sample cell. We obtained the homodyne intensity–intensity correlation function $G(q,t)$, with

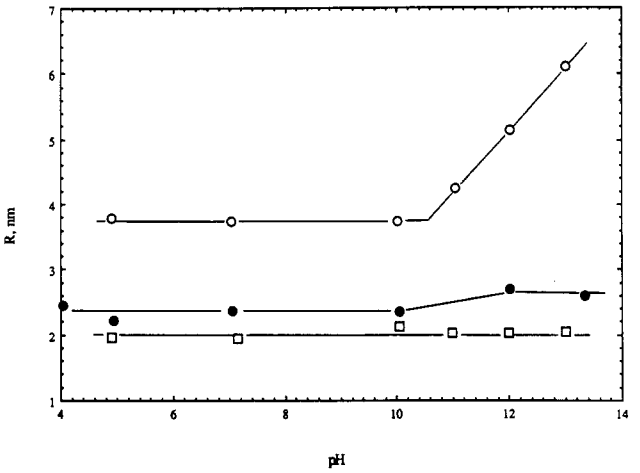

Figure 2. Apparent Stokes radius vs pH of (from top to bottom) BSA (○), RNAse (●), and lysozyme (□), all in 0.10 M NaCl.

q (the amplitude of the scattering vector) given by $q = (4\pi\tilde{n}/\lambda)(\sin(\theta/2))$, where $\tilde{n}$ is the refractive index of the medium, λ is the wavelength of the excitation light in a vacuum, and θ is the scattering angle. $G(q,t)$ is related to the time correlation function of concentration fluctuations $g(q,t)$ by

$$G(q,t) = A(1 + bg(q,t)^2) \quad (1)$$

where A is the experimental baseline and b is the fraction of the scattered intensity arising from concentration fluctuations. The quality of the measurements was verified by determining that the difference between the measured value of A and the calculated one was less than 1%. The distribution of diffusivities, and hence the size distribution function, was obtained from $g(q,t)$ by inverse Laplace transformation using the program CONTIN.

Results and Discussion

QELS was carried out in order to ensure that the solution conditions chosen for this study did not induce protein denaturation, and the results are shown in Figure 2. It appears that the native states of BSA, RNAse, and lysozyme are stable in basic solution at least up to pH 10.2, 10.4, and 13.5, respectively, while all are stable in acid down to less than pH 4. In a separate work, we have found that attempts to form complexes of BSA and PDMDAAC at pH > 11 lead to irreversible formation of amorphous precipitates, which appears to be a consequence of protein unfolding.[22] However, pH-induced unfolding should not take place under the conditions employed for polyelectrolyte–protein studies here.

Figure 3 shows turbidimetric titration curves for BSA in the presence of cationic (PDMDAAC, LBN52b, and LBN66) and anionic (PAMPS, NVP-AMPS, PVS, and high- and low-MW PSS) polymers with concentrations of polymer and protein of 0.2 and 1.0 g/L, respectively, and ionic strength 0.10 M. The plots of Figure 3, along with similar results for RNAse and lysozyme, are converted to turbidity vs the net protein charge (Z) in Figures 4–6. Z, which is pH-dependent, is calculated from published pH titration curves.[23–25]

We first consider the turbidity results for BSA in Figures 3 (vs pH) and 4 (vs net protein charge). All the curves display an abrupt increase in turbidity, corresponding to phase separation, the onset of which typically occurs at a transmittance of 90–97% (i.e., $3 < 100 - \%\ T < 10$). The pH (or net protein charge) at which this takes place for a given protein–polymer combination is defined as pH_ϕ (or Z_ϕ). This phase-separation point is approached as the pH progresses from the direction of the polyion–protein like charge state (i.e., from low pH for polycations and from high pH for polyanions). Prior to phase separation

