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Short Communication

Chromatographic evaluation of the binding of lysozyme to poly(dimethyldiallylammonium chloride)

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Abstract

Size-exclusion chromatography on Superose columns was used to examine the binding of lysozyme to a strong polycation, poly(dimethyldiallylammonium chloride). A modified Hummel–Dreyer method was employed to determine the number of protein molecules bound per polymer chain as a function of protein concentration, in 0.325 *M* buffer, at pH 9.0. Even though this pH is smaller than the isoelectric point, the protein binds to this polycation. The binding data could be fit to Hill's equation, and the resulting fitting parameters indicate that the binding is cooperative.

1. 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 coacervates [3–6] and 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 polyelectrolytes and proteins [16–19] particularly in regard to the mechanism of the complex formation and complex composition. For the complexation of bovine serum albumin (BSA), ribonuclease and lysozyme with both polycations and polyanions, in solutions of moderate ionic strength, Dubin and co-workers [16,19] proposed formation of non-stoichiometric soluble complexes prior to phase separation. Kokufuta *et al.* [20], employed colloidal titration to study complexation between human serum albumin (HSA) and poly(dimethyldiallylammonium chloride) (PDMDAAC) and potassium poly(vinyl alcohol sulfate) (KPVSD) in pure water. Titrating the protein with the polyelectrolytes, they found turbidity maxima (referred to as end points) corresponding to conditions under which the mole numbers of quaternary

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ammonium groups in PDMDAC and sulfates groups in KPVS were approximately identical to the contents of the acidic and basic groups in HSA. Therefore, it was concluded that complexation between HSA and PDMDAC and KPVS involves "stoichiometric" binding.

The stoichiometric structure of protein–polyelectrolyte complexes has been studied by methods such as sedimentation [14,21], X-ray scattering [14], light scattering [23] and size-exclusion chromatography (SEC) [17,24]. Among these methods, the former three are rather complicated with regard to both technique and the requirements of special equipment. In SEC, the relatively simple so-called Hummel–Dreyer (HD) method [25] may be used to determine the binding of one solute ("ligand") to a larger one, from which it is resolved chromatographically. However, the application of the HD method to the study of protein–polymer complexation is quite recent [17,24] and relatively underutilized. In this study, we use the HD method to study the association behavior of lysozyme with poly(dimethyldiallylammonium chloride).

2. Experimental

2.1. Materials

Poly(dimethyldiallylammonium chloride), a commercial sample "Merquat 100" with nominal molecular mass (M_r) of $2 \cdot 10^5$ and polydispersity of $M_w/M_n \approx 10$ (M_w = weight-average molecular weight, M_n = number-average molecular mass) was obtained from Calgon (Pittsburgh, PA, USA). The commercial sample was fractionated via SEC prior to use. The fractionation of PDMDAAC was carried out using a mobile phase of 0.5 *M* $NaNO_3$ buffered with 25 m*M* NaOAc of pH 6.5, which has been found to sufficiently repress adsorption effects. A 40.0-mg amount of polymer was applied to a Sephacryl S400 gel column via a 2.0-ml sample loop. The mobile phase was eluted through the column at a velocity of 2.0 ml/min, and the eluent was monitored using a R401 differential refractometer (Waters). The injected sample was separated into 30 fractions, collected at 4.8-ml intervals following the beginning of sample elution. The fraction with M_r of $1.97 \cdot 10^5$ and $M_w/M_n = 1.2$, characterized by light scattering and SEC, is used in this study. Lysozyme was obtained from Sigma as 95–99% pure with a p*I* of 11.0. All salts used in the present work were analytical-reagent grade and obtained from Sigma. Distilled and deionized water was used in all experiments.

2.2. Turbidimetric titration

Turbidimetric titrations were carried out at 22°C in solutions of the desired ionic strength. A 2-ml micro-buret (Gilmont) was used to deliver titrant (0.10 *M* NaOH) and the turbidity was followed with a Brinkmann PC600 probe colorimeter (420 nm, 2 cm pathlength). Solutions were always stirred, and turbidity values were obtained after several minutes of stabilization. In "type I" titrations NaOH was added to an initial solution of PDMDAAC, lysozyme and NaCl at a pH around 6. A pH electrode connected to Beckmann Φ34 pH meter was used to monitor pH change during the titration and the turbidity was recorded as a function of pH.

2.3. Size-exclusion chromatography

SEC was carried out on an apparatus comprised of a Minipump (Milton Roy), a Model 7012 injector (Rheodyne) equipped with a 100-μl sample loop, an R401 differential refractometer (Waters), and a Model 120 UV detector ($\lambda = 254$ nm) (Gilson). A Superose-6 column (30 cm × 1 cm OD) (Pharmacia) was eluted at 0.34 ml/min. Column efficiency, determined with acetone, was at least 12 000 plates/m.

