

# Roles of Electrostatic Interaction and Polymer Structure in the Binding of $\beta$ -Lactoglobulin to Anionic Polyelectrolytes: Measurement of Binding Constants by Frontal Analysis Continuous Capillary Electrophoresis

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Frontal analysis continuous capillary electrophoresis was used to measure the binding of  $\beta$ -lactoglobulin (BLG) to sodium poly(styrenesulfonate) (PSS) and sodium poly(2-acrylamido-2-methylpropanesulfonate) (PAMPS), two strong polyanions with similar linear charge densities. The binding isotherms obtained were well-fit by the McGhee–von Hippel equation, yielding the intrinsic binding constant,  $K_{\text{obs}}$ , and the binding site size,  $n$ , representing the number of polymer segments per bound protein. Two opposite ionic strength ( $I$ ) dependencies of  $K_{\text{obs}}$  for BLG–PSS were found depending upon pH, that is, increase of  $K_{\text{obs}}$  with  $I$  at pH 7.0, and decrease of  $K_{\text{obs}}$  with  $I$  at pH 6.3. The opposite  $I$  dependencies reflected the roles of electrostatic interactions for systems with heterogeneously charged components, but also demonstrated the inapplicability of a simple formulation ( $\log K_{\text{obs}} = \log K^\circ - Z\varphi \log [M^+]$ ) put forward for the binding of protein to DNA.  $K_{\text{obs}}$  for PAMPS was always much smaller than that for PSS at equal pH. In addition,  $n$  for BLG–PSS was small and independent of  $I$  and pH, while  $n$  for PAMPS was large and increased with  $I$  and pH, both results consistent with “tighter” binding of BLG to PSS than to PAMPS. This marked contrast may arise from the effects of polymer persistence length or from hydrophobic interactions.

## Introduction

The quantitative study of interactions between synthetic polyelectrolytes and proteins is relevant to several meaningful subjects.<sup>1</sup> First, since the hydrophobicity, charge density, and chain stiffness of synthetic polyelectrolytes can be systematically altered, such studies may lead to a better understanding of the binding between proteins and natural polyelectrolytes, such as DNA. Second, there are practical consequences to such insights, for example in the use of polyelectrolytes for protein separation,<sup>2–5</sup> protein purification,<sup>6,7</sup> or immobilization and stabilization of enzymes.<sup>8,9</sup> Thus, a detailed knowledge of the parameters that control the binding of proteins to polyelectrolytes is of interest, vis-à-vis models for protein–DNA binding, and is directly relevant to the technological applications of protein–polyelectrolyte complexation.

The intrinsic binding constant for protein–polyelectrolyte complex formation,  $K_{\text{obs}}$ , is an important parameter

in any systematic binding study, and its measurement is central to accurate evaluation of binding conditions. However, there have been so far few reports of  $K_{\text{obs}}$  for protein–synthetic polyelectrolyte systems. Gao et al.<sup>10</sup> developed a method for obtaining the protein–polyelectrolyte binding isotherm by “frontal analysis continuous capillary electrophoresis” (FACCE). FACCE data for bovine serum albumin (BSA) or BSA with sodium poly(styrenesulfonate) (PSS)<sup>11</sup> were then analyzed to yield  $K_{\text{obs}}$  using the McGhee and von Hippel<sup>12</sup> site binding model.

Recently, Hallberg and Dubin<sup>13</sup> studied the effect of pH on  $K_{\text{obs}}$  of  $\beta$ -lactoglobulin (BLG)–PSS and assessed the applicability of the theoretical treatment of Record and co-workers.<sup>14,15</sup> Their equation,

$$\log K_{\text{obs}} = \log K^\circ - Z\varphi \log [M^+] \quad (1)$$

effectively accounted for the  $I$  dependence of the binding between pentyllysine (L) and DNA (D). Here,  $K^\circ$  is the equilibrium constant for the reaction  $L + D = L-D + Z\varphi(M^+)$ , where  $Z$  is the ligand charge and  $\varphi$  is the fraction of counterion ( $M^+$ ) “thermodynamically associated with the polyelectrolyte”.  $\varphi$  contains contributions from condensed and screening ions, and arises from a thermodynamic treatment based on Manning’s counterion condensation theory.<sup>16</sup> Although the pH dependence of  $K_{\text{obs}}$  for

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BLG-PSS was found in ref 13 to follow the semilogarithmic dependence of  $K_{\text{obs}}$  on  $Z$  predicted by eq 1, the magnitude of  $Z_{\text{p}}$  found via eq 1 was unrealistic. Since the binding in ref 13 was observed on the "wrong side" of the isoelectric point ( $\text{pI} > \text{pH}$ ), Hallberg and Dubin assumed that  $Z$  in eq 1 can be replaced with  $fZ$ , where  $f$  represents the relationship between an effective local charge  $Z_{\text{eff}}$  and the net charge  $Z$  as  $f = |Z_{\text{eff}}/Z|$ .