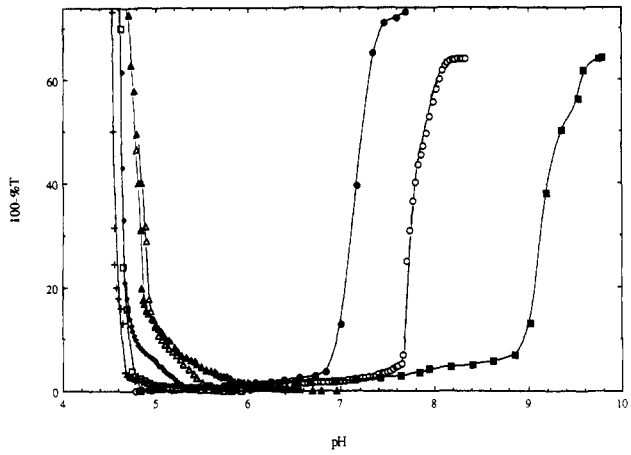

Figure 3. Turbidimetric titrations of BSA in the presence of (from left to right) PSS1 (+), PVS (□), NVP-AMPS (◇), PSS2 (▲), PAMPS (△), LBN52b (●), PDMDAAC (○), LBN66 (■), all in 0.10 M NaCl.

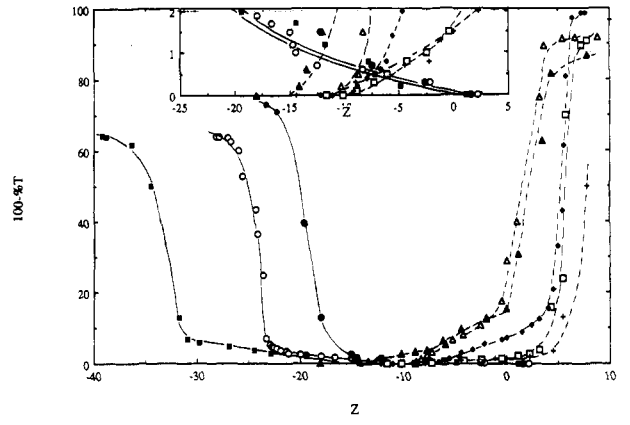

Figure 4. Data of Figure 3, after conversion of pH to the net protein charge. From left to right: LBN66 (■), PDMDAAC (○), LBN52b (●), PAMPS (△), NVP-AMPS (◇), NaPSS2 (▲), PVS (□), PSS1 (+). Solid lines are for polycations; broken lines are for polyanions. The inset shows an expanded plot in the low-turbidity range, used to determine Z_c^τ values in Table I.

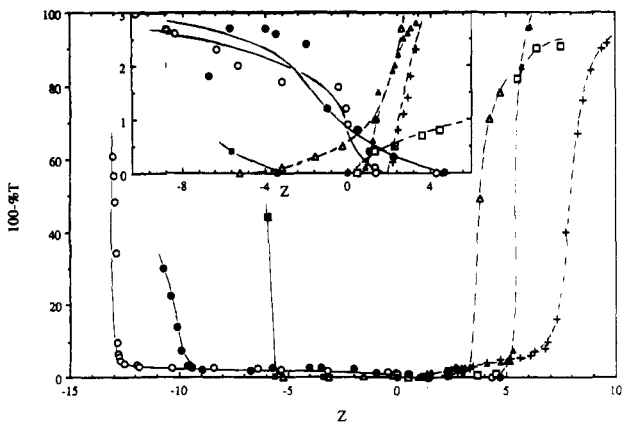

Figure 5. Turbidimetric titrations of RNAse with (from left to right) PDMDAAC (○), LBN52b (●), LBN66 (■), NaPSS2 (▲), PVS (□), PAMPS (△), and PSS1 (+). Solid lines are for polycations; broken lines are for polyanions. The inset shows an expanded plot in the low-turbidity range, used to determine Z_c^τ values for Table I.

