Electrophoretic and Quasi-Elastic Light Scattering of Soluble Protein–Polypelectrolyte Complexes

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Complexation between globular proteins (bovine serum albumin, bovine pancreas ribonuclease, and chicken egg lysozyme) and a number of synthetic polyelectrolytes was studied by quasi-elastic light scattering (QELS) and electrophoretic light scattering in dilute electrolyte solution. For each polyelectrolyte–protein pair, there is a well-defined critical pH at which binding commences (pHc). At this pH, QELS reveals fast and slow diffusion modes corresponding to free protein and complex, respectively; the relative amplitude of the latter increases with pH in the case of polycations, with opposite pH dependence for polyanions. Further pH change produces phase separation at a second well-defined point (pHw). The electrophoretic mobility of the polymer begins to change at pHc and moves toward zero as pH approaches pHw. These results are discussed in terms of (1) the role of protein “charge patches” as binding sites and (2) the alternative possibilities of intra- and inter-polyion complex formation.

Introduction

Proteins interact strongly with natural and synthetic polyelectrolytes, mainly through electrostatic forces. These forces may lead to the formation of soluble complexes,1,2 complex conserves,3,4 and amorphous precipitates.5,6 Early studies of protein–polypelectrolyte complex formation were carried out by Morawetz et al.7,8 in the 1950s. These studies describe the precipitation of liver catalase by some synthetic polyelectrolytes. Subsequently, the purification of proteins by polyelectrolytes was proposed. So far, protein–polypelectrolyte precipitation has been successfully used to separate and isolate whey proteins, to fractionate egg white proteins, and to remove nucleic acids from Baker’s yeast.12,13 Beside the application of polyelectrolytes to protein purification,14–16 polyelectrolytes have also been found to inhibit enzymatic activity of some proteins.18,19 Some synthetic polyelectrolytes have recently been shown to have significant inhibitory effects on bacteria, fungi, viruses, tumors, and enzymes when given to test animals prior to viral or tumoral challenge.20,21 Finally, understanding the details of these interactions at the molecular level may help clarify the mechanism of protein–nucleic acid approach and binding. For such varied reasons, studies of the interactions between synthetic polyelectrolytes and proteins could clarify the fundamental roles of the polyelectrolyte in inter alia protein purification, enzyme activity modification, and host resistance to a variety of pathophysiology.

An understanding of the properties of macroscopic protein–polypelectrolyte complexes requires insight into the formation and structure of soluble complexes. On the basis of studies of the complexation of bovine serum albumin (BSA) and quaternized poly(4-vinylpyridines), Kabanov22 proposed a model in which the polycation is wound around an asymmetrical, approximately cylindrical stack of BSA molecules. A number of free polyelectrolyte loops maintain a hydrophilic zone along the surface of the stack promoting the solubility of the particles of the complex. More recently, Dubin et al.16 studied the complexation of poly(dimethylamino)ammonium chloride (PDMDAAC) with different proteins and proposed the existence of a “primary” soluble complex in which a single polymer chain encompasses and entraps a number of proteins.

Quasi-elastic light scattering (QELS) and electrophoretic light scattering (ELS) have proven to be powerful techniques in the study of macromolecular solutions.23–25 However, the application of QELS to the study of protein–polypeptide complexation is quite recent.16,26,27 and the corresponding use of ELS has not been explored to the best of our knowledge. In this study, we use QELS and ELS techniques to study the association behavior of proteins with polyelectrolytes of different linear charge densities as part of a continuing effort to understand interactions and structure in protein–polyelectrolyte systems.28