The initial motivation of the present study was to determine the  $I$  dependence of  $K_{\text{obs}}$  for BLG-PSS at various pHs in order to consider the role of electrostatic interactions and thus further test the validity of eq 1 and its modifications. Although quantitative interpretation of the  $I$  dependence of  $K_{\text{obs}}$  must be based primarily on electrostatic interactions and Coulomb forces, the difference in  $K_{\text{obs}}$  for BLG-PSS versus BLG-PAMPS was striking. Thus, we also discuss the possible roles of persistence length and hydrophobic interactions in the large  $K_{\text{obs}}$  for PSS.

### Experimental Section

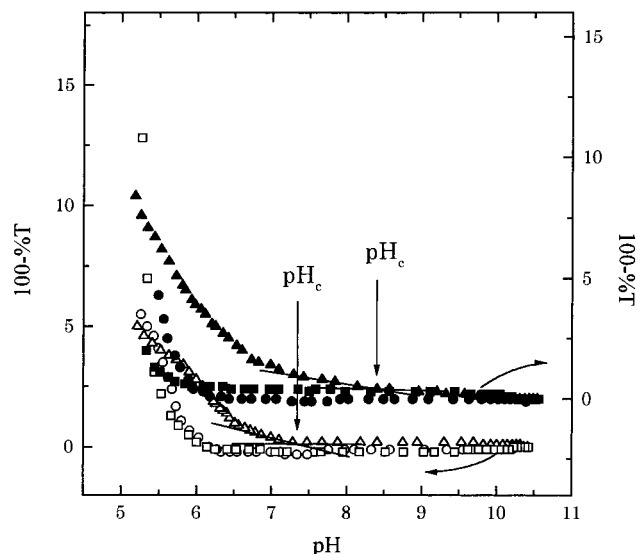
**Reagents and Solutions.**  $\beta$ -Lactoglobulin A&B (BLG) (Catalog no. L-2506) was purchased from Sigma Chemical Co. (St. Louis, MO). To avoid effects due to incomplete polymer sulfonation, sodium poly(styrenesulfonate) (NaPSS) ( $M_w = 2.5 \times 10^5$ ), prepared by polymerization of sodium styrenesulfonic acid, was used as previously described.<sup>13</sup> Sodium poly(2-acrylamido-2-methylpropanesulfonate) (NaPAMPS) ( $M_w = 5.0 \times 10^5$  and  $2.5 \times 10^5$ ), a gift from Dr. J. S. Tan,<sup>17</sup> was prepared by free radical polymerization in water, followed by stepwise fractionation using aqueous NaCl/ethanol.

Sample solutions were made from freshly prepared stock solutions of BLG and polyelectrolyte that were dissolved in CE run buffer solution. An adequate amount of 0.1 M hydrochloric acid was added to the stock solution of BLG to adjust the pH. The run buffer solution contained 10 mM phosphate salt as the pH buffer, and  $I$  was further adjusted with the addition of sodium chloride. The concentration range of BLG was 0.2–2.0 g/L, and the concentration of polyelectrolyte was constant at 0.2 g/L. All solutions were made from Milli-Q water.

**Method of Acid-Base Titration.** Sample solutions (20 mL of sample volume) containing adequate amounts of BLG, the polyelectrolyte, sodium hydroxide, and sodium chloride were titrated with 0.1 M hydrochloric acid at room temperature under  $N_2$ , using a 2.0 mL Gilmont microburet. The pH and transmittance of sample solution were simultaneously monitored. The pH was measured with a Corning pH meter 240 with an Orion 91-05 combination pH electrode. The transmittance was measured using a Brinkman PC 800 colorimeter equipped with a 420 nm filter and a 2 cm path length optical probe. All titrations were carried out with gentle magnetic stirring, and the observed potential and transmittance were determined when the values became stable for at least 1 min.

**Apparatus and Procedure for Frontal Analysis Continuous Capillary Electrophoresis.**<sup>10</sup> Capillary electrophoresis was performed using a Beckman (Fullerton, CA) P/ACE 5500 CE with a UV detector, operating at 8 kV and 25 °C. The fused silica capillary (Polymicro Technologies Inc., Phoenix, AZ) of dimensions  $50 \mu\text{m} \times 27 \text{ cm}$  (effective length 20 cm) was prepared prior to each set of experiments by washing with 0.1 N sodium hydroxide (NaOH) for 10 min followed by a 10-min wash with Milli-Q water.