we observe a more gradual increase in turbidity, usually corresponding to $100 - \%T < 10$. We may identify this region of low but measurable turbidity as the region of *soluble complex formation* and define its onset as pH_c (or Z_c). The nature of these complexes has been explored only superficially,[16] but it seems likely that they are in-

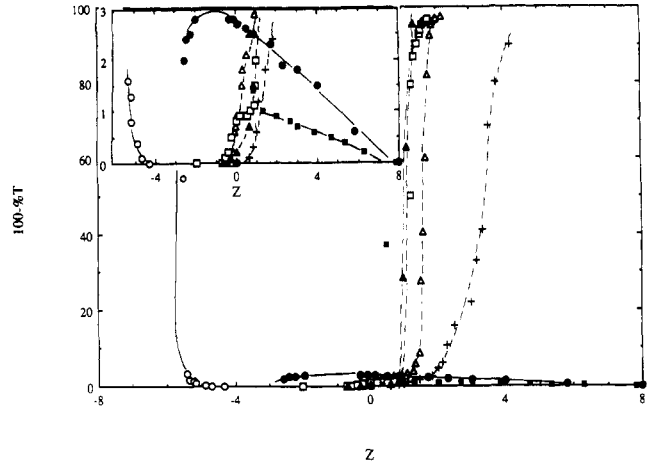

Figure 6. Turbidimetric titrations of lysozyme with (from left to right) PDMDAAC (○), LBN52b (●), PAMPS (△), PSS2 (▲), PVS (□), and PSS1 (+). Solid lines are for polycations; broken lines are for polyanions. The inset shows an expanded plot in the low-turbidity range, used to determine Z_c^τ values in Table I.

trapolymer species which are stabilized with respect to further association by a nonzero net charge of the same sign as the polyion. On the basis of preliminary QELS results, this complex appears to have dimensions similar to those of the protein-free polymer. As the pH is progressively changed (increased in the case of polycations and decreased for polyanions), the protein gains charge opposite to that of the polyion, so that the complex charge may approach electroneutrality, allowing for higher order association and, eventually, phase separation. Thus, pH_c is seen as the condition for the initiation of molecular binding of proteins to polyions, while pH_ϕ describes conditions under which these complexes undergo extensive higher order aggregation.

It is interesting to note the pronounced lack of symmetry for polyanions vs polycations in both phase separation and complex formation with BSA, shown in Figure 4. Qualitatively, we can state that the linear charge densities of the polycations are in the order LBN52b $\gg$ PDMDAAC $\cong$ LBN66. For the polyanions, the sequence of linear charge densities is NVP-AMPS $\ll$ PSS = PVS = PAMPS. Initially, we might have expected the curves to be centered about $Z = 0$, with Z_c for polyanions being approximately equal to $-Z_c$ for polycations of similar linear charge density. This is clearly not the case. Complex formation and phase separation for BSA in the presence of polycations requires the development of a significant negative net charge on the protein ($-30 < Z_\phi < -15$); but in the presence of polyanions, phase separation occurs close to the isoelectric point, and complex formation occurs even when the net charge of the protein is the same as that of the polyanion (i.e., $pH_c >$ IEP)! A second notable difference between the behavior of BSA with polyanions vs polycations is in the effect of the linear charge density. For polycations, the point of phase separation, Z_ϕ, displays a progression toward more negative values as the linear charge density decreases, but no clear trend is evident for the polyanions.

