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MODEL OF THE POLYELECTROLYTE PRECIPITATION OF GENETICALLY ENGINEERED ENZYMES POSSESSING CHARGED POLYPEPTIDE TAILS

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ABSTRACT

A model is presented for the polyelectrolyte precipitation of proteins possessing charged fusion tails. The model is based on multiple equilibria binding and accounts separately for the binding of the fusion polypeptide. The predictions of the model are compared to experimental results obtained with monomeric and multimeric fusion proteins. The enzymes investigated were various fusions of glucoamylase from *Aspergillus niger* and β-galactosidase from *Escherichia coli*, respectively. Electrostatic cooperativity is not evidenced for the binding of these negatively charged proteins to positively charged, highly branched polyethyleneimine. Qualitative agreement is achieved between the model and experimental results for the behavior of the association constants of the protein and fusion polypeptide with respect to the number of polypeptide charges, ionic strength, and polymer dosage. For the precipitation of multimeric proteins, it is proposed that each of the fusion polypeptides acts as a strong electrostatic interaction site which can preferentially bind the enzyme to multiple polyelectrolytes, resulting in a tightly bound, crosslinked matrix. Increasing the ionic strength leads to a reduction in the electrostatic repulsion within the protein–polyelectrolyte complex. The combination of reduced electrostatic repulsion and the strong binding of the tails results in enhancement of the precipitation as the ionic strength is increased.

INTRODUCTION

The accurate modeling of separation methods offers two advantages: gaining an understanding of the mechanisms and their relative importance in effecting the separation, and accurate prediction of the conditions which will result in a desired separation. Many models of various separation processes exist. The accuracy of these models varies depending on the complexity of the process they are attempting to describe. Each of the various general methods of precipitation have been modeled to some degree [1, 2]. Authors who have modeled the various aspects of polyelectrolyte precipitation of proteins include Clark and Glatz [3] and Fisher and Glatz [4]. Due to the complexity and vast number of proteins, not to mention other cellular components, these models focus on defined systems.

Our work has focused on the enhancement of polyelectrolyte precipitation through the genetic fusion of charged polypeptides [5–8]. A model to account for the enhancement of precipitation as a result of these polypeptide tails is developed here. Development of the model closely follows that of Clark and Glatz [3]. Before proceeding with the deliberation of the model itself, the effects which the model is intended to account for will be presented. In their work involving the precipitation of the egg white proteins lysozyme and ovalbumin by carboxymethyl cellulose (CMC), Clark and Glatz [9] came to several conclusions concerning the effects of pH, polymer dosage, and ionic strength on protein recovery and fractionation:

1. Only proteins possessing a charge opposite to that of the polyelectrolyte are precipitated, and those of higher charge density are precipitated preferentially.
2. The efficiency of precipitation increases with protein charge; less polyelectrolyte is needed to achieve precipitation of proteins possessing higher charge.
3. Up to an optimal polymer dosage, protein removal increases with polyelectrolyte dosage. At dosages higher than the optimum, protein removal decreases. Highly charged proteins are less susceptible to the latter effect.
4. An increase in ionic strength increases the required polymer dosage to effect the same protein removal, reduces the maximum possible precipitation, and reduces the effect which precipitation pH has on protein recovery.
5. Fractional precipitation can be attained by the proper adjustment of pH or polymer dosage. If the target protein is highly charged, the efficiency of fractionation can be improved by increasing the ionic strength; the target protein will be purified to a higher degree.

An initial attempt at modeling the precipitation process as a soluble analog to ion exchange was made by Clark [10]. The model was based on Carlson's [11] model for protein–ion exchange. The model incorporates phase equilibria criteria and assumes the polyelectrolyte to be entirely in the solid phase. The latter assumption has been found by Shieh [12] to be true for poly(acrylic acid) (PAA) and CMC precipitations of egg white proteins and pure lysozyme over nearly the entire dosage range. Slight deviations were observed at high and low dosages. Hill and Zadow [13, 14] found the assumption to be not entirely accurate for precipitations with

CMC. Clark's model was found to have several failings, even though qualitative prediction was obtained up to the optimum polyelectrolyte dosage:

1. The model predicted a significantly lower protein recovery than was found experimentally.
2. The predicted effect of ionic strength on the protein removal was found to be much greater than experimental results.
3. The increased solubility of protein–polymer complexes at polyelectrolyte dosages greater than the optimum was not predicted by the model.

The model finally proposed by Clark and Glatz [3] assumes multiequilibrium and cooperative binding. The phenomenon where a substrate binds a variable number of ligands is defined as multiequilibrium. Cooperative binding accounts for the effect which ligands already bound to a substrate have on subsequent binding. Whether further binding is enhanced or deterred is termed positive or negative cooperativity, respectively [3, 15, 16]. Cooperativity is an effect often observed in biological systems. A modification of the Debye–Huckel theory was used to account for the electrostatic effects responsible for cooperative binding.

DEVELOPMENT OF THE MODEL

To account for the binding of multiple ligands to a macromolecule, the theory of multiple equilibria is used. Multiple equilibria defines the case in which multiple ligand molecules can bind to each macromolecule. The model is based on the fact that the macromolecules will bind various amounts of ligand. For the purposes of modeling polyelectrolyte precipitation, the polyelectrolyte, X, will be defined as the macromolecule which has n binding sites for a protein ligand, P. The polyelectrolyte can exist in $n + 1$ forms if interactions other than polyelectrolyte–protein interactions are neglected. Since the macromolecule can exist in many states, an average number of ligands bound to the macromolecule is often used to express the multiple equilibrium binding. The average binding number, ν, is described by

$$\nu = [\mathrm{P}]_{\mathrm{Bound}}/[\mathrm{X}]_{\mathrm{Total}} \tag{1}$$

where the square brackets denote molar concentrations. The binding would thus be described by n types of reactions and their respective association constants:

$$\begin{aligned} &\mathrm{X} + \mathrm{P} \leftrightarrow \mathrm{XP} && K_1 = [\mathrm{XP}]/[\mathrm{X}][\mathrm{P}] \\ &\mathrm{X} + 2\mathrm{P} \leftrightarrow \mathrm{XP}_2 && K_2 = [\mathrm{XP}_2]/[\mathrm{X}][\mathrm{P}]^2 \\ &\qquad\vdots && \\ &\mathrm{X} + n\mathrm{P} \leftrightarrow \mathrm{XP}_n && K_n = [\mathrm{XP}_n]/[\mathrm{X}][\mathrm{P}]^n \end{aligned} \tag{2}$$

It follows from Eq. (1) that the expression which describes this type of binding is given by

$$\nu = \sum_{i=1}^{n} i[\mathrm{XP}_i] / \sum_{i=0}^{n} [\mathrm{XP}_i] \tag{3}$$

which, upon comparison with Eq. (2), yields

$$\nu = \sum_{i=1}^{n} iK_i[\mathrm{P}]^i / \sum_{i=0}^{n} K_i[\mathrm{P}]^i \tag{4}$$

Tanford [17] and Van Holde [15] have shown that for identical and independent binding sites, multiple equilibria binding can be described by one average association constant, reducing Eq. (4) to

$$\nu = \frac{nK[\mathrm{P}]}{1 + K[\mathrm{P}]} \tag{5}$$

where K is the association constant for the binding of a protein molecule to an unoccupied site on a protein–polyelectrolyte complex and [P] is the molar concentration of unbound protein. This equation assumes that each of the binding sites on the macromolecule possesses the same affinity for the ligand as any other, i.e., the binding is noncooperative. Solving for the association constant yields

$$K = \frac{\nu}{[\mathrm{P}](n - \nu)} \tag{6}$$

which corresponds to a Gibb's free energy change of

$$\Delta G = \Delta G^\circ + RT \ln K \tag{7}$$

Extension to Ligands Possessing Multiple Intrinsic Affinities

The genetically engineered enzymes which we are investigating possess a high charge density polypeptide tail on the surface of the enzyme, which might be expected to have a different affinity toward the macromolecule than the protein surface in general. A schematic of the precipitation of such fusion enzymes by polyelectrolytes is depicted in Fig. 1. An equation for the binding between a large molecule and two ligands (small molecules or ions, in the original development), each of which possess different affinities for the large molecule, was developed by Tanford [17] and Van Holde [15]. The general expression which describes such binding is

$$\nu = \frac{n_p K_p[\mathrm{P}]}{1 + K_p[\mathrm{P}]} + \frac{n_t K_t[\mathrm{P}]}{1 + K_t[\mathrm{P}]} \tag{8}$$

where the subscripts p and t denote the two different ligands. As used here, a single protein ligand possesses two different affinities for the polyelectrolyte, depending on whether binding is dominated by the protein itself (subscript p) or by the charged fusion tail (subscript t).

In the absence of the tail, $n_t = 0$ and Eq. (8) reduces to Eq. (6). The latter part of Eq. (8) represents the additional protein bound to the polyelectrolyte through interactions with the tail. It then follows from Eq. (8) that the determination of K_t is made relative to the control enzyme which lacks the fusion tail. K_t/K_p would thus be an indication of the strength of binding of the tail relative to that of the control protein. The increased binding strength resulting from the presence of the charged fusion peptides has already been demonstrated using ion-exchange chromatography

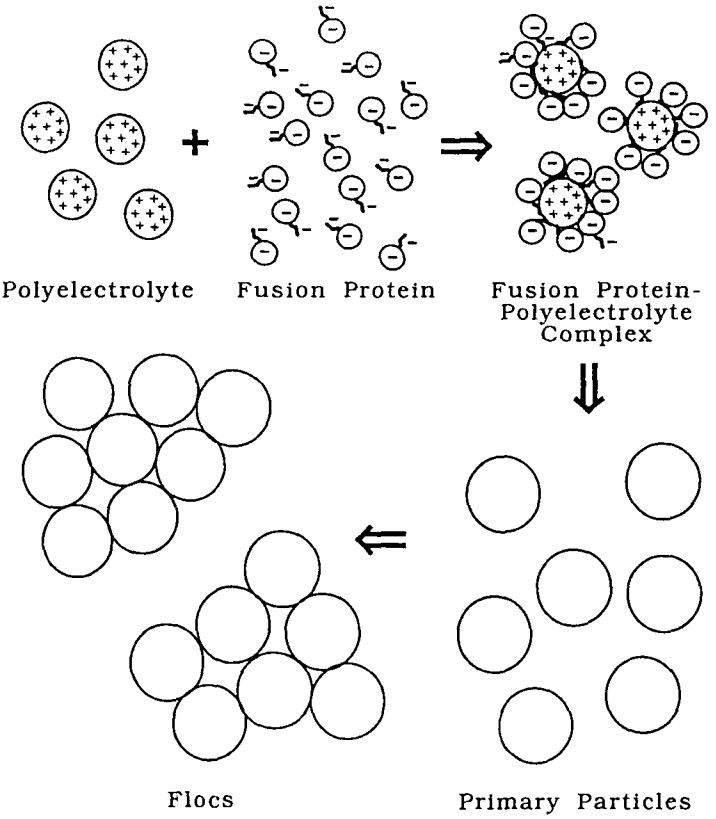

FIG. 1. Schematic of polyelectrolyte precipitation for enzymes possessing charged fusion polypeptides.