The FACCE experiment was initialized by equilibrating the capillary with buffer solution for 5 min. The inlet end of the capillary was then placed in a vial containing the equilibrated sample solution, and the outlet end was placed into a vial containing buffer solution. Constant voltage was applied, and separation, manifested in continuous plateaus, was observed. The first eluting plateau was the unbound protein, and the second was the mixture of unbound protein and protein-polyelectrolyte complex. Although a multiple-peak pattern sometimes appeared



**Figure 1.** Turbidimetric titration of a mixture of BLG and polyelectrolyte: (□) 1.0 g/L of BLG (blank); (Δ) with 0.2 g/L PSS; (○) with 0.2 g/L PAMPS at  $I = 0.01 \text{ M NaCl}$ ; (■) 1.0 g/L of BLG (blank); (▲) with 0.2 g/L PSS; (●) with 0.2 g/L PAMPS at  $I = 0.05 \text{ M NaCl}$ .

instead of the second plateaus, the first plateau was always obtained. After each electrophoretic run, a 2-min wash with 0.1 N NaOH followed by a 2-min wash with water was performed. The concentration of unbound protein was determined from the height of the first plateau, using a calibration curve constructed by measuring the plateau height of known concentrations of protein obtained under the same experimental conditions as those for the protein-polyelectrolyte mixture. The extent of binding, that is, the number of protein molecules bound per polymer residue ( $\nu$ ) was determined from the amount of unbound BLG and the total amount used.

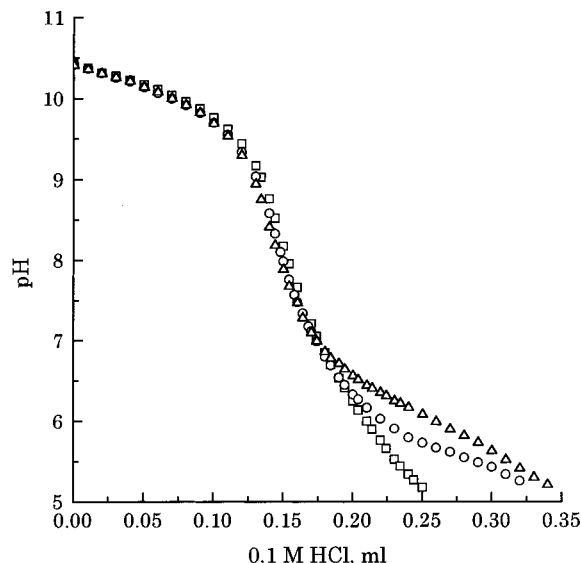
### Result and Discussion

**Acid-Base Titration.** While FACCE is used in this study to provide quantitative binding data, other techniques must be applied first to establish appropriate conditions for FACCE. Qualitative information about the binding of BLG-PSS and BLG-PAMPS can be obtained from acid-base titrations of the mixture of BLG and polyelectrolyte by using turbidimetry and potentiometry. These methods were performed at  $I = 0.01$  and  $0.05 \text{ M}$ .

Solution turbidity changes when polyelectrolyte complexes are formed with a large protein such as BSA.<sup>18</sup> Figure 1 shows turbidimetric titration curves as 100%-transmittance versus pH. The pH at the onset of complexation ( $\text{pH}_c$ ) is observed to be about 8.5 at  $I = 0.05 \text{ M}$  and 7.3 at  $I = 0.01 \text{ M}$ . Although an increase in  $I$  would be expected to screen Coulombic interactions and weaken complexation, we find binding occurs more readily (i.e. at higher pH) for larger  $I$ . BSA has been observed to bind with several polyanions and polycations,<sup>18</sup> However, those results indicated that the  $\text{pH}_c$  of the complexation with polyanions decreased with increasing  $I$  and that the  $\text{pH}_c$  for polycations increased with increasing  $I$ . That is, binding decreased with increasing  $I$ , presumably due to the screening of an attractive Coulomb force. This anomalous  $I$  dependence of the binding behavior of BLG-PSS will be interpreted below in terms of the measured binding constants. The turbidimetric titration curves for PAMPS at  $I = 0.01$  and  $I = 0.05$  may indicate  $\text{pH}_c \approx 6.4$ , but this result is obscured by the change of turbidity for polymer-free BLG due to protein aggregation.