The fact that strong polyanions complex with BSA even when the net protein charge is negative resembles a previous observation of the phase separation lysozyme by PDMDAAC at pH *below* the isoelectric point.[11] We are compelled to recognize the protein's nonuniform charge distribution, such that—in the case of BSA—some region rich in basic residues interacts strongly with polyanions regardless of the presence of a net negative charge. At the ionic strength employed, the Debye length κ^{-1} is 10 Å, and

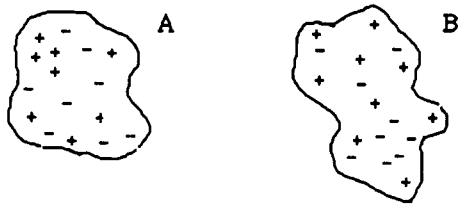

Figure 7. Schematic depiction of proteins with positive (A) and negative (B) charge patches near the isoelectric point.

it is not unrealistic to imagine that polyion repeat units in the vicinity of such a hypothetical "positive charge patch" would be ionically shielded from charges on other parts of the protein. This is especially true since these polymers—for which the bare Kuhn lengths are of the same order as the protein radii—are not so flexible as to be able to "wrap around" the protein in order to form numerous multiple interactive contacts. The existence of protein charge patches has been deduced from the ionic strength dependence of the retention of proteins on ion-exchange chromatography columns.[26] The latter situation is analogous to the present system if one recognizes that the ion-exchange stationary phase is typically a cross-linked polyelectrolyte. If the polyion–protein binding site is relatively small (results obtained for systems comprised of strong polyelectrolytes in the presence of oppositely charged micelles suggest that the cooperatively bound sequence of polyion residues contains six or less repeat units[21]), it is the protein charge density within this "patch" that dictates conditions for polyion binding. Thus, the results for BSA suggest the existence of some region on the order of perhaps 100 Å^2 with a substantial net positive charge, near the isoelectric point. According to this hypothesis, the carboxyl groups are more homogeneously distributed than the basic amino acids, and a substantial negative *net* charge must be developed before the *local* charge density attains a sufficient magnitude to bind polycations (see Figure 7).

Previously reported quasielastic light scattering studies[16] revealed the formation of soluble complexes of BSA and PDMDAAC about 1 pH unit below the phase-separation point. The first strongly scattering species observed has a similar diffusivity to PDMDAAC, suggesting the formation of an intrapolymer complex, which then self-aggregates further en route to coacervation. On closer inspection, the turbidity data (insets of Figures 4–6) can be used to approximately identify the pH for this complexation (pH_c^{τ}) with a reproducibility of ±0.1 pH, corresponding to an uncertainty of ±1–3 units in Z_c^{τ}. These values for various proteins and polymers are shown in Table I.

In order to support the hypothesis that the initial appearance of turbidity revealed in the insets of Figures 4–6 corresponds to the onset of polyelectrolyte–protein interactions, we carried out limited quasielastic light scattering (QELS) experiments. Solutions containing 5 g/L of protein and 1 g/L of polyelectrolyte were combined in 0.10 M NaCl at pH > 11 (in the case of polyanions) and pH < 4 (in the case of polycations). Incremental changes in pH in the direction of phase separation were carried out followed by successive QELS measurements. In the case of NaPSS, PAMPS, NVP–AMPS, PDMDAAC, LBN66, or LBN52b, the onset of complexation was signaled by the appearance of a slow-diffusion mode (R_S^{app} > 30 nm) in addition to the fast-diffusion mode corresponding to protein ($R_S^{app} \cong$ 2–4 nm). The pH at which this occurred, which we call pH_c^{Q}, could be more precisely identified than pH_c^{τ} or pH_c^{ϕ} (within ±0.2 pH units). In the case of PVS/BSA, for which R_S^{app} is smaller for the polymer than for the protein, pH_c^{Q} corresponded to an increase in R_S^{app} from 4–5 to 7 ± 1 nm. The results of the QELS experiments (superscript Q) are given along with those by turbidity (superscript τ) in Table I. There is general agreement between the turbidity and QELS values for Z_c, except for the high charge density PVS (see below).