[8]. Higher ionic strengths were required to elute the tailed enzymes from the ion-exchange column.

The first term on the right-hand side of Eq. (8) describes the interaction of the unmodified protein with the polyelectrolyte. It is assumed that there is no interaction between the protein and the tail, hence the values for n_p and K_p would be the same in the presence or absence of the tail. The calculation of n_p based on charge equivalence through the assumption of a neutral complex, as was made by Clark and Glatz [3] in their model, is not made here because the size of the enzymes will sterically limit binding. The effect of steric hindrance can be demonstrated for the two enzymes studied. The hydrodynamic radii of the 470 kDa β-galactosidase and the 65 kDa glucoamylase were estimated to be 84.1 and 37.8 Å, respectively, using the correlation of Teller [18] for monomeric and multimeric proteins. The highly branched form of polyethyleneimine (PEI) used in the precipitations has been shown to assume a spherically symmetric compact shape in solution [19]. The hydrodynamic radius of the 55 kDa polyelectrolyte was estimated to be 130 Å at I = 0.1 M, using the correlation between molecular weight and size from Lindquist and Stratton [20] and Hostetler and Swanson [19]. The maximum number of proteins

which could sterically interact with the polyelectrolyte was estimated by treating both the protein and polyelectrolyte as hard spheres to determine how many proteins could pack on the surface of the polyelectrolyte. This number was approximated as the number of proteins which could pack (square-pitch) on a flat surface whose area was equivalent to that of a sphere with a radius equal to the combined radii of the protein and polyelectrolyte. Using the hydrodynamic sizes given above, n_p was found to be sterically limited to 20 for β-galactosidase and 61 glucoamylase. These estimates are well below the $n_p = 28$ and 300, respectively, found by assuming charge equivalence of the protein and PEI at the experimental pH (see Table 1).

Cooperativity as a Result of Electrostatic Interactions

If the binding of a ligand to one site does influence the affinity of other sites, the binding is said to be cooperative. Tanford [17] proposed that K be defined in terms of an intrinsic association constant, K_{int}, and a cooperativity function, Φ. The advantages of this definition are that it accounts for the effects of cooperative binding through a single function, and that the equilibrium behavior can be described in terms of a single association constant rather than n association constants. The form of the cooperativity function will depend on the nature of the interaction. For negative cooperativity, the strength of binding, K, will decrease as the number of bound sites, ν, increases.

Tanford developed a model for the case where cooperativity was the result of electrostatic binding between charged species where all electrostatic interactions vanish when the net average charge of the complex, Z, is zero. Adapted to this situation [3], the polyelectrolyte is assumed to have n identical binding sites for the protein, each of which displays cooperative binding. For this system it is convenient to define a cooperativity function with an intrinsic association constant, K_{int}, as

$$K = K_{int}e^{-\Phi(Z)} \tag{9}$$

TABLE 1. Estimated Net Charge for β-Galactosidase, Glucoamylase, and PEI. Net Protein Charges Were Estimated using the Henderson-Hasselbalch Equation and Amino Acid pK Values from Stryer [21]. The Charge on PEI Was Estimated from the Titration Data of Kokufuta [22]

pH	Molecule	Estimated net charge
5.7	BGCD1	−22.5
	BGCD5	−37.7
	BGCD11	−60.6
	PEI	625
4.5	GACD0	−2.5
	GACD5	−5.3
	GACD10	−8.1
	PEI	750

where K_{int} is the limiting value of K when all of the binding sites are filled ($\nu = n$, $Z = 0$). K_{int} will now have a corresponding intrinsic standard free energy change, ΔG°_{int}, such that

$$\Delta G^\circ = \Delta G^\circ_{int} + RT\Phi(Z) \tag{10}$$

As defined, $\Phi(Z)$ is a positive function of Z if the macromolecule and ligand possess like charge, and a negative function of Z if the macromolecule and ligand possess an opposite charge.

Determination of $-\Phi(Z)$ and K_{int}

To evaluate the functionality of $\Phi(Z)$, Tanford [17] proposed that it is directly related to the change in electrostatic free energy of the complex. Clark and Glatz [3] used a modification of the Debye-Huckel theory by Melander and Horvath [23], which incorporates Kirkwood's expression [24] to accommodate for high ionic strength effects, to yield for the cooperativity of binding:

$$\Phi(Z) = ZZ_p\left(A - \frac{B\sqrt{I}}{1 + C\sqrt{I}}\right) \tag{11}$$

The constants were evaluated for aqueous solutions at 25°C to be

$$A = 7.122/R_i \tag{12}$$

$$B = 2.341 \tag{13}$$

$$C = 0.3287a \tag{14}$$

where I is in mol/L, and R_i and a are in Å. The distance of closest approach, a, is determined from the center-to center distance of the macromolecule and the ligand.

The expression for K_{int} incorporated Halicioglu and Sinanoglu's [25] expression for the free energy change of cavity formation upon transferring a solute molecule into solution [3]:

$$RT \ln K_{int} = F - \Delta G^{\circ\prime} + \frac{Z_p^2}{2}\left(A - \frac{B\sqrt{I}}{1 + C\sqrt{I}}\right) + (\Omega - \Lambda)IRT \tag{15}$$

where

$$F = [NH + 4.8N^{1/3}(\kappa^e - 1)V^{2/3}]\omega \tag{16}$$

$$\Omega = [NH + 4.8N^{1/3}(\kappa^e - 1)V^{2/3}]\sigma/RT \tag{17}$$

The latter term on the right-hand side of Eq. (15) contains the terms Λ and Ω, which are commonly referred to as the intrinsic salting-in and salting-out coefficients, respectively. Equation (15) is valid for either K_p or K_i.

Crosslink Formation

The above treatment describes a situation where precipitation results from the increasing hydrophobicity of the protein/polyelectrolyte complex. For a multimeric fusion enzyme such as β-galactosidase, an alternative solubility criteria should be considered. The versions of the enzyme possessing fusion tails would contain multi-

ple strong electrostatic interaction sites. These sites would be capable of forming crosslinks between enzyme-polyelectrolyte complexes by the binding of a single enzyme to multiple polyelectrolytes. The result would be a tightly bound matrix of enzyme and polyelectrolyte, analogous to the picture of affinity matrix formation in the affinity precipitation of multimeric enzymes by bis-ligands [26, 27]. A schematic of this process is shown in Fig. 2. In this scenario, increasing amounts of polyelectrolyte lead to larger complex sizes of decreasing solubility. The formation of an insoluble matrix would then be expected to be dependent on the amount of polyelectrolyte relative to the protein in solution, which is termed the dosage. Furthermore, these complexes would be expected to have a relatively high net charge as a result of steric limitations to binding discussed earlier. One would therefore expect the complexes to be soluble until they reach a high molecular weight through matrix formation. The binding of the polyelectrolyte to the protein would still be governed by the same equations as developed in the previous sections, yet the criteria for precipitation could now be viewed as the formation of a gel matrix of very large molecular weight.

The polyelectrolyte dosage required for gel formation/precipitation can be estimated from the theory of gel formation in polymerization reactions. In such a reaction, successively higher conversion of two types of multifunctional monomers to polymer, in which at least one monomer possesses a functionality greater than 2, increases the probability of forming a network. In the network, all of the monomers of higher functionality in the reaction mass are interconnected. The point at which the statistical probability for the formation of such a network becomes 1 is termed the gel point. The conversion at this point has been derived for the reaction in which one type of molecule is bifunctional and the other has a functionality of $f >$ 2 [28–30]. For such a reaction, the fraction of higher functionality groups that have reacted at the gel point, p_f, is given by

$$(1/p_f)^2 = r(f - 1) \tag{18}$$

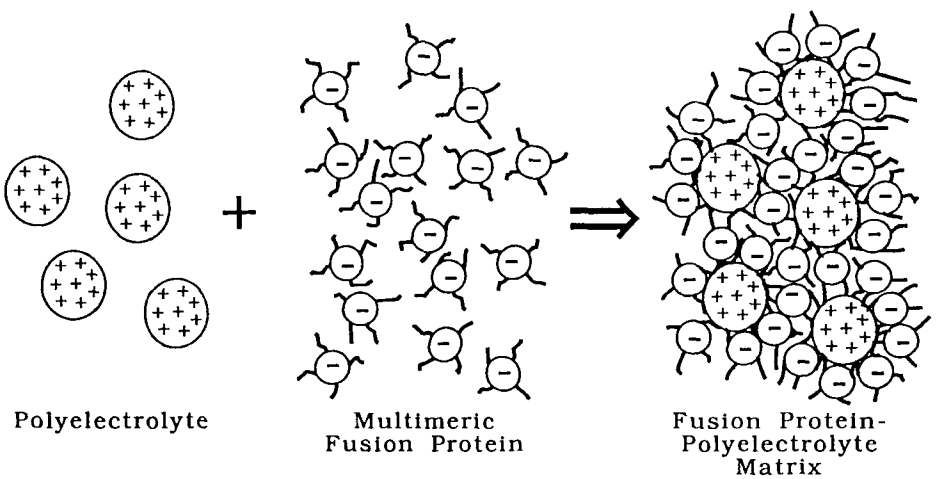

FIG. 2. Precipitation resulting in matrix formation for the polyelectrolyte complexation of multimeric enzymes possessing multiple charged fusion polypeptides.

where r is the molar stoichiometric ratio of the two types of reacting groups. Precipitation would be expected to occur at a point equal to or less than the gel point as a result of the high molecular weight of the complexes formed. Experimentally, precipitation was found by Bobalek et al. [31] to occur prior to the formation of a gel in polymerization reactions.