Injections were performed in mobile phase of 0.325 *M* ionic strength (*I*) and pH 9.0, under which conditions PDMDAAC and lysozyme form soluble complexes. To determine complex stoichiometry, we have used the HD method. HD experiments were carried out employing 0.25 *M* NaOAc buffer as mobile phase, and the protein concentration in the mobile phase varied from 0.09 to 0.66 g/l. Higher protein concentrations could not be used because of a loss of detector sensitivity at high optical density. Poly-

mer samples (2.0 g/l) were filtered by Gelman 0.2-μm syringe filters before injection.

3. Results and discussion

Fig. 1 shows a Type 1 turbidimetric titration curve of PDMDAAC at polymer concentration of 0.06 g/l in 0.5 g/l lysozyme solution, in 0.325 *M* NaCl. The titration curve displays an abrupt increase in turbidity at pH 11.4, about 0.4 pH units above the isoelectric point of lysozyme, corresponding to colloidal complex formation. Prior to colloid formation, we observe a *ca.* 1% turbidity increase at pH 8.2 for the polymer–protein solution. This small turbidity increase is due to the initial formation of the soluble complex: particles with a size (35–46 nm) larger than either lysozyme (5 nm) or PDMDAAC (25 nm) are detected at this pH by quasielastic light scattering [19]. It is also interesting that the soluble complex is formed at conditions where the protein has the same charge sign as the polymer. This has been interpreted as arising from the existence of non-uniform charge distribution or "surface charge patches" on the protein [19].

The HD SEC method was used to evaluate the binding of lysozyme to PDMDAAC at $I = 0.325$ *M*, $8.5 < \text{pH} < 10$, conditions under which soluble complexes are formed (see Fig. 1). PDMDAAC was injected onto an SEC column in which the mobile phase is a buffer, adjusted to the desired pH and ionic strength, and containing some appropriate concentration of lysozyme. As an example, a UV (254 nm) chromatogram resulting from the injection of PDMDAAC into a mobile phase of pH 9.20, 25 m*M* $Na_2B_4O_7$ and 0.25 *M* NaCl buffer, containing 0.093 g/l lysozyme, is displayed in Fig. 2. The initial UV-absorbing peak is observed at a smaller elution volume than that of protein alone, and must correspond to soluble complex. Its elution volume, 17.9 ml, is found to be independent of the protein concentration over the range studied. This result is consistent with highly cooperative binding (see below). Based on a calibration of the SEC column with pullulan standards, the apparent hydrodynamic radius $R_{SEC(pul)}$ of the complex is quite small, about twice the diameter of the protein. However, it is unlikely that the retention of the complex is free of solute-packing interaction effects; indeed, the very small $R_{SEC(pul)}$ observed for PDMDAAC (upper trace) is strong evidence for adsorption of the polycation. Interpretation of retention volumes in terms of size are therefore inappropriate.

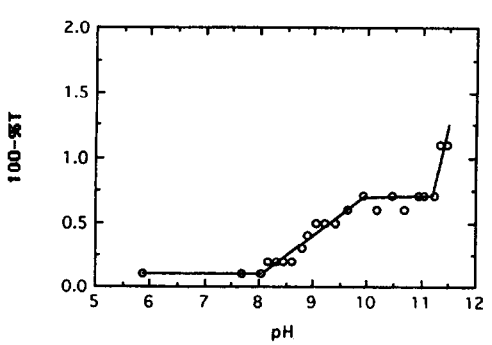

Fig. 1. Type I turbidimetric titration of 0.06 g/l PDMDAAC and 0.5 g/l lysozyme in 0.325 *M* NaCl, showing pH-dependence of % transmittance.

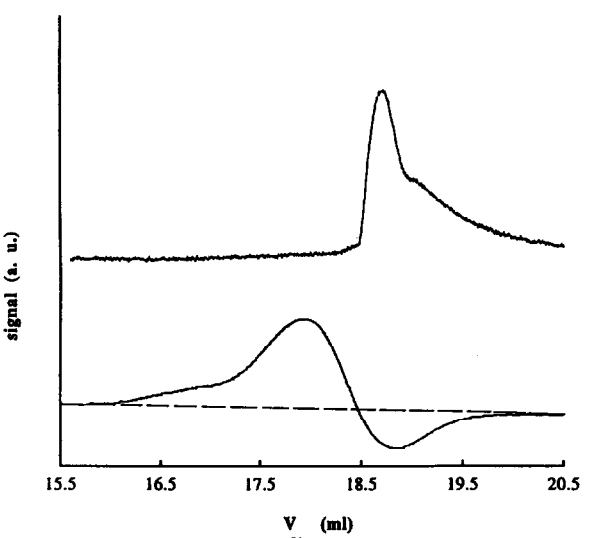

Fig. 2. UV (254 nm) chromatogram (Superose 6) of PDMDAAC in mobile phase comprised of 0.25 *M* NaCl + 25 m*M* $Na_2B_4O_7$, pH 9.2 buffer, containing 0.093 g/l lysozyme. Broken line shows baseline used for peak integration. For purposes of comparison, the chromatogram of PDMDAAC in protein-free mobile phase (monitored by refractive index) is shown above. Note that PDMDAAC itself is UV-inactive and so makes no contribution to the lower chromatogram.