It is useful to first contrast the results relevant to soluble complex formation for BSA and lysozyme in Table I. The former evidently complexes with polyanions at pH well above the isoelectric point (i.e., at large net negative charge), but for complexation with polycations, the protein net charge can be negative or very near its IEP. This suggests a highly nonuniform distribution of positive charges for BSA at the IEP, whereas the negative charges are more homogeneously distributed. For lysozyme, complexation with polyanions occurs at $-7 < Z_c^{\tau} < -1$, while complexation with polycations can occur when the protein has an even large positive charge.[16] This finding indicates an inhomogeneous distribution of both carboxylate and ammonium ions in the protein near the IEP, with greater heterogeneity for the former. Indeed, computer graphics visualization of this protein using the program DelPhi (Biosym Technologies)[27] reveals a region of negative electrostatic potential at neutral pH. RNAse appears to occupy a position intermediate to the other two proteins with regard to complexation with both polyanions and polycations.

It is not clear that the linear charge density of a polyion effectively predicts the intensity of its binding to proteins. For example, PVS is consistently seen to bind proteins more strongly than the other homopolyanions (i.e., the onset of binding, observed by either QELS or turbidimetry, occurs at a more negative net protein charge than that for PSS or PAMPS), although the linear charge densities are the same. With regard to the polycations, the linear charge density of PDMDAAC is very similar to that of LBN66, yet LBN66 binds the proteins BSA and lysozyme more strongly (at a more positive Z_c^{Q}) than does PDMDAAC. Therefore, as an alternative measure of polymer charge density, we employ part of the model of Davis and Russell in which the polyion is treated as a cylinder bearing a continuous smeared charge[29] to calculate the "geometric surface charge densities" (GCD) of the polymers. However, we do not take into account, as does ref 28, counterion condensation; this is because we believe that these same counterions will be displaced from the polyion, upon binding to protein.[30] The relative GCD values for the polycations (with units of Å^{-2}) are +0.010, +0.008, and +0.005, for LBN52b, PDMDAAC, and LBN66, respectively. For polyanions we obtain −0.034, −0.010, −0.010, and −0.005 for PVS, PSS, PAMPS, and NVP–AMPS, respectively. In many cases, the GCD values closely follow the structural linear charge densities. The most dramatic exception is NaPVS, which attains a large GCD because of its relatively small molecular diameter. A somewhat more subtle effect is the increase in the charge density of PDMDAAC relative to the vinyl polycations, which occurs by virtue of the greater proximity of the charge to the polymer backbone for the cyclopolymer. The most highly charged polycation, LBN52b, displays for all the proteins, as expected, the most positive values for Z_c^{Q}; i.e., the protein does not need to develop a substantial net negative charge to bind. Considering the relationship between Z_c and the charge density for the polyanions, it becomes even more important to rely on the QELS measurements: as we can see, turbidimetry underestimates the absolute value of Z_c for PVS, and this is clearly because

Table I
Net Protein Charge at the Points of Initial Polymer Binding (Z_c)[a] and Phase Separation (Z_ϕ)

	BSA			RNAse			lysozyme		
	Z_c^Q	Z_c^τ	Z_ϕ	Z_c^Q	Z_c^τ	Z_ϕ	Z_c^Q	Z_c^τ	Z_ϕ
				Polyanions[b]					
NVP-AMPS [-0.005]	-6 ± 3	-9	+4						
PSS1 [0.010]	-4 ± 3	-9	+5	-4 ± 1	+2	+7	-1.5 ± 1	+0.5	+2
PSS2 [-0.010]	-12 ± 4	-12	-1	-3 ± 1	+1	+5	-2.6 ± 1	-0.7	+0.7
PAMPS [-0.010]	-14 ± 3	-12	-2	-4 ± 1	-3.5	+2	-2.6 ± 1	-0.7	+1.3
PVS [-0.034]	-25 ± 3	-10	+3	-8 ± 1	+0.5	+5	-7 ± 1	-0.9	+1.3
				Polycations[b]					
LBN66 [+0.005]	-2 ± 2	-2 ± 3	-30	-3 ± 1	-3.5	-6	+6 ± 1	+6	+1
PDMDACC [+0.008]	-6 ± 2	0 ± 3	-23	-0.5 ± 1	+1	-13	-0.3 ± 1	-4	-5.5
LBN52b [+0.010]	-1.5 ± 3	0 ± 3	-18	+4 ± 1	+4	-9.5	+7 ± 1	+6	c