The polymerization scenario is very similar to the formation of polyelectrolyte–protein complexes in that the polyelectrolyte and multimeric protein both act as polyfunctional monomers in a polymerization reaction. For the purpose of modeling the formation of an interconnected matrix, or gel, in polyelectrolyte precipitation, Eq. (18) could be used as an approximation of the crosslinking occurring between a dimeric protein and a polyelectrolyte. If all four tails of the tetrameric β-galactosidase could take part in network formation, lower values for p_f would result. Equation (18) can be extended to account for this higher functionality, g, of the second "monomer":

$$(1/p_f)^2 = r(f - 1)(g - 1) \tag{19}$$

where $f > g > 2$. It can be seen that for $g = 2$, Eq. (19) reduces to Eq. (18). The gel point can thus be calculated from Eq. (19) by allowing f to be the maximum number of enzymes which can bind to a polyelectrolyte ($f = n$) and inserting the value of r, which is a function of the dosage, D:

$$r = D\left(\frac{M_p}{M_x}\right)\left(\frac{f_x}{f_p}\right) = D\left(\frac{M_p}{M_x}\right)\left(\frac{f}{g}\right) \tag{20}$$

Rearranging Eq. (19) to solve for p_f and inserting Eq. (20) yields

$$p_f = \left[D\left(\frac{M_p}{M_x}\right)\left(\frac{f}{g}\right)(f - 1)(g - 1)\right]^{-1/2} \tag{21}$$

Model Predictions

Equation (9) predicts that ln K_p should vary linearly with Z, having a slope of $-\Theta(Z)/Z$ and an intercept of ln $K_{\text{int},p}$. From Eq. (11), the slope $-\Phi(Z)/Z$ should have a positive value for the interaction of a negatively charged protein or polypeptide tail with a positively charged polyelectrolyte. The absolute value of the slope would be expected to decrease as the ionic strength increases, reaching a constant value at high ionic strength. Since K_t is a modification of K_p, to account for the presence of the tail it should follow the same behavior as K_p. In the case of multiple-tailed enzymes, precipitation (and K_t, if binding was equated with precipitation) would furthermore be expected to be dependent on the polyelectrolyte dosage.

MATERIALS AND METHODS

Two enzymes, glucoamylase and β-galactosidase, were genetically modified and used in the precipitation studies of purified enzymes. Purified enzyme solutions were used to avoid interference from other proteins. The tails of all enzymes were constructed primarily of poly(aspartic acid) and located at the carboxyl terminus of the enzyme. All precipitations were carried out in sodium acetate buffer of various

ionic strengths. PEI of 55 kDa was used as the precipitant. Details as to the construction and production of the enzymes can be found elsewhere [5, 7, 8].

Glucomylase from *Aspergillus niger* was used as the monomeric enzyme for the precipitation studies. Three different fusion enzymes were constructed from a shortened version of the enzyme. All versions were found to retain full activity. The fusion enzymes included a control with no poly(aspartic acid) tail (GACD0), a tail containing 5 aspartic acid residues (GACD5), and a tail containing 10 aspartic acid residues (GACD10). The purification, characterization, and polyelectrolyte precipitation of these enzymes have been published elsewhere [7].

β-Galactosidase from *Escherichia coli* was used as the multimeric enzyme for the precipitation studies using pure enzymes. Three different enzymes were constructed, including a control with no poly(aspartic acid) tail (BGCD1), a tail containing 5 aspartic acid residues (BGCD5), and a tail containing 11 aspartic acid residues (BGCD11) (Zhao et al., 1990). The purification, characterization, and polyelectrolyte precipitation of these enzymes have been published elsewhere [8].

Zeta potential measurements were performed on β-galactosidase/PEI precipitates using a Lazer Zee Meter model 500, Zeta-Potential Instrument (Pen-Kem, Inc.). For these measurements, precipitation with PEI was carried out using commercial (wild-type) β-galactosidase (WTBG) from the Sigma Chemical Co. WTBG was dialyzed to 100 mM NaOAc, pH 5.7, and adjusted to 0.50 mg/mL prior to complexation with PEI (55 kDa, Polysciences). PEI in 100 mM NaOAc, pH 5.7, was added to WTBG at various dosages. The final protein concentration was 0.25 mg/mL.

RESULTS AND DISCUSSION

Application of the model to precipitation studies will be discussed in two separate parts. The first section deals with monomeric enzymes which contain only one polypeptide tail, whereas the second section looks at tetrameric enzymes containing four tails. The system is defined with PEI as the macromolecule and the enzyme as the ligand. The amount of protein precipitated is taken as $[P]_{Bound}$. In order to compare the data to model predictions, the assumption had to be made that all of the PEI in the system was contained in the solid precipitate. This assumption has been shown to be fairly accurate with various polyelectrolytes up to the optimal dosage [3, 8, 12–14]. It then follows from Eq. (1) that ν is actually an apparent binding number based on the amount precipitated. The association constants obtained are then also apparent association constants for the same reason. For simplicity, however, the term "apparent" will be dropped from the results and discussion which follow. It should, however, be kept in mind that the results are based on the observed precipitation and are not a true measure of the binding, i.e., they are only a measure of the binding which results in precipitation.

Application to Monomeric Enzymes: Glucoamylase

The precipitation curves obtained experimentally for the GACD fusion enzymes at the various ionic strengths studied are given in Fig. 3(a), 4(a), and 5(a). Also shown in these figures are the respective binding numbers calculated from Eq. (1).

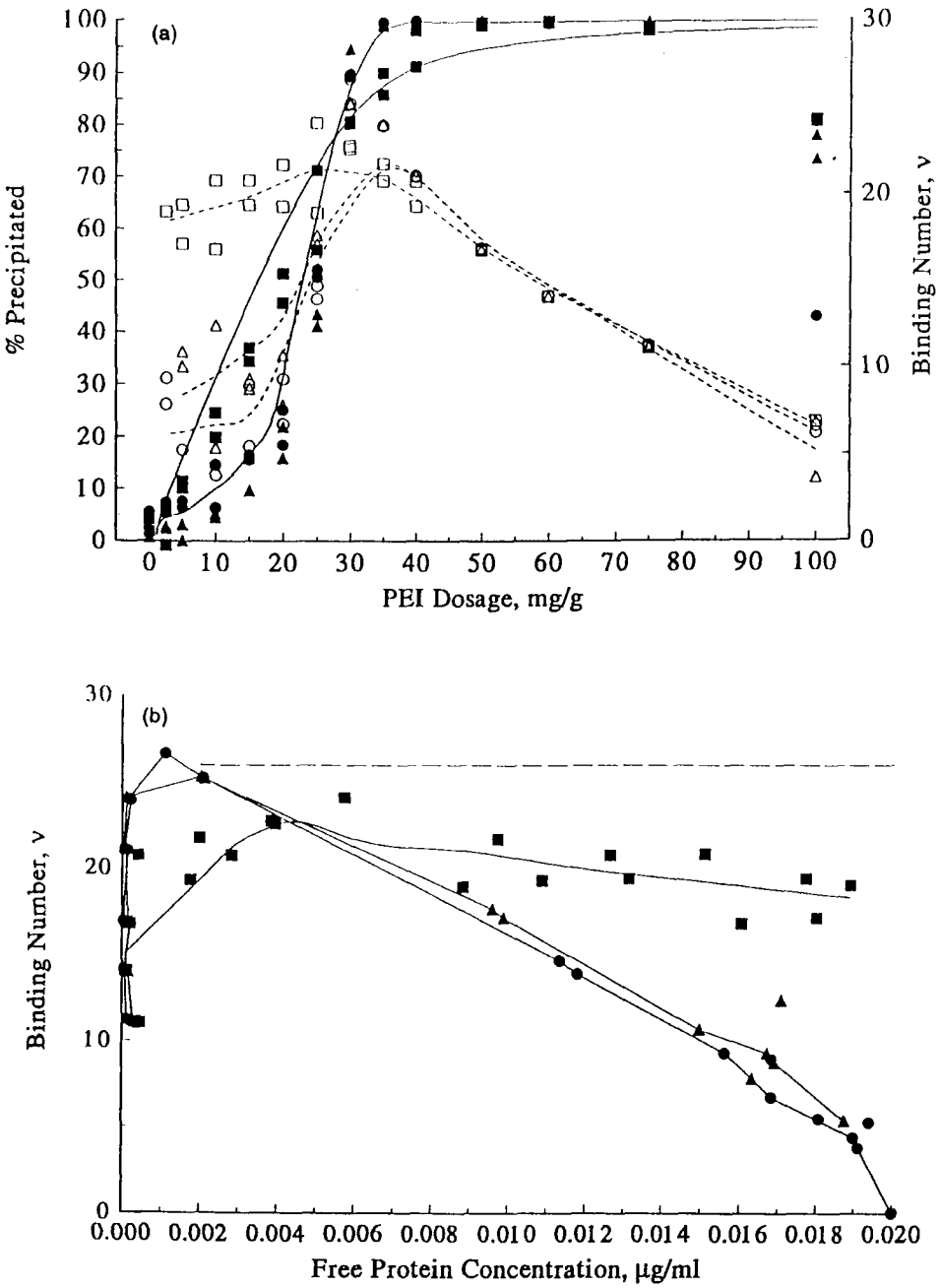

FIG. 3. The complexation of glucoamylase with PEI at I = 20 mM, pH 4.5: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data; (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, GACD0; ▲, △, GACD5; ●, ○, GACD10. The dashed line indicates an adjusted ν for the tailed enzymes.