Experimental Section

Materials. Structures of the synthetic cationic and anionic polymers are shown in Figure 1. Poly(dimethylamino)ammonium chloride (PDMDAAC) was a commercial sample of “Merquat 100” from Calgon Corporation (Pittsburgh, PA), possessing a nominal molecular weight (MW) of 2 × 106 and having a reported polydispersity of Mn/Mw ≥ 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.) LBN 52b, a homopolymer of the acrylate of (trimethylamino)ethyl chloride (CMA), and LBN 66 (50% of CMA with acrylamide) were both kindly supplied by Dr. F. Lafuma from the laboratory of Dr. R. Audebert, University Pierre et Marie Curie, Paris. The values of Mn for LBN 56 and LBN 52b are 5.6 × 103 and 2.8 × 105, respectively, and the polydispersities are estimated at ca. 2 from size exclusion chromatography.29 Sodium poly(styrenesulfonate) (NaPSS) was from Pressure Chemical Company (Pittsburgh, PA) with nominal MW of 354 000 and polydispersity of less than 1.1, and used as received.30 Sodium poly(vinyl sulfate) (PVS) with nominal MW 2000 was obtained from Polyscience Inc. (Warrington, PA) as a 5% aqueous solution. Sodium poly(2-acrylamidomethylpropyl sulfate) (PAMPS) and the copolymer of AMPS with N-vinylpyrrolidone (50 mol %) (NVP-AMPS), with MWs of 2.4 × 106 and 2 × 105, respectively, were prepared by free radical polymerization by D. W. McQuigg of Reilly Industries (Indianapolis, IN). The polydispersities of PVS, PAMPS, and NVP-AMPS were not determined. Bovine serum albumine (BSA), bovine pancreas ribonuclease (RNAse), and chicken egg lysozyme were obtained from Sigma Chemical as 95–99% pure lyophilized proteins, with pI values of 4.9, 9.0, and 11.0, respectively.

Sample Preparation. All solutions were prepared with deionized water that was made from glass distilled water subsequently passed

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through one carbon and two ion-exchange filters. Scattering solutions were made dust-free by filtration through 0.2-μm Acrodisc filters. The concentrations of protein and polyelectrolyte in I = 0.1 M NaCl solution were 5 and 1 g/L, respectively. These concentrations correspond to excess protein concentration in terms of the number of proteins per polymer chain, except for the case of the very low MW PVS where the number of polymer chains is greater than that of proteins. 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.

Methods. Quasi-Elastic Light Scattering. Quasi-elastic light scattering measurements were made at scattering angles from 30° to 150° with a Brookhaven (Holtsville, NY) 72-channel BI-2030 AT digital correlator and using a Jodon 15-mW He-Ne laser (Ann Arbor, MI). We obtain the homodyne intensity-fluctuations. The quality of the measurements was verified by determining that the difference between the measured value of the fraction of the scattered intensity arising from concentration fluctuations. The cosine term is due to simultaneous correlation of the program QUANTA and version 21.3 of CHARMm, where E (V/cm) and \( \mu \) are the applied electric field strength and electrophoretic mobility, respectively. There-
the solution, respectively. The relationship between the mobility
\( u \) and the surface potential \( \xi \) is

\[ \xi \approx \phi = 4\pi \left( \sigma / \varepsilon \right) \]

where \( \sigma \) is the surface charge density of the particle, and \( \varepsilon \) and \( \kappa \) are the dielectrical constant and Debye–Hückel parameter of the solution, respectively. The relationship between the mobility \( (u) \) and the \( \xi \) potential is

\[ \xi = 4\pi (\eta u / \varepsilon) \]
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Figure 4. Distribution functions of LBN 66–lysozyme complex obtained by CONTIN program from the autocorrelation functions at different pH (corresponding net protein charge): 8.07 (+6.4), 8.45 (+6.0), 8.96 (+5.7), 9.62 (+4.7), and 10.09 (+1.0) (from top to bottom).

to a bimodal distribution. To make sure that both relaxation times resolved by CONTIN are due to diffusion, we carried out angular dependence measurements, as shown in Figure 5 for LBN 66–lysozyme at pH 8.96. The observation that the curves in Figure 5 for both fast and slow modes of relaxation are linear and with zero intercept confirms that the bimodal distribution corresponds to two diffusion modes.