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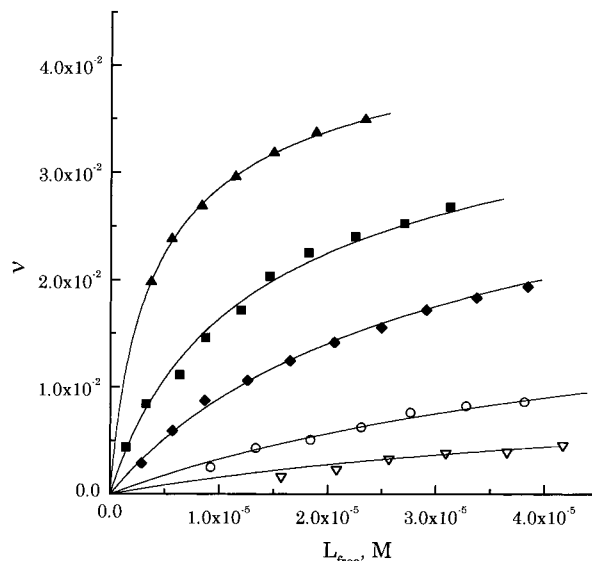


**Figure 2.** pH titration curves of mixture of BLG and polyelectrolyte at  $I = 0.01$ : (□) 1.0 g/L of BLG (blank); (○) with 0.2 g/L PAMPS; (△) with 0.2 g/L PSS.

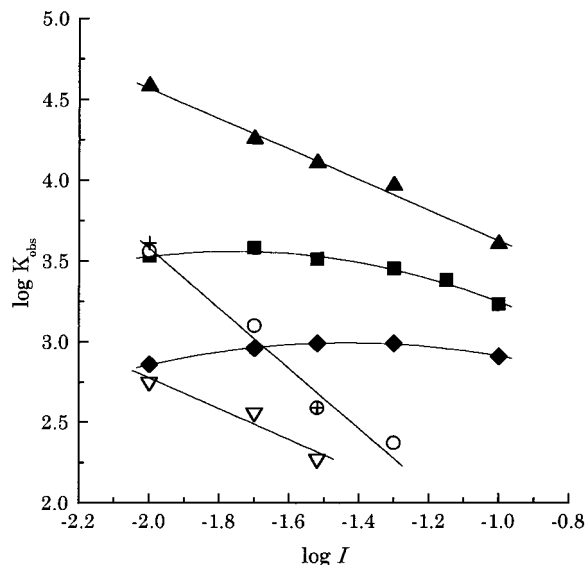
Complexation of protein with polyelectrolyte can also be observed by potentiometric titration of the proteins' acidic and basic groups. Figure 2 shows the pH titration curves of BLG with and without polyelectrolyte at  $I = 0.01$  M. The addition of the polyelectrolyte increases the amount of  $H^+$ -consumption (basicity) of titratable groups (i.e. an increase in pH) at  $pH < 7.0$  for PSS and at  $pH < 6.2$  for PAMPS. This is due to an apparent  $pK_a$  shift of the protein's amino acid residues.<sup>19</sup> Therefore, the complexation of BLG with PAMPS at  $pH \approx 6.5$  can be detected by potentiometry but not by turbidimetry. Since the net charge of BLG is negative at  $pH > 5.2$  (pI), binding of BLG to PSS and PAMPS at  $pH > 5.2$  corresponds to complexation "on the wrong side" of pI. The release of  $[H^+]$  arising from addition of the polyelectrolyte ( $\Delta[H^+]$ ) is larger for BLG-PSS than for BLG-PAMPS, signifying "stronger" binding. Similar pH titration curves were obtained at  $I = 0.05$  M NaCl. Each  $\Delta[H^+]$  was smaller at  $I = 0.05$  M than that at  $I = 0.01$  M, indicating that an increase of  $I$  depresses the binding force between BLG and the polyelectrolytes.

**Binding Isotherms by FACCE.** Binding isotherms of BLG-PSS and BLG-PAMPS at  $I = 0.03$  M and at various pH values are shown in Figure 3. Here  $\nu$  is the number of protein molecules bound per polymer residue, and  $L_{free}$  is the concentration of unbound BLG. The amount of BLG bound depends strongly on the pH and decreases with pH in a manner similar to that found previously.<sup>13</sup> The diminution of the binding with pH must be the result of increased repulsion due to both the increasing negative charge and the decreasing positive charge of BLG. Most remarkably, as shown in Figure 3, the amount of BLG bound to PAMPS at pH 6.3 is much smaller than that for PSS; that is, BLG binds far more strongly to PSS than to PAMPS. This difference agrees with the results of the acid-base titration.

The McGhee and von Hippel<sup>12</sup> equation ( $\nu/L = K_{obs}(1 - m\nu)/(1 - m\nu)/(1 - (n - 1)\nu)^{n-1}$ ) based on the "overlapping binding site" model was used to interpret these binding isotherms. Two binding model parameters (observed binding constant ( $K_{obs}$ ) and binding site size ( $n$ )) were simultaneously obtained from the curve of  $\nu/L_{free}$  versus



**Figure 3.** Binding isotherms of BLG-PSS and BLG-PAMPS at  $I = 0.03$ : (◆) BLG-PSS at pH 7.0; (■) BLG-PSS at pH 6.7; (▲) BLG-PSS at pH 6.3; (▽) BLG-PAMPS at pH 6.3; (○) BLG-PAMPS at pH 6.1.