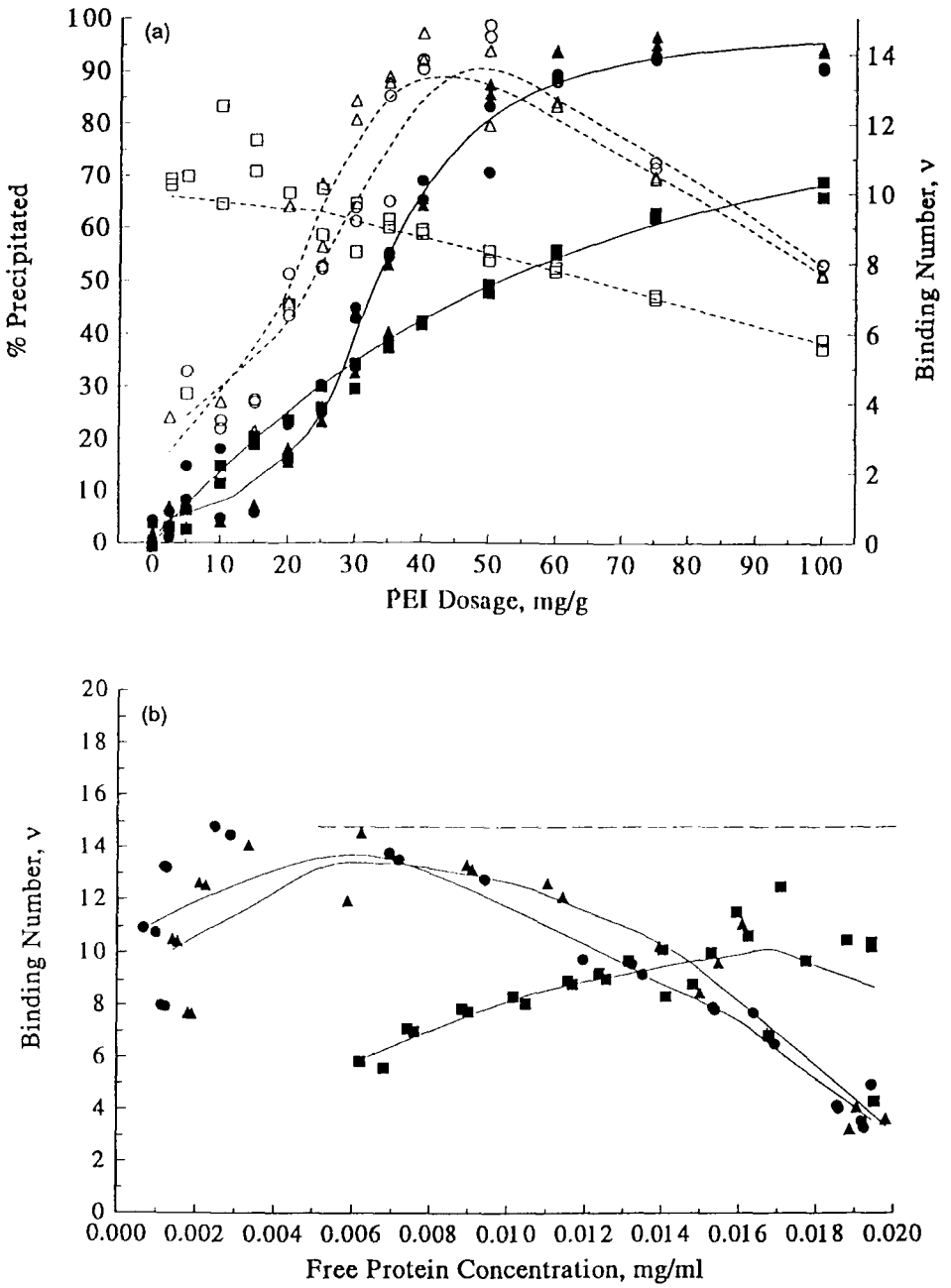

FIG. 4. The complexation of glucoamylase with PEI at I = 100 mM, pH 4.5: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data; (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, GACD0; ▲, △, GACD5; ●, ○, GACD10. The dashed line indicates an adjusted ν for the tailed enzymes.

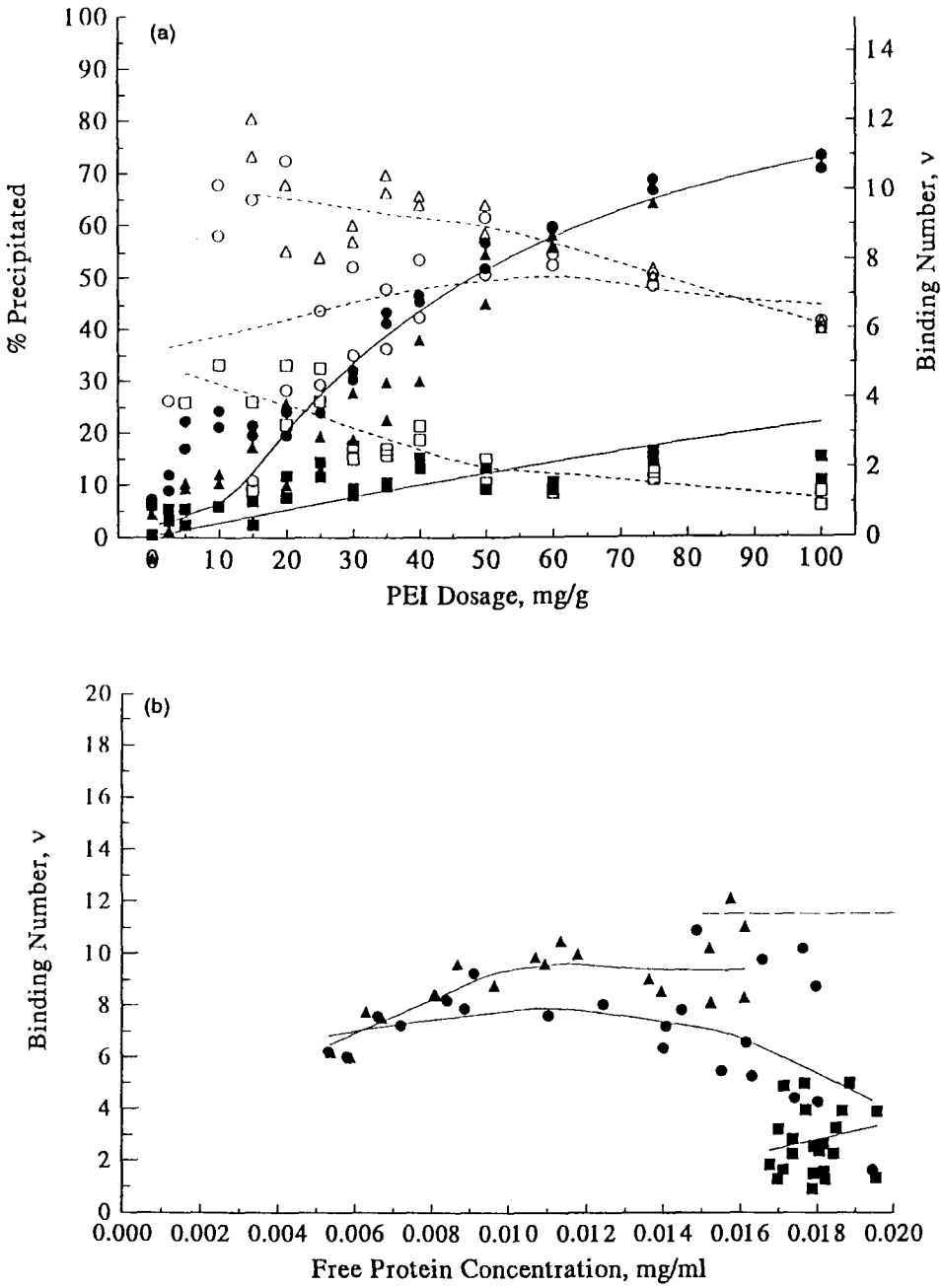

FIG. 5. The complexation of glucoamylase with PEI at I = 200 mM, pH 4.5: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data; (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, GACD0; ▲, △, GACD5; ●, ○, GACD10. The dashed line indicates an adjusted ν for the tailed enzymes.

Maximum Binding Number

Determination of the maximum binding number, n, was made by using Eq. (1) to calculate values for ν at various dosages. Binding plots were constructed by plotting the binding number, ν, versus the free protein concentration, [P], for the various fusion enzymes at each ionic strength, as shown in Figs. 3(b), 4(b), and 5(b). In contrast to the control enzyme, the binding curves for the tailed proteins show that ν decreases at higher [P] rather than leveling off as expected. Typically, the binding number should increase at low [P] to a constant value at high [P] [15, 17]. The most likely explanation for the decrease in ν upon increasing [P] is that not all of the polyelectrolyte is present in the precipitate. At least some of the polyelectrolyte remains in solution as soluble protein-polyelectrolyte complexes until nearly all of the protein has been precipitated. The polyelectrolyte concentrations in the solution could not be determined experimentally as a result of the extremely low amounts used in the precipitations. The sensitivity of the assay for PEI would have had an error of greater than 40% if all of the PEI were present in the solution and not in the precipitate.

Because of the decrease in ν at higher [P], the maximum value obtained for ν on each binding plot was taken as the maximum binding number for that enzyme at that ionic strength. Since the binding number was found to decrease with ionic strength, an absolute maximum binding number, n_{abs}, was determined by extrapolating n out to zero ionic strength. Figure 6 shows n_{abs} to be approximately 28 for all the versions of glucoamylase. This is considerably lower than the estimates of 60

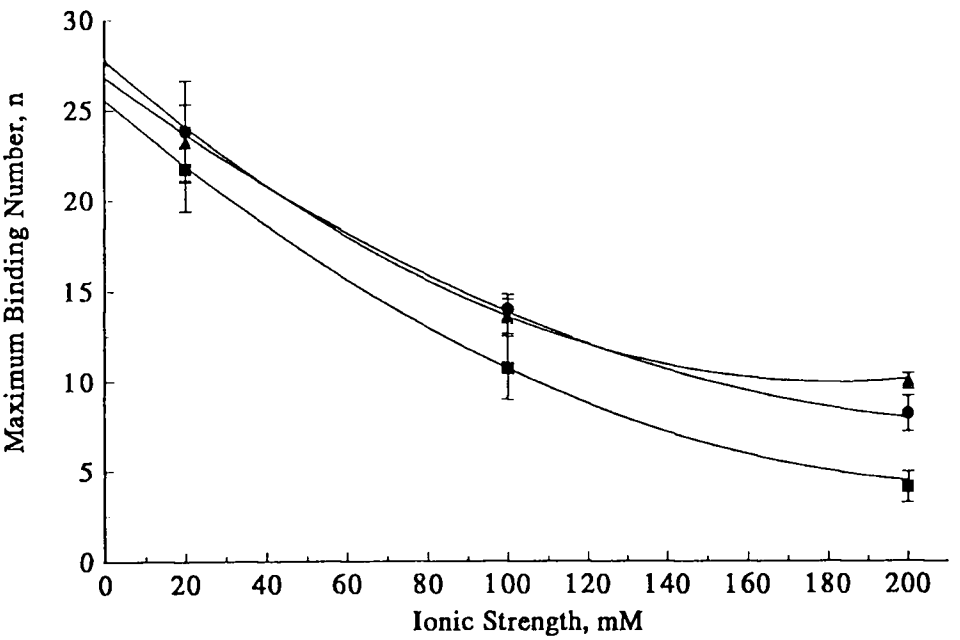

FIG. 6. Determination of the absolute maximum binding number for glucoamylase at pH 4.5: ■, GACD0; ▲, GACD5; ●, GACD10.

and 300 found by the steric hindrance and electrostatic neutrality calculations, respectively. The higher maximum binding numbers observed for the tailed versions of the enzymes at the ionic strengths investigated are a result of the high charge density of the fusion tails being less susceptible to interference from ionic shielding than would the scattered charges on the surface of the protein.