The diffusion due to pure polyelectrolyte was not resolved because of the low polyelectrolyte concentration and because the refractive index of the polymer is close to that of the solvent (see Figure 4). At pH < 8.07, one diffusion mode was resolved with a relaxation time constant identical to that of the pure protein. The bimodal distribution was first resolved at pH 8.50, with a fast mode of the same relaxation time as the single distribution observed at pH ≤ 8.07. While the fast mode decreases in intensity with increasing pH, the slow mode increases with respect to both intensity and relaxation time. The slow modes have slower diffusion than that of the polymer. These results suggest that the bimodal distribution corresponds to the diffusion of the pure protein and the complex formed by the protein and the polymer. The onset of slow modes corresponding to complex formation is defined as pHc9 (or Zc9 in terms of net protein charge). With increasing pH, binding and soluble complex formation are enhanced. At pH 10.02, the fast mode disappears and the solution becomes cloudy, corresponding to phase separation. This phase separation is initiated by a colloidal solution, which does not at once form two totally separate phases. The colloidal particles aggregate over a period of time without further change in pH. This pH is defined as pH, (phase separation pH) (or Zc).

The range of diameters of soluble complexes for various pairs of proteins and polymers is shown in Table IV. One notes that in each case the initial observed complex at pHcq has a size similar to that of the corresponding polymer and that the final soluble complex at pHc has more than twice this diameter. The sizes corresponding to pHc were measured for the colloidal solution, at the point of incipient phase separation, prior to physical coacervation. Static light scattering studies of the soluble complex formed between BSA and PDMDAAC support this hypothesis.

### Table III: Net Protein Charge at Initial Polymer Binding (Zcq) and Phase Separation (Zc)

<table>
<thead>
<tr>
<th>Polyelectrolytes</th>
<th>Zcq</th>
<th>Zc</th>
<th>BSA</th>
<th>RNase</th>
<th>Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP-AMPS</td>
<td>-6</td>
<td>-8</td>
<td>+4</td>
<td>-2</td>
<td>-1.5</td>
</tr>
<tr>
<td>NaPSS</td>
<td>-12</td>
<td>-15</td>
<td>-1</td>
<td>-3</td>
<td>-3</td>
</tr>
<tr>
<td>PAMPS</td>
<td>-14</td>
<td>-15</td>
<td>-2</td>
<td>-4</td>
<td>-5</td>
</tr>
<tr>
<td>NaPVS</td>
<td>-25</td>
<td>-26</td>
<td>+3</td>
<td>-8</td>
<td>-7.5</td>
</tr>
<tr>
<td>LBN 66</td>
<td>-2</td>
<td>-2</td>
<td>-30</td>
<td>-3</td>
<td>-2</td>
</tr>
<tr>
<td>PDMDAAC</td>
<td>-6</td>
<td>-5</td>
<td>-23</td>
<td>-0.5</td>
<td>-1</td>
</tr>
<tr>
<td>LBN 52b</td>
<td>-1.5</td>
<td>0</td>
<td>18</td>
<td>+4</td>
<td>+3</td>
</tr>
</tbody>
</table>

* Zcq by QELS; Zc by ELS.
TABLE IV: Range of Diameters (nm) of Soluble Complexes

<table>
<thead>
<tr>
<th>polymer</th>
<th>BSA</th>
<th>RNase</th>
<th>lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>polyanions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaPVS</td>
<td>6–45</td>
<td>6–46</td>
<td>6–48</td>
</tr>
<tr>
<td>NaPSS</td>
<td>46–63</td>
<td>46–60</td>
<td>46–57</td>
</tr>
<tr>
<td>PAMPS</td>
<td>30–110</td>
<td>30–85</td>
<td>30–98</td>
</tr>
<tr>
<td>NVP-AMPS</td>
<td>40–67</td>
<td>40–84</td>
<td>40–87</td>
</tr>
<tr>
<td>polycations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDMDAAC</td>
<td>20–48</td>
<td>20–68</td>
<td>20–87</td>
</tr>
<tr>
<td>LBN 52</td>
<td>28–81</td>
<td>28–88</td>
<td>28–75</td>
</tr>
<tr>
<td>LBN 66</td>
<td>31–87</td>
<td>31–86</td>
<td>31–99</td>
</tr>
</tbody>
</table>

* The change of diameter from pH_1 to pH_2.

**Figure 7.** Electrophoretic mobility of RNase–LBN 66 in 0.10 M NaCl as a function of pH. Note the charge neutrality at the phase separation point. The mobility measurements at pH > pH_0 were made over a short time period on stable stirred suspensions and were stable and reproducible.