**Figure 4.** Effect of ionic strength on binding constants of BLG-PSS and BLG-PAMPS: (◆) BLG-PSS at pH 7.0; (■) BLG-PSS at pH 6.7; (▲) BLG-PSS at pH 6.3; (▽) BLG-PAMPS ( $M_w = 5.0 \times 10^5$ ) at pH 6.3; (○) BLG-PAMPS ( $M_w = 5.0 \times 10^5$ ) at pH 6.1; (+) BLG-PAMPS ( $M_w = 2.5 \times 10^5$ ) at pH 6.1.

$\nu$  by nonlinear curve fitting. Although a parameter for cooperative binding<sup>11</sup> could also be included, the binding isotherms in the present study were well-fitted without this parameter. The solid curves in Figure 3 calculated from the two parameters,  $K_{obs}$  and  $n$ , conform very well to the experimental points.

**Binding Constants.** The binding constants of BLG-PSS and BLG-PAMPS are presented as  $\log K_{obs}$  versus  $\log I$  in Figure 4. As shown in a previous study,<sup>11</sup>  $K_{obs}$  of PSS was independent of molecular weight ( $M_w$ ) at  $M_w > 10^5$ , so the difference in  $M_w$  between PSS and PAMPS need not be considered. The distinctive features of Figure 4 are the following: (1)  $K_{obs}$  decreases with pH for both BLG-PSS and BLG-PAMPS; (2) at low pH (pH 6.1 and 6.3),  $\log K_{obs}$  decreases linearly with  $\log I$  for both BLG-PSS and BLG-PAMPS and the magnitude of the effect of  $I$  on  $\log K_{obs}$  is amplified with decreasing pH; (3) at pH 7.0,  $K_{obs}$  of BLG-PSS increases with  $I$  at  $I < 0.3$  but

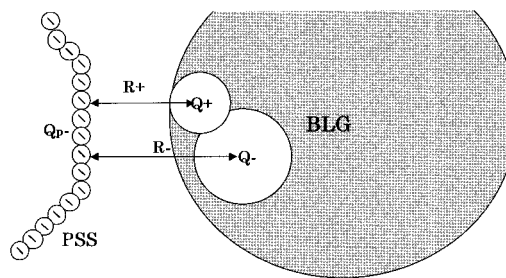
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decreases at  $I > 0.3$ ; (4)  $K_{\text{obs}}$  of BLG–PSS is larger than that of BLG–PAMPS at pH 6.3; (5) at pH 6.3, the  $I$  dependence of the binding constant for BLG–PSS is nearly equal to that for BLG–PAMPS.

The binding of proteins to polyelectrolytes is mainly driven by electrostatic interactions, although in some cases hydrogen bonding<sup>20</sup> and hydrophobic interaction<sup>21</sup> may contribute. In the present case hydrogen bonding cannot occur, leaving hydrophobic interactions to be considered. But, while Coulomb forces are strongly diminished with increasing  $I$  at low  $I$ , hydrophobic interactions appear to be sensitive to the concentration of electrolyte ions only at the ionic strengths larger than those used here.<sup>23,24</sup> Thus, despite the net negative charge on BLG, electrostatic interactions must be primarily responsible for the  $I$  dependence of  $K_{\text{obs}}$ .

**Electrostatic Interaction.** The substantial effect of pH on the decrease of  $K_{\text{obs}}$  for both PSS and PAMPS must be related to the increase of BLG's negative charge, which promotes Coulombic repulsion between BLG and the polyelectrolytes. In a previous report,<sup>13</sup> the linear relationship between  $K_{\text{obs}}$  for BLG–PSS and the net protein charge suggested that a modification of Record's model, that is, eq 1, could be applied to the binding of BLG to PSS at pH  $> pI$ . In the present work, the relationship between  $\log K_{\text{obs}}$  and  $\log I$  for BLG–PSS and BLG–PAMPS is linear at low pH, so eq 1 may be appropriate. However, at high pH, eq 1 is inconsistent with the reverse  $I$  dependence. One might take into account in the case of PSS the somewhat controversial consideration of this polymer's hydrophobicity (see below), since hydrophobic forces may increase with added salt, in contrast to electrostatic effects, as seen in salting-out and hydrophobic interaction chromatography.<sup>24</sup> However, these effects are only seen at ionic strengths much larger than those studied here. Therefore, we must consider whether the unusual  $I$  dependence could arise from electrostatic considerations. Rubinstein and co-workers<sup>17</sup> have provided a useful model for this interpretation.