Electrostatic Cooperativity and Intrinsic Association Constants

Cooperativity in binding was not observed for the precipitation of glucoamylase with branched PEI. As a result of the relatively constant binding numbers obtained for GACD0, Z was found to vary relatively little and therefore $\Phi(Z)$ could not be determined. As was evidenced by Clark and Glatz [3], the values of $|Z|$ were found to be quite high. Average values of Z for GACD0 were estimated to be 482, 636, and 710, at 20, 100, and 200 mM ionic strength, respectively. These high Z values indicate that there are an excess of unbound imine groups on the polyelectrolyte. This result agrees well with the hypothesis that branched PEI behaves as a compact sphere. The relatively large proteins would not have access to the internal charge of PEI. In the absence of cooperative binding, K and K_{int} become essentially equivalent and K_{int} is therefore not analyzed separately.

Association Constants

When combining n_{abs} with Eq. (6), a single value for the association constant K_p was found to accurately describe the observed precipitation at each ionic strength for GACD0 (Figs. 3a, 4a, and 5a). The predicted dependence of K_p on I was found to qualitatively agree with experimental results in that K_p decreases as the ionic strength is increased (Fig. 7).

For both tailed enzymes, however, the binding number increases to a maximum and then decreases significantly at higher free protein concentrations, as can be seen in Figs. 3(b), 4(b), and 5(b). Again, this can be explained by the hypothesis that some PEI exists as soluble complexes with protein until nearly complete precipitation is obtained. Taking into account this possibility, K_t values could be calculated by estimating the amount of polyelectrolyte involved in soluble complexes. This amount was determined by assuming that $\nu = n$ over the entire range of precipitation (dashed line on Figs. 3b, 4b, and 5b), as was observed for the control protein. The amount of polyelectrolyte in the precipitating complex is then calculated by substituting n for ν in Eq. (1). Taking into account the amount of soluble PEI complexes, K_t values (Fig. 7) for the precipitating complexes were calculated from fits to the experimental precipitation results (Figs. 3a, 4a, and 5a) using the adjusted values for ν.

A constant value for K_t was found to accurately model the experimental results at each ionic strength (Figs. 3a, 4a, and 5a). A single value for K_t was determined for the two fusion enzymes as a result of the nearly identical precipitation curves. As the theory predicted, K_t was found to decrease as I increases (Fig. 7). Furthermore, K_t was found to be much greater than K_p, indicating that the binding was dominated by tail–polyelectrolyte interactions.

As for the predicted behavior of K_t, the theory could not directly account for the crossover in precipitation behavior of the tailed enzymes when compared to the control enzyme (see Figs. 3a, 4a, and 5a). The tailed enzymes displayed only trace

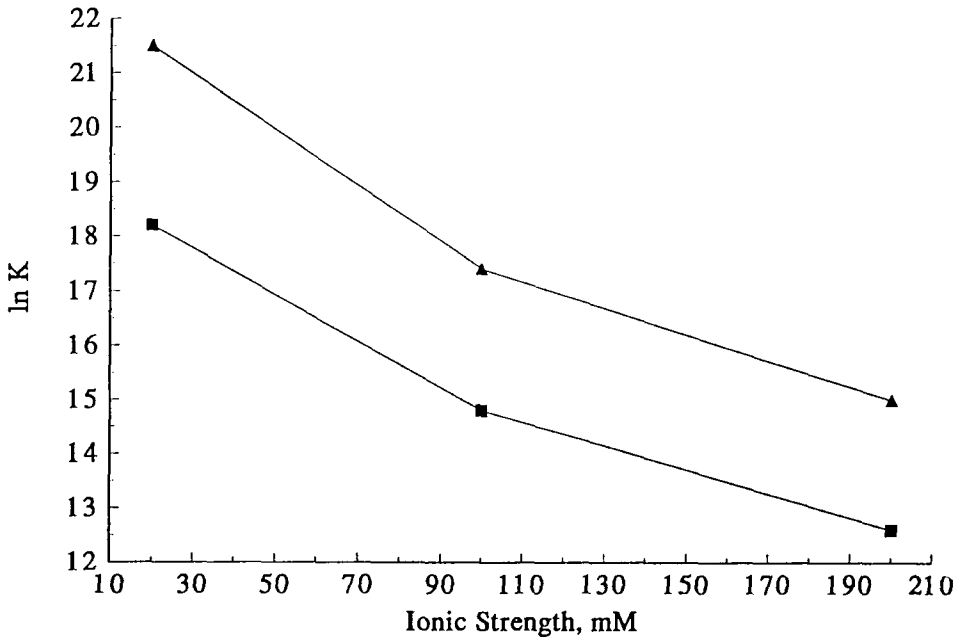

FIG. 7. Dependence of the association constants for glucoamylase on the ionic strength: ■, K_p; ▲, K_t for GACD5 and GACD10.

amounts of precipitation at low dosages, then precipitated to completion over a relatively narrow range of dosages. A possible explanation would be the presence of a critical dosage as is present in bridging theory [32–34]. At low dosages, the polyelectrolyte is saturated with the fusion protein and remains soluble. As the dosage is increased to the point where the polyelectrolyte begins to encounter other complexes before becoming completely saturated with protein, the complexes would begin to form bridges. The resulting increase in complex size and reduction in complex solubility would lead to precipitation.

The assumption that the polyelectrolyte is saturated with protein at low dosages is supported by estimates of the maximum binding number. If all the protein present in solution were to bind to the polyelectrolyte, the steric limit to binding ($\nu = 60$) would be exceeded at dosages lower than 0.014. The experimental limit to binding ($\nu = 28$) obtained by extrapolation of experimental data (Fig. 6) would be exceeded at dosages lower than 0.030. These values do, in fact, cover the range where the presence of the tails was found to increase the solubility of the complex (Figs. 3a, 4a, and 5a).

Application to Multimeric Enzymes: β-Galactosidase

The precipitation curves obtained experimentally for the BGCD fusion enzymes at the various ionic strengths studied are given in Figs. 8(a), 9(a), and 10(a). Also shown in these figures are the respective binding numbers calculated from Eq. (1).

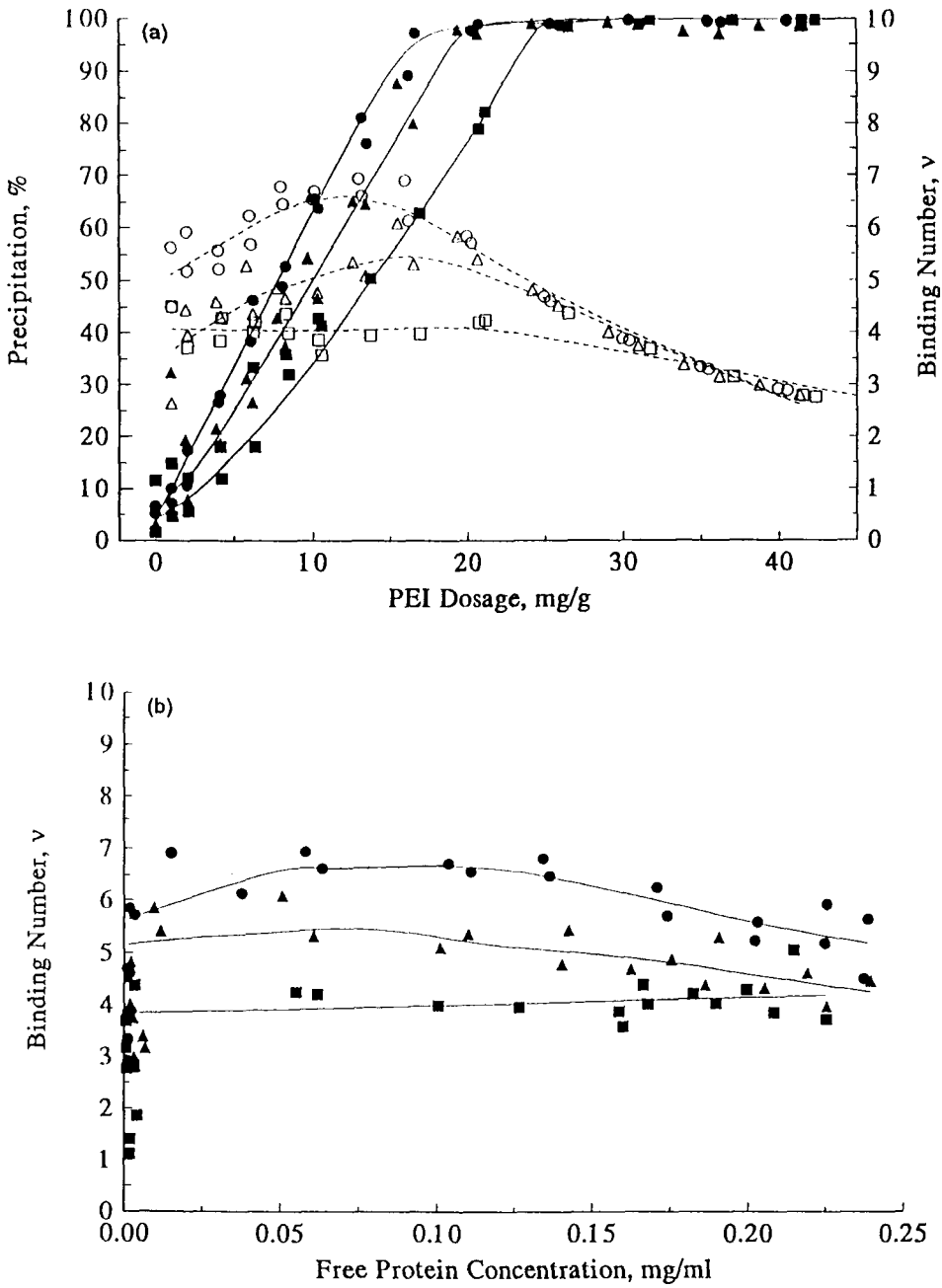

FIG. 8. The complexation of β-galactosidase with PEI at I = 20 mM, pH 5.7: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data; (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, BGCD1; ▲, △, BGCD5; ●, ○, BGCD11.