[a] Z_c^Q by QELS; Z_c^τ by turbidity. [b] Geometric charge densities given in brackets; see text for explanation. [c] Resolubilizes.

binding of this low-MW polyelectrolyte to the protein can occur without measurable change in turbidity. Then, on inspection of the data for BSA with NVP-AMPS, PAMPS, and PVS, we observe that as the geometric charge density of the polyion increases from -0.005 to -0.010 to -0.034, Z_c^Q progresses from -6 to -14 to -25. Thus, the critical pH (and hence, the net negative protein charge at incipient polyion binding) increases with the polyion negative charge density. This perhaps counterintuitive result may only be explained by recognizing that the increased electrostatic attraction between the more densely charged polyanions and a *local* protein positive region overcomes possible repulsion between the polyanion and the global protein charge.

Correlations between polyion charge density and critical protein charge are less clear for the polycations, perhaps because the range of polymer charge densities is smaller. As noted above, LBN52b does exhibit the most positive Z_c^Q values for all three proteins; on the other hand, PDMDAAC, with an intermediate GCD, does not typically display values of Z_c^Q or Z_c^τ intermediate to the other polycations. The apparent relative weakness of PDMDAAC binding (i.e., typically more negative values of Z_c^Q or Z_c^τ compared to LBN66) is more consistent with the lower linear charge density of the former polymer and makes it difficult to conclude that the geometric surface charge density is the more relevant charge density parameter.

Correlation between the polymer charge density and Z_ϕ is observed for BSA with polycations (the smaller the GCD, the more negative Z_ϕ) but is obscure for all other cases, indicating that phase-separation behavior is more complicated than the initial binding. Phase-separation effects are subject to several interpretations. The point of phase separation may be viewed as the condition for charge neutrality of a soluble complex. The self-association of such species to form larger particles and eventually coacervate would be inhibited by the presence of a substantial net charge. We may write[11] for the net charge of the protein-polyelectrolyte complex

$$Z_T = Z_P + \bar{n}Z_{pr} \tag{2}$$

and at the point of phase separation

$$Z_T = Z_P + \bar{n}_\phi Z_\phi = 0 \tag{2a}$$

where Z_P is the formal charge of the polyion (i.e., for homopolymers, the degree of polymerization), $\bar{n}$ is the average number of protein molecules bound per polyion chain, and Z_{pr} is the net charge of a bound protein molecule, defined as Z_ϕ at the point of phase separation and taken to be opposite in sign to Z_P. In support of this postulate, we previously found that $\bar{n}_\phi$ deduced from eq 2a for BSA-PDMDAAC was in good agreement with the value obtained by a variant of the Hummel-Dreyer size-exclusion chromatography method, namely, 25 ± 5.[11] However, the effect of the polyion linear charge density on phase separation may be more complicated. An increase in the linear charge density at constant chain length increases Z_P, which, according to eq 2a, with $|Z_\phi| = Z_P/\bar{n}$, increases $|Z_\phi|$. On the other hand, an increase in the linear charge density may also increase the protein-binding affinity and so increase $\bar{n}$ under any given set of conditions of pH, ionic strength, and polymer-protein stoichiometry. This would reduce $|Z_\phi|$. From the results in Table I, we can discern no clear dependence of $|Z_\phi|$ on polymer charge density and therefore cannot resolve between these two possible effects.