Nonuniform positive sites on the protein must be the "patches" that bind to the polyelectrolyte;<sup>25</sup> that is, the binding domain probably contains both positive and negative charges. Thus, attraction and repulsion arise simultaneously. Structural information about the distribution of positive and negative sites of the protein and the location of the polyelectrolyte is thus necessary to analyze the electrostatic interaction. Several theoretical treatments have appeared for electrostatic interactions in the cases of charged surface–polyelectrolyte,<sup>26–28</sup> charged surface–protein,<sup>29–31</sup> and protein–polyelectrolyte.<sup>14,15,32,33</sup> A particularly simple calculation based upon the distances



**Figure 5.** Model of electrostatic binding of BLG–PSS.

between charged sites was used by Bowman et al.<sup>17</sup> to interpret the stoichiometry of a polyelectrolyte–gelatin complex measured by light scattering. Figure 5 depicts conceptually this model, with  $R_+$  the average distance between the protein's positive sites and the polyelectrolyte's (negative) sites, and  $R_-$  the average distance between the protein's negative sites and the polyelectrolyte's sites. The presence of salt leads to Coulombic screening by the factor  $\exp(-R/R_D)$ , where  $R_D$  is the Debye screening length<sup>34</sup> ( $R_D = 1/\kappa$ ). Therefore, when finite-size factors of charged particles are neglected, the potential energy ( $U$ ) for the electrostatic interaction of the complex can be described<sup>17</sup> by

$$U = -\frac{Q_p}{2\epsilon} \left( \frac{Q_+}{R_+} e^{-R_+/R_D} - \frac{Q_-}{R_-} e^{-R_-/R_D} \right) \quad (2)$$

where  $Q_p$  is the charge of the segment of polyanion associated with the protein molecule which contains  $Q_+$  positive charges and  $Q_-$  negative charges, and  $\epsilon$  is the dielectric constant. The criterion for "patch binding" must be  $R_+ < R_-$  and  $Q_+ < Q_-$ . If  $Q_+$ ,  $Q_-$ ,  $R_+$ , and  $R_-$  are independent of  $I$ , the electrostatic interaction depends only upon  $R_D$ . At low  $I$ ,  $R_+ < R_- < R_D$  and neither attraction nor repulsion are screened. At intermediate  $I$ ,  $R_+ < R_D < R_-$  and only repulsion is screened. At high  $I$ ,  $R_D < R_+ < R_-$  and both attraction and repulsion are screened. Thus, the condition of  $R_+ < R_D < R_-$  will produce a maximum of  $U$  with respect to  $I$ .

The maximum in  $U$  can be calculated from differentiation of eq 2 by  $\kappa$ , so  $\partial U/\partial \kappa = 0$  yields the following:

$$\kappa = -\frac{1}{(R_- - R_+)} \ln \left( \frac{Q_+}{Q_-} \right) \quad (3)$$

Here, a meaningful value of  $\kappa$  in eq 3 can be obtained only for  $Q_+ < Q_-$  and  $R_+ < R_-$ . Thus, a maximum in  $U$  at an arbitrary  $I$  can be obtained only on the condition of "patch binding". According to ref 35, the positive and negative charges of BLG are 42 and 56, respectively, so  $Q_+/Q_- \approx 1/1.3$ , leading to a maximum point of  $U$  at  $I = 0.03$  at  $R_- - R_+ = 0.45$  nm. Adopting, as one possible case,  $R_- = 2.45$  nm and  $R_+ = 2$  nm, we obtained the potential energy function  $U/2\epsilon Q_p Q_+$  calculated from  $\kappa = 3.288\sqrt{I} \text{ nm}^{-1}$  at 25 °C. As shown in Figure 6, this function effectively reproduces the features of BLG–PSS's  $K_{\text{obs}}$  in Figure 4. Moreover, when pH increases, and  $Q_+/Q_-$  decreases, the maximum point of  $U/2\epsilon Q_p Q_+$  occurs at lower  $I$ . The values  $R \approx 2$  nm must be considered very approximate because the simple model of Figure 5 lacks structural detail and charge multiplicity, both of which would be required for more rigorous study using computational modeling. However, the present simple model does reproduce with some fidelity the pH and  $I$  dependence of  $K_{\text{obs}}$  for BLG–PSS.