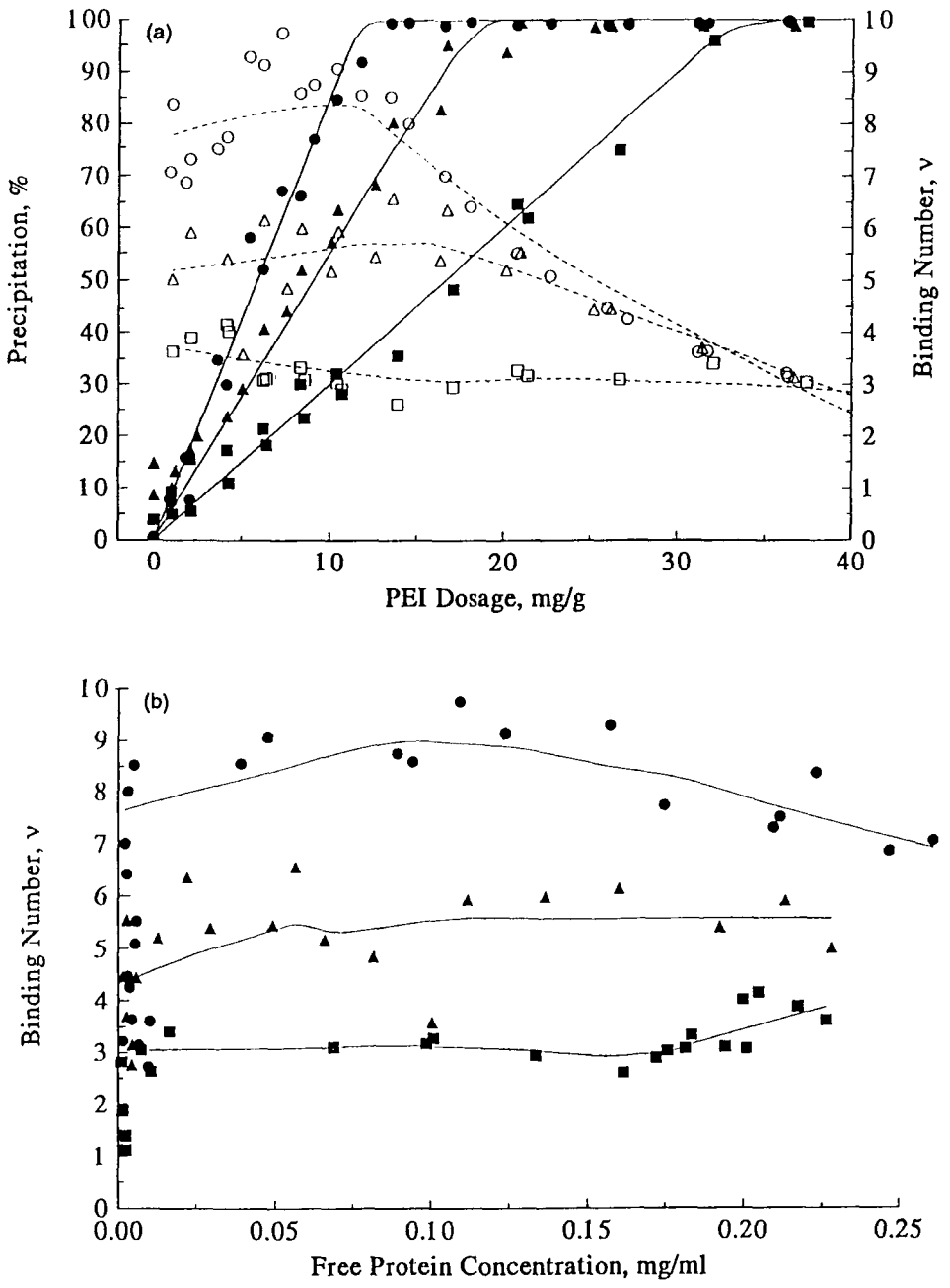

FIG. 9. The complexation of β-galactosidase with PEI at I = 100 mM, pH 5.7: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data; (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, BGCD1; ▲, △, BGCD5; ●, ○, BGCD11.

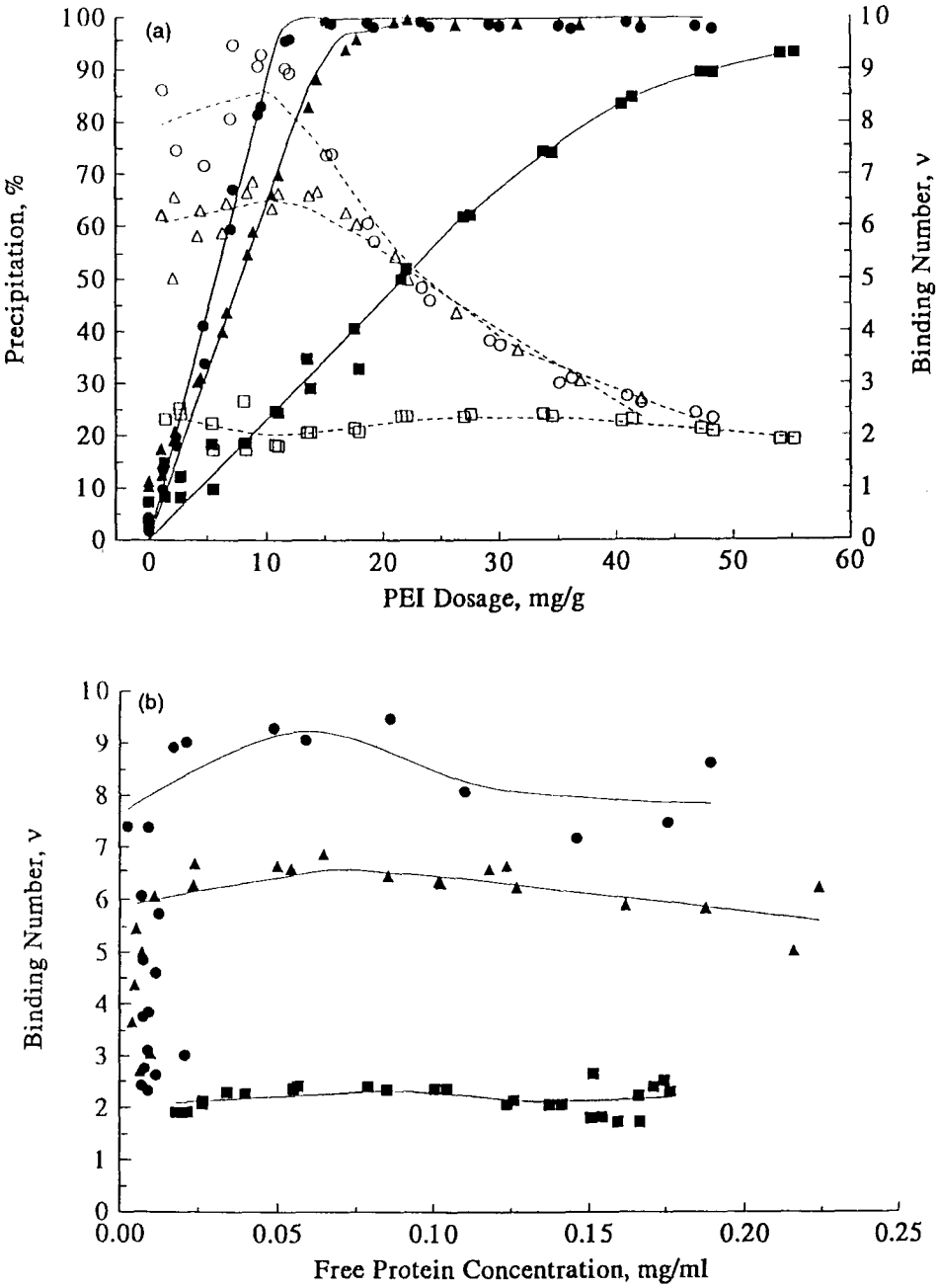

FIG. 10. The complexation of β-galactosidase with PEI at I = 200 mM, pH 5.7: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data; (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, BGCD1; ▲, △, BGCD5; ●, ○, BGCD11.

Maximum Binding Number

The binding curves obtained for the β-galactosidase fusion proteins reveal much lower binding numbers than were obtained for glucoamylase. The binding curves are shown in Figs. 8(b), 9(b), and 10(b). These lower binding numbers result from the greater size of β-galactosidase (470 kDa versus 65 kDa) which would decrease the number of enzymes able to bind to a polyelectrolyte molecule. At a constant ionic strength, the binding numbers were found to be constant for the control protein (BGCD1), and decreased only slightly at higher [P] for the tailed proteins. An unexpected effect was encountered with the dependence of n on I. As I was increased from 20 to 100 mM, n actually increased for both of the fusion tail enzymes (Fig. 11). The untailed version displayed the expected decrease in n upon increasing I. The value of n_{abs} for β-galactosidase was determined to be 10 from Fig. 11. This value for n is considerably less than the estimates of 20 and 28 from the steric limitation and electrostatic neutrality calculations, respectively.

Electrostatic Cooperativity

Cooperativity as a result of electrostatic interactions was not evidenced for the precipitation of β-galactosidase with branched PEI. No correlation between the net complex charge and the binding constants could be determined as the result of a relatively constant binding number, which in turn yields a relatively constant complex charge. High average Z values were once again observed: 534, 549, and 577 for 20, 100, and 200 mM ionic strength, respectively.

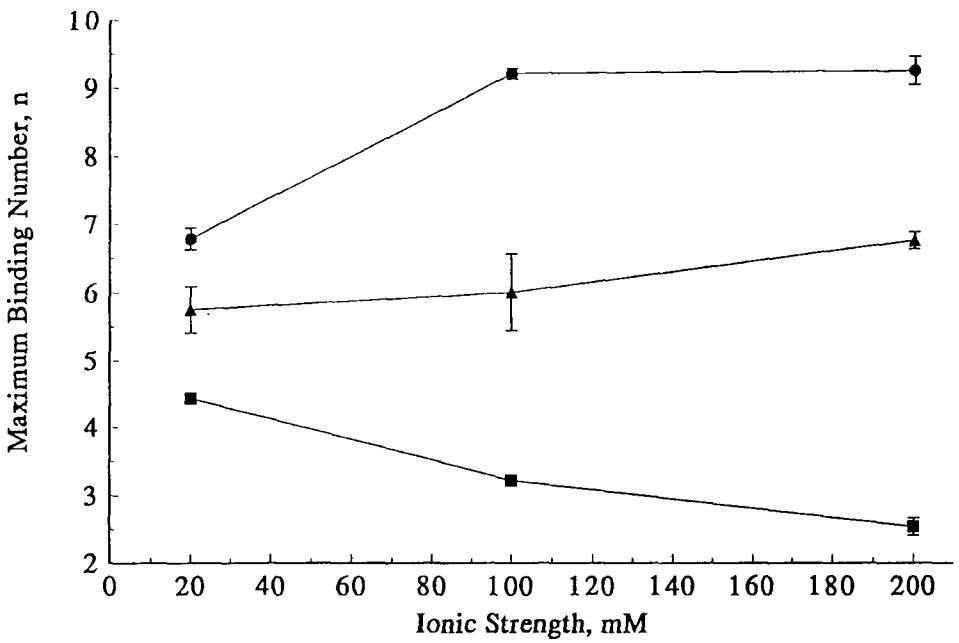

FIG. 11. Determination of the absolute maximum binding number for β-galactosidase at pH 5.7: ■, BGCD1; ▲, BGCD5; ●, BGCD11.