Attempts to compare the results for different proteins with a single polymer are complicated by the enormous diversity of protein structure and behavior. However, we do note rather empirically that the more basic the protein (viz., lysozyme), the more readily it both binds to and coacervates with polycations (see Table I). We also note that the difference between Z_c and Z_ϕ is smallest for the most basic protein. For the polyanions, the trend is not as obvious. Proper interpretation of such effects would require an understanding of the dependence of the binding behavior of the proteins on Z, but such information is currently lacking.

The complexity of the data with polyanions arises in part from the wide range of MWs employed. The importance of chain length is made evident by comparison of the curves for BSA with 364K (PSS2) and 1.6K (PSS1) polymers. For the high-MW polyanion, complex formation first occurs at $Z < -12$, followed by phase separation at $Z \cong 0$ (the curve closely follows that for high-MW PAMPS with a similar charge density). For the low-MW PSS, phase separation takes place at $Z \cong +5$, rather close to the result for PVS. It is evident that phase separation occurs closer to the IEP with the higher MW polymer. The explanation for this cannot be found in eq 2a, because both Z_P and $\bar{n}$ are expected to increase with MW. Here, it is instructive to compare the turbidity curves for PVS and NVP-AMPS, the former being high charge density and low MW, and vice versa for the latter. The respective curves are congruent in the phase-separation region but not at lower Z. The turbidities seen with PVS are relatively low at $Z < Z_\phi$, a feature which is shared with 1.6K PSS; on the other hand, precoacervation turbidities are much larger for all of the high-MW polyanions. Soluble complexes of these polyanions with BSA in which numerous protein molecules may be bound can scatter strongly, whereas the binding of one polyelectrolyte molecule (e.g., 1.6K PSS with a degree of polymerization ca. 8) to BSA would be expected to yield only a small increase in scattering. These "primary complexes" must associate in

large numbers to produce strong turbidity, and this appears to occur when the net protein charge resembles the polyion total valency. On the other hand, the relatively large size of (multiprotein) complexes with 354K PSS, PAMPS, and NVP–AMPS enhances the formation of higher order aggregates, even in the vicinity of the IEP.

The data in hand do not allow us to draw firm conclusions about the role of non-Coulombic short-range interactions, which would require at this stage some speculations about the proximity of the polymer segments to the protein in the bound state. Since the bare Kuhn length of, e.g., PSS, is about 2.5 nm, i.e., of the same order as the radius of the largest protein of this study, we may not visualize the polymer as wrapped around the protein. This observation does not, however, exclude the possibility of some intimate contact between one or two repeat units and some complementary region of the protein, so that the possibility of ion-pair formation or hydrophobic interactions may not at this point be completely dismissed. Thus, it is plausible that the number of site-specific charge-neutralization interactions between protein and polymer could be enhanced in polymers with charged groups on long side chains. Crevices or other discontinuities on the surface of the protein could fortuitously interact with apolar moieties present in some of the polymers to strengthen the polymer–protein interaction. Polymers of variable side-chain length but consistent charge group identity might help to more fully address these questions; however, steric and hydrophobic effects could prove difficult to deconvolute.

Conclusions

The foregoing results are in close parallel to "retention maps" reported by Regnier and co-workers[26] for pH dependences of ion-exchange chromatographic (IEC) capacity factors for various proteins. Proteins that bind to cation-exchange resins above the isoelectric points of the former are held to contain positive charge patches, while those that bind to anion-exchange resins below their isoelectric points are believed to have localized regions of dense negative charge. According to models put forward to explain the IEC behavior, the orientation of the protein with respect to the chromatographic stationary phase is highly specific. In the present case, the flexibility of the soluble polyelectrolyte should allow for an increase in the surface area of the protein involved in binding. Semiempirical relationships deduced elsewhere from studies of the interactions of polyelectrolytes with uniformly charged colloids[31] may further elucidate the behavior of polyelectrolyte and proteins. Finally, computational modeling of the electrostatic domain around the protein[27,28] offers the possibility of quantitative comparisons between the dimensions of the regions of large potential and the polymer length scales.

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