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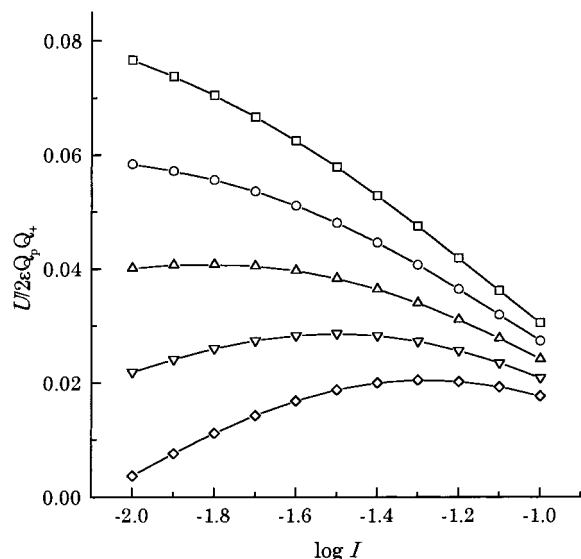
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**Figure 6.** Electrostatic potential energy of the BLG-PSS complex versus  $I$  at  $R_+ = 2$  nm and  $R_- = 2.45$  nm: (□)  $Q_-/Q_+ = 1:1$ ; (○)  $Q_-/Q_+ = 1:1.1$ ; (△)  $Q_-/Q_+ = 1:1.2$ ; (▽)  $Q_-/Q_+ = 1:1.3$ ; (◇)  $Q_-/Q_+ = 1:1.4$ .

**Effect of Polyelectrolyte Stiffness.** Since the positive sites of the protein and the negative sites of the polyelectrolyte are not complementary, the binding force must also depend on the flexibility of the polymer, a more flexible polyelectrolyte conforming more readily to a bound configuration of lower energy and thus having a larger  $K_{\text{obs}}$ . The flexibility of polymers is expressed via the persistence length ( $L_p$ ). For polyelectrolytes,  $L_p$  depends on  $I$  because of intramolecular electrostatic forces, but the role of such forces in short-range binding is unknown. In this case, the intrinsic  $L_p$  (independent of  $I$ ) must be considered. The values of  $L_p$  of PSS and PAMPS at infinite ionic strength are 1.4 and 2.4 nm, respectively.<sup>36</sup> Therefore, it is possible that the large  $K_{\text{obs}}$  of PSS in comparison with that of PAMPS is partly due to its greater chain flexibility.

**Effect of Hydrophobic Interaction.** The large  $K_{\text{obs}}$  of PSS raises the question of hydrophobic interaction. Since PSS has a "backbone" including phenyl residues and PAMPS has polar peptide residues near the vinyl groups, PSS could be regarded as more hydrophobic than PAMPS. BLG certainly is a hydrophobic protein,<sup>37</sup> and hydrophobic pockets have been reported, one of them possibly related to dimer formation at pH 7.<sup>38</sup> However, the hydrophobicity of PSS is not clear, and indeed there are two mutually exclusive descriptions of PSS in the literature. The majority view contains the implicit perspective that PSS is a model polyelectrolyte, and in fact experimental support for a great part of polyelectrolyte theory rests on measurements with PSS. On the other hand, a number of workers have alluded to the hydrophobicity of PSS in order to explain a variety of observations, including fluorescence probe properties,<sup>39,40</sup> surfactant and dye binding,<sup>41,42</sup> and retention in size exclusion chromatography.<sup>43,44</sup>

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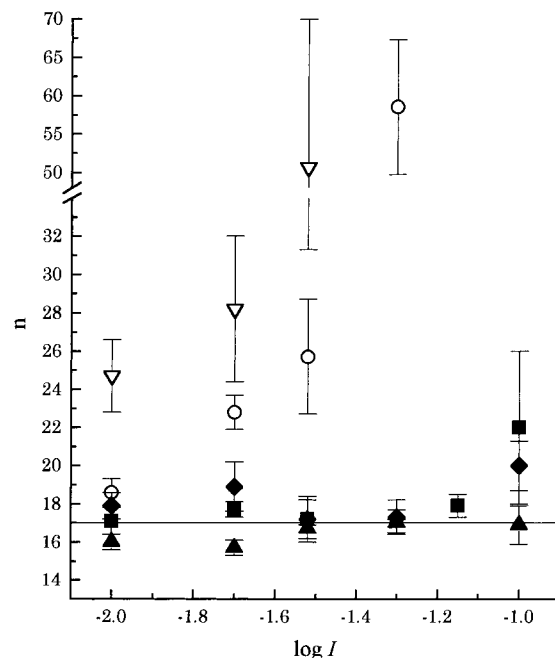
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**Figure 7.** Effect of ionic strength on the number of binding sites: (◆) BLG-PSS at pH 7.0; (■) BLG-PSS at pH 6.7; (▲) BLG-PSS at pH 6.3; (▽) BLG-PAMPS at pH 6.3; (○) BLG-PAMPS at pH 6.1. The solid line is  $n = 17$ .