Association Constants

As was stated previously, we hypothesize that the high charge density tails act as selective electrostatic interaction sites which enable multiple polyelectrolytes to bind per enzyme. Since increasing amounts of polyelectrolyte would lead to larger complex sizes, the formation of the matrix should be dependent on the dosage. Association constants were calculated using Eq. (6) and the apparent ν from Eq. (1). It was found that ln K_p and ln K_t both displayed a linear dependence on the dosage (Figs. 12, 13, and 14) and hence can be described as

$$K = K_0 e^{mD} \tag{22}$$

with an intercept of ln K_0 and a slope of m. Fitted curves to the data, shown in Figs. 8(a), 9(a), and 10(a), were calculated using Eq. (5) and association constants from linear fits of ln K_p and ln K_t versus D.

The dependence of K_p on I follows the expected trend for polyelectrolyte precipitation without matrix formation from Eq. (15): K_p decreases as I increases (Fig. 12). For the fusion tails, however, K_t increases upon increasing ionic strength (Figs. 13 and 14). This is understandable if the increase in I is not sufficient to disrupt protein-polyelectrolyte binding but can reduce the electrostatic barrier to formation of a matrix of complexes carrying a net charge of Z. This could well describe the situation for the fusion enzymes, which have shown strong binding on a diethylaminoethyl ion-exchange column [8]. In those experiments, also conducted at pH 5.7, ionic strengths of 0.55, 0.61, and 0.68 were required to elute the BGCD1,

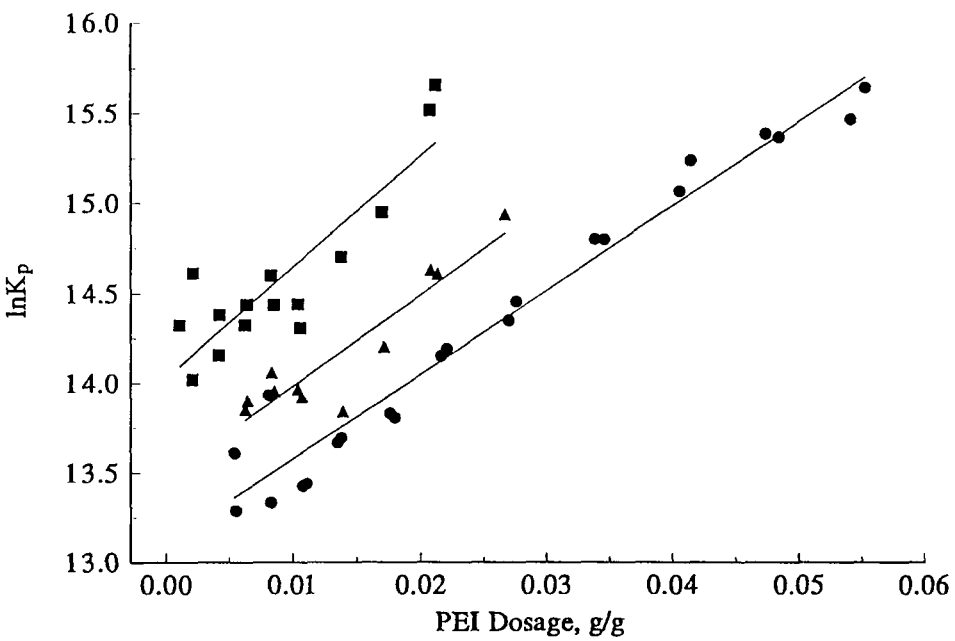

FIG. 12. Effect of dosage and ionic strength on the BGCD1 association constant, pH 5.7: ■, I = 20 mM; ▲, I = 100 mM; ●, I = 200 mM.

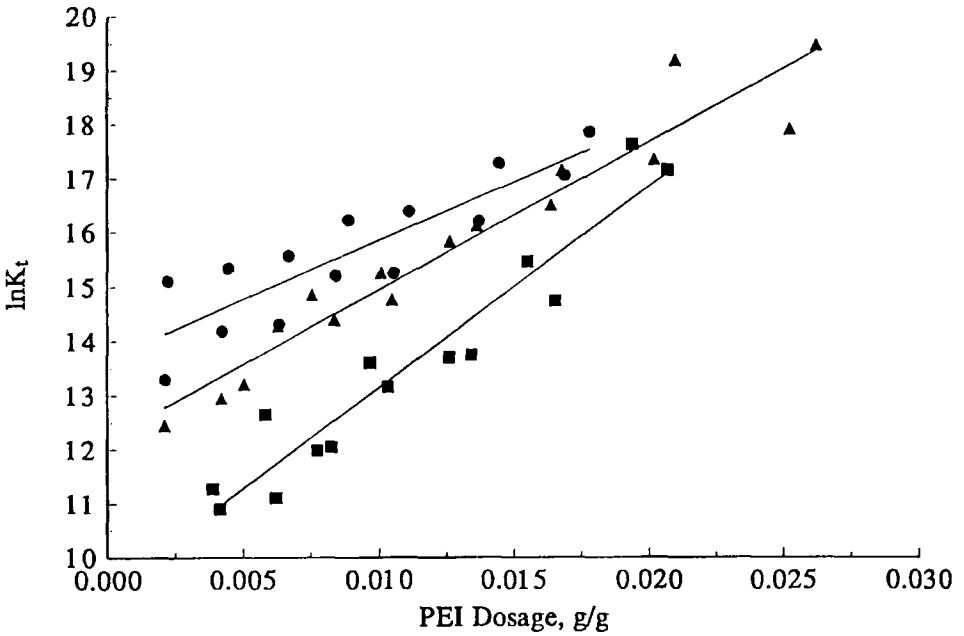

FIG. 13. Effect of dosage and ionic strength on the BGCD5 association constant, pH 5.7: ■, I = 20 mM; ▲, I = 100 mM; ●, I = 200 mM.

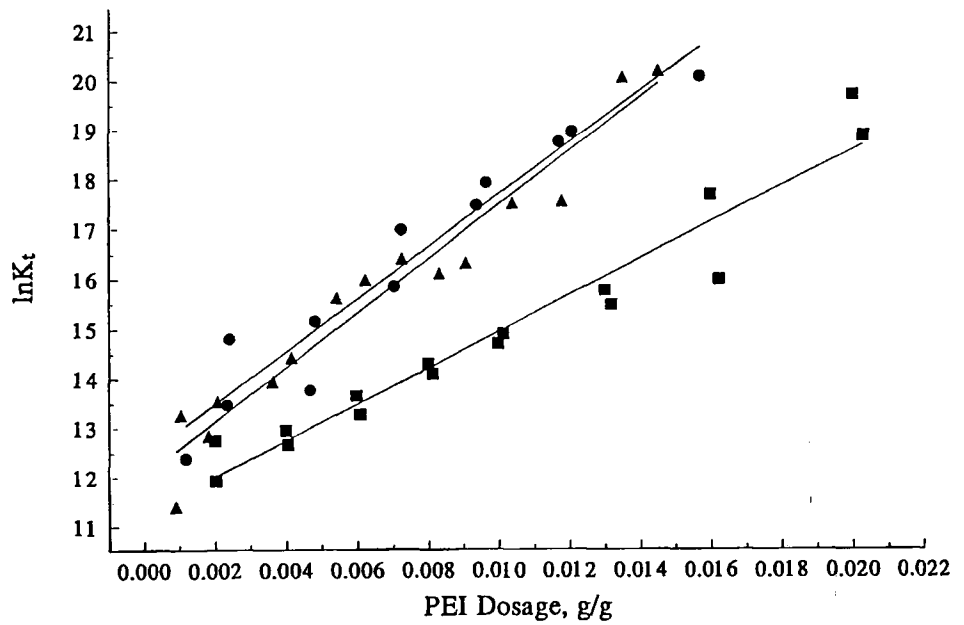

FIG. 14. Effect of dosage and ionic strength on the BGCD11 association constant, pH 5.7: ■, I = 20 mM; ▲, I = 100 mM; ●, I = 200 mM.

BGCD5, and BGCD11, respectively. These ionic strengths are all higher than those investigated in the precipitation studies.

An increase in precipitation upon increasing the ionic strength can also be found in analogous situations where two highly charged components form complexes: the precipitation of polyelectrolytes by polyelectrolytes [35] or the flocculation of silica with PEI [20]. In both cases, the increase in precipitation with ionic strength was said to be the result of an increase in ionic shielding which decreased the electrostatic repulsion between the protein–polyelectrolyte complexes.

Net Charge Considerations

Although the net charge of an enzyme is a good indication as to the degree of interaction with polyelectrolytes, localized charges also play a significant role. The extent to which the potential of these charges extends into solution is given by the thickness of the double layer [36]. The thickness of the double layer was calculated to be 22, 9.6, and 6.8 Å at 20, 100, and 200 mM ionic strength, respectively. Although the net charge of the complexes was calculated to be positive, the exterior charge which is available for interaction should be negative as a result of the relatively small thickness of the double layer when compared to the size of the protein, if the polyelectrolyte is surrounded by the negatively charged protein. As is shown in Fig. 15, the zeta potential of the flocs at 100 mM ionic strength is negative at low dosages where the polyelectrolyte is saturated by protein, indicating that the interacting surfaces of the flocs indeed do have a negative character. As the dosage is raised past the point where the protein is completely removed from solution, the

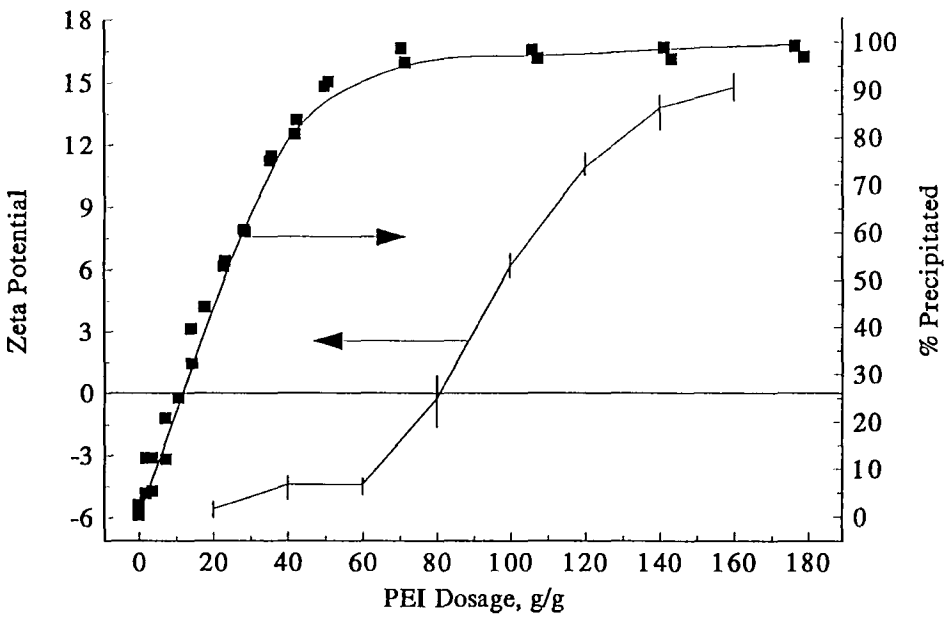

FIG. 15. Effects of the polyelectrolyte dosage and the percent protein precipitated on the zeta potential for commercial β-galactosidase in pH 5.7, 100 mM NaOAc.