**Figure 8.** Electrophoretic mobility of RNase with (a) PDMDAAC (Δ), LBN 66 (○), LBN 52 (□) and (b) PAMPS (△), NaPVS (○) (axis below); NaPSS (△) and NVP-AMPS (+) (axis above), all in 0.10 M NaCl.

**Figure 9.** Electrophoretic mobility of BSA with (a) PDMDAAC (Δ), LBN 66 (○), LBN 52 (□) and (b) PAMPS (△), NaPVS (○) (axis below); NaPSS (△) and NVP-AMPS (+) (axis above), all in 0.10 M NaCl.

**Figure 10.** Electrophoretic mobility of lysozyme with (a) PDMDAAC (Δ), LBN 66 (○), LBN 52 (□) and (b) PAMPS (△), NaPVS (○) (axis below); NaPSS (△) and NVP-AMPS (+) (axis above), all in 0.10 M NaCl.

**Electrophoretic Light Scattering Study of the Complexation Process.** The electrophoretic light scattering spectra obtained at four different angles for RNase–LBN 66 complex at pH 9.10 are shown in Figure 6. The average of the spectra gives a mobility of 0.7 (μm cm/V s). In contrast to QELS, ELS does not resolve a bimodal distribution at the complexation pH because the low charge and smaller size of the protein renders it "invisible". The mobility obtained at different pH is plotted in Figure 7. It is interesting to observe that there is no discontinuity in the plot at pH_0. This may suggest (a) that the mobility of the coacervate is the same as the mobility of the soluble aggregate with which it is in equilibrium or (b) that the charge-to-size ratio of the coacervate is too small for it to make a significant contribution to the measured mobility. In any event, one notes that the mobility of the complex starts decreasing around 9.10 as the pH progresses toward phase separation. When pH ≤ 9.10 the measured values are identical to the mobility of LBN 66 polymer. The onset of the mobility change at pH 9.10 can be easily understood as a result of initial protein–polymer complexation by considering the motion of a complex in an electric field.

The motion of the complex at steady state in a field of strength $E$ (V cm$^{-1}$) can be described by balancing the electrostatic force, $F_e$, and frictional force, $F_f$:

$$ F_e = F_f $$

(13)

The electrostatic and frictional forces can be given by
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Figure 1. Electrostatic potential of RNAse in solution calculated as described in text. The blue and red contours represent the potential ranges from 0.2 to 1.0 kcal/mol and from -0.2 to -1.0 kcal/mol, respectively; each range consists of five contour levels using an interval of 0.2 kcal/mol. The protein backbone is highlighted by a yellow ribbon. The view is looking down on to the protein active site. The protein has a large net positive charge at this pH, but the negative charge patch (red) is easily visualized.

\[
F_e = (q_p + nq_{pc})E
\]

\[
F_i = v(f_p + \beta n f_{pc})
\]

where \(q_p\) and \(q_{pc}\) are the net charge of a bound protein and total polymer charge, respectively; \(f_p\) and \(f_{pc}\) are the friction coefficients of the bound protein and the polymer, respectively, which are assumed to combine additively; \(\beta\) is the friction coupling factor; \(E\) is the external field strength; \(v\) is the center-of-mass velocity of the complex; and \(n\) is the number of bound proteins per polymer chain. Substituting eqs 14 and 15 into eq 13, we have for the mobility of the complex

\[
u = \frac{v}{E} = \frac{q_p + nq_{pc}}{f_p + \beta n f_{pc}}
\]

The pH dependence of the complex mobility is expected from eq 14 since the net protein charge \(q_{pc}\) is a function of pH, as is \(n\). Equation 16 is also reduced to the pure polymer mobility at \(n = 0\). Therefore, the mobility change with pH may be used to characterize the initial protein–polyelectrolyte complexation via eq 16.