In addition to the positive peak for the complex, one also sees the loss of protein from the mobile phase, as evidenced by the negative peak at the retention volume of lysozyme (*ca.* 18.9 ml). The area under this peak is proportional to the amount of protein bound to the injected polymer sample. The width of the two peaks may be in part a manifestation of some degree of adsorption of solutes on the column, as noted above, which at this pH surely bears some negative surface charge (although one cannot exclude the possible contribution of slow exchange between free and bound protein as a contributing effect). The moderate ionic strength of the mobile phase would be expected to diminish, but not eradicate such adsorptive band spreading. Even though the chromatographic peaks are larger than would be expected —at least for the unbound protein— the separation was sufficient to allow for quantitation of bound protein.

At $I = 0.325$ (25 m*M* $Na_2B_4O_7 + 0.25$ *M* NaCl), the amounts of protein corresponding to the negative peak area were determined from a calibration plot of peak area *vs.* protein concentration. The broken line in Fig. 2 illustrates the extrapolation of the baseline required for determination of the peak areas. Various detector level sensitivities were employed at different protein concentrations in order to optimize the accuracy of this measurement. Two injections were made for each protein concentration and the results were virtually identical in all cases. These results were used to calculate the degree of binding n, the molar ratio of bound lysozyme to polymer, *i.e.*, the number of protein molecules bound per polymer chain. Shown in Fig. 3 is n as a function of lysozyme concentration, obtained at $I = 0.325$ and pH 9.20.

In general, binding of ligands to macromolecules can be described by Hill's equation [22]

$$n = \frac{[L]_{bound}}{[P]} = \frac{c_1[L]^z}{1 + c_2[L]^z} \quad (1)$$

where n is the degree of binding, [L] is the free protein concentration, $[L]_{bound}$ is the bound protein concentration and [P] is the total polymer concentration, and c_1 and c_2 are constants related to the intrinsic binding constant and the number of sites. z is an empirical exponent, called Hill's coefficient. The quantity z is a measure of cooperativity; if $z = 1$, non-cooperativity is observed, *i.e.*, a single binding constant governs the independent binding of all ligands. If $z > 1$, the binding is cooperative, which means that the second ligand binds more readily than the first. If $z < 1$, then the binding is anti-cooperative.

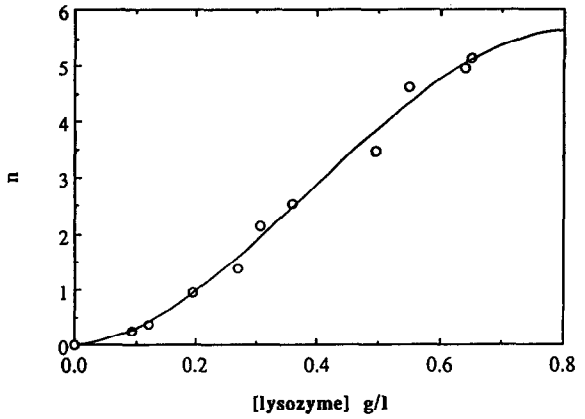

Fig. 3. Degree of binding n as a function of mobile phase lysozyme concentration ($I = 0.325$, pH 9.2, PDMDAAC concentration = 2.0 g/l). The solid line corresponds to Eq. 1 with $z = 2$.

In cooperative or anti-cooperative binding, the initial binding usually induces a conformation change in the macromolecule, thus affecting subsequent binding. For non-cooperative binding, c_1 and c_2 are identical to the binding constant. In the case of cooperative binding, c_1 and c_2 have no clear-cut physical definition because the overall cooperative binding constant is a function of both the intrinsic binding constant and the number of binding sites. The case dealt with here is of course more complicated than the model underlying Eq. 1 inasmuch as the polymer itself is capable of drastic conformational change accompanying the binding of the ligand —lysozyme— and the binding sites are not particularly well-defined. Nevertheless, the Hill equation may be considered a zeroth-order approach to analysis of the current binding results.

The binding data in Fig. 3 are fitted to Eq. 1, as shown by the solid curve, yielding $c_1 = 29.2$, $c_2 = 3.5$ and $z = 2$. The value of z suggests a cooperative association between lysozyme and PDMDAAC at the conditions of this study. On the other hand, the number of proteins bound per polymer chain at saturation conditions is only 6, which is considerably less than values found for other protein-polyelectrolyte pairs, such as BSA-PDMDAAC [23]. This may be a reflection of the strong repulsive electrostatic interactions that arise when lysozyme, at pH < p*I*, is complexed with a strong polycation. It is somewhat surprising that, despite these repulsive interactions, the binding is nevertheless cooperative. One possibility is that extension of the polymer chain upon binding enhances the binding of subsequence proteins. Such chain expansion during protein binding to a polyelectrolyte has been observed elsewhere [23].

In summary, we have shown, by both turbidimetric titration and HD SEC, the existence of stable, soluble complexes formed by lysozyme and PDMDAAC in pH 9.2 and $I = 0.325$ *M* supporting electrolyte. The binding of lysozyme to PDMDAAC to form soluble complexes is cooperative.

4. Acknowledgements

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5. References

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