TABLE 2. Minimum Dosages Which Would Result in the Formation of a Gel

	g	
p_f	2	4
1.0	0.0026	0.0017
0.5	0.00015	0.00010

zeta potential increases to become positive. This is consistent with the hypothesis that the positively charged polyelectrolyte continues to bind to the exterior of the complexes after all the protein has been incorporated into the flocs.

Formation of the Complex

Figures 12, 13, and 14 show that K_p and K_t increase as the dosage increases. That the slope for the control enzyme was not zero may be attributed to the fact that the control enzyme contains one negatively charged aspartic acid residue per fusion tail. It is therefore not a true control, but rather possesses a "minimal" tail and would be capable of matrix formation.

The minimum dosage which would be necessary for gel formation can be estimated by setting $p_f = 1$ in Eq. (21) if the measured n_{abs} is the true limit to binding. However, the steric estimates of the maximum binding number were considerably higher. Setting $p_f = n_{experimental}(n_{steric})^{-1}$, which yields $p_f = 0.5$, would be a measure of the saturation actually realized. Estimates of the dosages which would result in the formation of a gel are given in Table 2 for two values of g. Any dosage greater than these values should result in the formation of a gel and therefore precipitation of the complex. These minimum values for D demonstrate the validity of using the matrix formation theory to estimate solubility criteria since precipitation was observed for β-galactosidase at $D > 0.002$.

CONCLUSIONS

A model based on multiple equilibrium binding has been developed which qualitatively predicts the polyelectrolyte precipitation of enzymes possessing charged fusion tails. The model incorporates a separate affinity to account for the binding of the charged tail to the polyelectrolyte. Electrostatic cooperativity is not evidenced for the binding of negatively charged proteins to branched PEI.

For the monomeric glucoamylase, the maximum number of enzymes which could bind per PEI molecule was found to be approximately 28, which is significantly lower than the steric estimate of 60. Both K_p and K_t were found experimentally to follow the model predictions: K_p and K_t decrease as the ionic strength is increased. K_t was found to be much greater than K_p, indicating that binding was dominated by the charged tails of the fusion enzymes.

For the multimeric β-galactosidase, which has a molecular weight approximately 8-fold that of glucoamylase, the absolute maximum binding number was

found to be approximately 10. This is significantly lower than the steric estimate of 20. The binding number of the tailed enzymes actually increased with increasing ionic strength. It was found that this behavior could be understood if the precipitate is viewed as an interconnected matrix with the multiple fusion enzymes strongly binding to and crosslinking multiple polyelectrolytes. Values for K_p were found to decrease as I increased, whereas values for the K_t of both fusion enzymes were found to increase. The apparent increase in the K_t values is proposed to be due to reduced intermolecular repulsion in the tightly bound matrix upon increasing I. Both K_p and K_t were found to increase with increasing dosage.

SYMBOLS AND ABBREVIATIONS

a	distance of closest approach (Å)
A	parameter in Debye–Huckel equation
B	constant in Debye–Huckel equation
BGCD	β-galactosidase with carboxy-terminal poly(aspartic acid) fusion
C	parameter in Debye–Huckel equation
CMC	carboxymethyl cellulose
D	dosage, g polyelectrolyte (g protein)$^{-1}$
f	functionality
F	parameter in Halicioglu and Sinanoglu's expression
g	functionality
G	Gibb's free energy (kcal·mol^{-1})
GACD	glucoamylase with carboxy-terminal poly(aspartic acid) fusion
H	molecular surface area of the solute (Å^2)
I	ionic strength (mol·L^{-1})
K	binding or association constant (L·mol^{-1})
m	slope
M	molecular weight
n	maximum binding number
N	Avagadro's constant
p	fraction reacted
P	unmodified (control) protein
PAA	poly(acrylic acid)
PEI	polyethyleneimine
r	stoichiometric ratio of reactive groups
R	ideal gas constant
R_i	radius of gyration of species i (Å)
T	temperature (°K)
V	molar volume of solvent (Å^3)
X	polyelectrolyte
Z	net charge
κ^e	dimensional correction for the macroscopic surface tension
Λ	intrinsic salting-in constant
ν	average binding number
σ	surface tension increment (dyn·cm^{-1})
Φ	cooperativity function

ω	surface tension of pure water ($dyn \cdot cm^{-1}$)
Ω	intrinsic salting-out constant

Superscripts

$^\circ$	standard state
$'$	uncharged

Subscripts

abs	absolute
f	multifunctional molecule
i	species i
int	intrinsic
o	intercept
p	protein
t	tail peptide
x	polyelectrolyte

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REFERENCES

[1] R. K. Scopes, *Protein Purification: Principles and Practice*, Springer, New York, 1987, p. 21.
[2] C. E. Glatz, in *Pharmaceutical Biotechnology: Solubility of Protein Pharmaceuticals* (T. J. Ahern and M. C. Manning, Eds.), Plenum Publishers, New York, 1992.
[3] K. M. Clark and C. E. Glatz, *Chem. Eng. Sci., 47*, 215 (1992).
[4] R. R. Fisher and C. E. Glatz, *Biotechnol. Bioeng., 32*, 786 (1988).
[5] J. Zhao, C. F. Ford, C. E. Glatz, M. A. Rougvie, and S. M. Gendel, *J. Biotech., 14*, 273 (1990).
[6] D. E. Parker, C. E. Glatz, C. F. Ford, S. M. Gendel, I. Suominen, and M. A. Rougvie, *Biotech. Bioeng., 36*, 467 (1990).
[7] I. Suominen, C. Ford, D. Stachon, H. Heimo, M. Niederauer, H. Nurmela, and C. Glatz, *Enzyme Microb. Technol., 15*, 593 (1993).
[8] M. Q. Niederauer, I. Suominen, M. A. Rougvie, C. F. Ford, and C. E. Glatz, *Biotechnol. Prog.*, Submitted.
[9] K. M. Clark and C. E. Glatz, In *ACS Symposium Series 419: Downstream Processing and Bioseparation* (J. F. Hamel, Ed.), ACS Press, Washington, D.C., 1990.
[10] K. M. Clark, Dissertation, Iowa State University, Ames, Iowa, 1988.
[11] A. Carlson, *React. Polym. Ion Exch. Sorbents, 6*, 21 (1987).
[12] J.-Y. Shieh, Master's Thesis, Iowa State University, Ames, Iowa, 1989.

[13] R. D. Hill and J. G. Zadow, *J. Dairy Res., 41*, 373 (1974).
[14] R. D. Hill and J. G. Zadow, *Ibid., 45*, 77 (1978).
[15] K. E. Van Holde, in *Foundations of Modern Biochemistry Series* (L. Hager and F. Wold, Eds.), Prentice-Hall, Englewood Cliffs, New Jersey, 1971, p. 51.
[16] K. A. Connors, in *Binding Constants: The Measurement of Molecular Complex Stability*, Wiley, New York, 1987.
[17] C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, New York, 1961, p. 526.
[18] D. C. Teller, *Nature, 260*, 729 (1976).
[19] R. E. Hostetler and J. W. Swanson, *J. Polym. Sci., Polym. Chem. Ed., 12*, 29 (1974).
[20] G. M. Lindquist and R. A. Stratton, *J. Colloid Interface Sci., 55*, 45 (1976).
[21] L. Stryer, *Biochemistry*, Freeman, New York, 1988.
[22] E. Kokufuta, *Macromolecules, 12*, 350 (1979).
[23] W. Melander and C. Horvath, *Arch. Biochem. Biophys., 183*, 200 (1977).
[24] J. G. Kirkwood, *J. Chem. Phys., 7*, 911 (1939).
[25] T. Halicioglu and O. Sinanoglu, *Ann. N.Y. Acad. Sci., 158*, 308 (1969).
[26] S. Flygare, M.-O. Månnsson, P.-O. Larsson, and K. Mosbach, *Appl. Biochem. Biotech., 7*, 59 (1982).
[27] M. Q. Niederauer and C. E. Glatz, in *Adv. Biochem. Eng./Biotech.*, Vol. 47 (A. Fiechter, Ed.), Springer, New York, 1992, p. 159.
[28] P. J. Flory, *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, New York, 1953.
[29] F. Rodriguez, *Principles of Polymer Systems*, McGraw-Hill, New York, 1982.
[30] S. L. Rosen, *Fundamental Principles of Polymeric Materials*, Wiley, New York, 1982, p. 109.
[31] E. G. Bobalek, E. R. Moore, S. S. Levy, and C. C. Lee, *J. Appl. Polym. Sci., 8*, 625 (1964).
[32] V. K. LaMer and T. W. Healy, *Rev. Pure Appl. Chem., 13*, 112 (1963).
[33] V. K. LaMer and T. W. Healy, *J. Phys. Chem., 67*, 2417 (1963).
[34] R. Hogg, *J. Colloid Interface Sci., 102*, 232 (1984).
[35] E. Tsuchida, K. Abe, and M. Honma, *Macromolecules, 9*, 112 (1976).
[36] M. A. Hughes, in *Solid–Liquid Separation*, 3rd ed. (L. Svarovsky, Ed.), Butterworths, New York, 1990, p. 78.