The plot of Figure 7, along with similar results for the other proteins and polyelectrolytes, is converted to mobility vs the net protein charge \(Z_c\) in Figures 8–10. Z, which is pH-dependent, is calculated from published pH titration curves.46–48 The net protein charge \(Z_c\) at initial binding for each of the protein–polymer pairs from Figures 8–10 is also summarized in Table III. One notes that these results are consistent with the QELS data, and both QELS and ELS results obtained for the polyanions show that the higher the linear charge density the stronger the complexation. The effect of polyelectrolyte charge density on the complexation has been discussed elsewhere.27

It is interesting to note the pronounced lack of symmetry for polyanions vs polycations in complex formation with proteins, as shown in Figures 8–10. Qualitatively, we can state that the linear charge densities, calculated by the Davis and Russell model,44 of the polycations are in the order LBN52b > PDMDAAC ≈ LBN66. For the polyanions, the sequence of linear charge densities is NVP-AMPS < PAMPS ≈ NaPSS < PVS. 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 for proteins in the presence of polyanions requires a negative net charge on the protein with a few exceptions; but in the presence of polyanions, complexation formation occurs even when the net charge of protein is of the same sign as the polyanion.

Previous turbidimetric and QELS studies of these protein–polyelectrolyte pairs also show that polyanions bind proteins more strongly than polycations even when the net protein charge is negative. This phenomenon can be understood in terms of nonuniform protein charge distribution or “surface charge
patches”. This interpretation is supported by electrophoretic mobility data as shown by Figures 8–10, where the mobility becomes more negative when the proteins and polyanions initially interact. This change in mobility proves that the polyanions gain negative charge upon complexation. To overcome the global electrostatic repulsion, the proteins must have strong “positive charge patch” to bind the polymer units. From eq 16, the mobility gained at initial complexation also suggests that the initial complex does not have an increased friction factor relative to the free polymer. Therefore, an intra-polymer structure for the initial complexes is expected. For the few cases where the positive proteins associate with polyanions, we did not observe the mobility increase at the point of initial binding. This could be a compensation of charge and friction effects.

The existence of nonuniform charge distribution or “surface charge patches” on the protein appears to play an important role in protein–polyelectrolyte interaction. An example of such patches is illustrated by the calculated electrostatic potential for RNase shown in Figure 11. Here the blue and red lines represent the ranges $\psi_f = 0.2$ to 1.0 kcal/mol and $\psi_f = -0.2$ to $-1.0$ kcal/mol contour levels, respectively. The amino acid sequence contains 18 basic and 10 acidic residues, of which 10 are lysine, 4 arginine, 4 histidine, 5 glutamic acid, and 5 aspartic acid. Given the side chain $pK_a$ values of these residues, one can conclude that the fully charged condition would be most closely approached at pH 6, which is well below the isoelectric point (IEP) of RNase. As described in the Methods section, this is the amino acid representation we have used to calculate the electrostatic potential surrounding RNase. As one would expect at pH 6, RNase is seen to have a large positive net charge. However, even under these conditions, negatively charged areas still exist on the protein surface. How these surface charge patches change and redistribute as conditions are computationally varied is currently under investigation and will be communicated later.

With regard to the mechanism of phase separation, we proposed that at the point of coacervation

$$Z_T = Z_p + n_p Z_p = 0$$  \hspace{1cm} (17)$$

where $Z_T$ is the net charge of the protein–polyelectrolyte complex, $Z_p$ is the formal charge of the polyanion, $n_p$ is the average number of protein molecules bound per polyanion chain, and $Z_p$ is the protein net charge at the point of phase separation.\(^{16}\) The requirement of neutrality at phase separation is only observed here for complexes formed from polycations, as shown by the curves crossing zero mobility in Figures 8–10. For the polyanions, phase separation occurs for complexes with a negative charge. Phase separation with polyanions seems complicated. Since soluble complexes exist only when the polyanion charge is between $Z_c$ and $Z_n$, the difference of $\Delta Z = |Z_c - Z_n|$ can be used to characterize the stability of the complexes. $\Delta Z_{RNAse} > \Delta Z_{RNAse}$ are obtained for all polymers in Table III.

Conclusions

QELS and ELS are powerful techniques to study the interactions between polyelectrolytes and proteins. Both QELS and ELS results are consistent with a mechanism that entails an initial binding of polyelectrolytes to proteins localized at protein surface charge patches. Upon change in pH, the interaction between polyelectrolyte and proteins leads first to soluble complex formation and then to phase separation. It is suggested that a transition from intra-polymer to interpolymer complexation, followed by further aggregation, accompanies the progression from soluble complex to phase separation.

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