The situation is greatly complicated by the fact that PSS as used in most studies, obtained from Pressure Chemical Co., has been generally found to have 80–90% sulfonation. The effect of unsulfonated styrene residues could be large if their distribution is inhomogeneous, but this issue is highly conjectural. Unfortunately, there are no ready techniques for detecting this compositional polydispersity, and many studies with PSS fail to even characterize the material with respect to % sulfonation. Regardless of the source of the polymer, statements about the aromatic "hydrophobic backbone" of PSS could be simplistic, since any space-filling model reveals that the styrene groups are inaccessible to solvent due to the bulky sulfonate groups, as it is difficult to imagine a greater effect on water structure by styrene (i.e. a hydrophobic effect) than by the more highly exposed sulfonate groups. Space-filling models in which the styrene residues are strongly stacked do exhibit regions of "exposed" carbon-carbon backbone,<sup>45</sup> but given the uncertainty of the force fields used to construct such models, the influence or existence of such regions is highly speculative. The fact remains that the binding strength of PSS to BLG is larger than that of PAMPS. We obtained identical binding isotherms for Pressure Chemical (ca. 85% sulfonation) PSS and for our principal material, which, being polymerized from sodium styrenesulfonate, is 100% sulfonated;<sup>13</sup> therefore, we cannot attribute this effect to the influence of unsubstituted styrene residues.

Gao and Dubin<sup>46</sup> studied the hydrophobic contribution to  $K_{\text{obs}}$  between BLG and a series of alternating copolymers of maleic acid and alkyl vinyl ethers and found that a minimum alkyl chain length of three or four methylenes was required for significant hydrophobic interactions to occur. Similar hydrophobic properties were also observed for the binding between BSA and hydrophobically modified

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polyacrylates.<sup>47</sup> It can be stated that BLG and BSA tend to bind to more hydrophobic polymers, but it must be understood that the term "more hydrophobic" refers to the accessibility of the polymer's alkyl chain. Thus, neither poly(acrylic acid) nor the copolymers of maleic acid with either methyl or ethyl vinyl ether show any indication of hydrophobic interaction with the proteins studied. On the other hand, for PSS, protein binding is increased when BLG is replaced by BSA which is considered to be a more hydrophobic protein.<sup>37</sup> Circumstantial evidence therefore suggests that some type of hydrophobic interaction is responsible for the large  $K_{\text{obs}}$  of BLG-PSS, although an unresolved question is the manner of contact between the apolar regions of polymer and protein.

**Binding Site Size.** Figure 7 shows the binding site size,  $n$  (number of polymer repeat units divided by number of proteins bound, per polymer chain), for BLG-PSS as a function of  $I$ . The value of  $n$  is approximately 17 and independent of  $I$  and pH, in disagreement with a previous result<sup>13</sup> in which  $n$  decreased with pH. As shown in Figure 7,  $n$  of BLG-PAMPS increases strongly to  $\sim 50$  with  $I$ . The absence of an  $I$  dependence and the small value of  $n$  correspond to large  $K_{\text{obs}}$  values of BLG-PSS, while for BLG-PAMPS the  $I$  and pH dependencies and large  $n$  correspond to small  $K_{\text{obs}}$  values. These results indicate that the binding of BLG-PSS is "tight" (efficiently ion-paired) and that the binding of BLG-PAMPS is "loose". Possibly, the electrostatic interaction between BLG and PAMPS is relatively loose because polymer stiffness (larger persistence length) prevents the chain configuration from adjusting to the charge array of the protein. This "loose" binding corresponds to large  $n$  and is susceptible to the effect of salt. In contrast, the electrostatic interaction between BLG and PSS is "tight", possibly due both to the increased polymer flexibility and to the hydrophobic interactions. This "tight" binding corresponds to small  $n$  and is less susceptible to the effect of pH and  $I$ .

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The value of the binding site size,  $n = 17$ , of BLG-PSS indicates the polymer contour length per bound protein is  $\sim 5$  nm. The Stokes radius of BLG is  $\sim 2.7$  nm.<sup>48</sup> Since the binding chain contour length is approximately equal to the diameter of BLG, PSS cannot "wind around" BLG. Such binding without any compaction or collapse of the polymer chain is in agreement with previous light-scattering results, which showed the BLG-PSS complex to be free-draining.<sup>11</sup> This strong but highly localized binding is consistent with the unique interaction of PSS with BLG.

## Conclusion

The ionic strength dependence of  $K_{\text{obs}}$  for BLG-PSS is complicated and cannot be explained by simple electrostatic treatments such as those of Record et al.,<sup>14,15</sup> which in any event cannot be easily applied to the binding between protein and polyelectrolyte "on the wrong side" of the isoelectric point. The complex ionic strength dependence of  $K_{\text{obs}}$  can, however, be qualitatively interpreted on the basis of Coulomb forces and taking into account protein charge heterogeneity. Striking contrasts between BLG-PSS and BLG-PAMPS, including the large  $K_{\text{obs}}$  for the former, are believed to be related to the chain flexibility and hydrophobicity of PSS, although the second effect is poorly understood.

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