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THERMODYNAMIC MODEL FOR ELECTROSTATIC-INTERACTION CHROMATOGRAPHY OF PROTEINS

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SUMMARY

A thermodynamic model derived by Record *et al.* [M. T. Record, Jr., *Biopolymers*, 14 (1975) 2137 and M. T. Record, Jr., C. F. Anderson and T. M. Lohman, *Q. Rev. Biophys.*, 11 (1978) 103] from Wyman's linkage theory [J. Wyman, *Adv. Protein Chem.*, 19 (1964) 223] using Manning's condensation model [J. Manning, *J. Chem. Phys.*, 51 (1969) 924] was extended to electrostatic interaction chromatography. Mixed, electrostatic and hydrophobic interactions of a model protein, ovalbumin were characterized by ion and water release.

INTRODUCTION

Retention properties of proteins on ion-exchange surfaces were explained qualitatively on the basis of the law of mass action by Boardman and Partridge[1] and applied to describe weak cation-exchange behavior by Arànyi and Boross[2]. Barford *et al.*[3] have also developed an ion-exchange theory applying the mass action law. Based on the work of Boardman and Partridge, a stoichiometric displacement model was introduced[4]. Barford *et al.*[3] pointed out the possibility of a "mixed-mode" mechanism occurring during protein adsorption. Mixed electrostatic and hydrophobic interactions of proteins were investigated in detail by Kennedy *et al.*[5] and Horváth *et al.*[6].

The importance of interactions of biopolymers with ions and ligands and their effects on macromolecular interactions was placed at the center of biothermodynamics as early as the late 1940s[7]. Wyman[8,9] developed a general thermodynamic approach to interpret the effects of ligands on the oxygenation of hemoglobin and the effects of ligands on hemoglobin dissociation. Tanford[10] extended Wyman's theory taking into account the Gibbs–Duhem relationship between solvent and ligand activities, *i.e.*, between solvation and ligand binding. Aune and Tanford[11,12] extended the binding analysis to cover cation and anion participation in a macromolecular equilibrium.

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Record *et al.*[13,14] utilized the results of Wyman, Aune and Tanford and established a unified theoretical approach to interpret the effects of electrolyte ions and solvent on macromolecular equilibria involving biopolyelectrolytes in solution. This theory includes solubility equilibria, binding and aggregation equilibria and conformational transitions.

Record *et al.*[14] focused, in particular, on salt-dependent macromolecular equilibria in which the phenomenological association constant expressed in terms of macromolecular concentration, increases dramatically with decreasing electrolyte concentration. These authors offered evidence that the complexes are stabilized partly by the formation of ionic interactions and the concomitant release of low-molecular-weight ions previously associated with the charged groups on the biopolymers. Since ions are released in these ionic interactions, the equilibrium shifts to favour complex formation when the salt concentration is reduced. Consequently, ion release, or in other words, reduction in electrolyte activity, can be considered to drive these reactions[15,16]. Reduction in electrolyte activity is an "entropic" phenomenon in its nature. According to Record *et al.*[14], there are at least five potential origins of the effect of an electrolyte (CA) on the phenomenological equilibrium quotient, K_{obs}.

$$\frac{\mathrm{d}\ln K_{obs}}{\mathrm{d}\ln a_{\pm}} = \Delta(n_C + n_{A^-} - \frac{pm}{55.5} n_W) + \frac{\mathrm{d}\ln \gamma_{O_{M_1}}\gamma_{O_{M_2}}}{\mathrm{d}\ln a_{\pm}} - \Delta n_L \frac{\mathrm{d}\ln a_L}{\mathrm{d}\ln a_{\pm}} \qquad (1)$$

These are: (1) differential cation binding ($\Delta n_C \neq 0$); (2) differential anion binding ($\Delta n_A \neq 0$); (3) differential hydration ($\Delta n_W \neq 0$) at high salt concentration, m; (4) differential screening (Debye–Hückel) effects of electrolyte on macroion charges reflected in a variation of the macromolecular activity coefficient ratio with $a_{\pm}$, and (5) the effect of $a_{\pm}$ on the ligand activity coefficient, γ_L.

In eqn. 1, $a_{\pm} = (a_C{}^{p+} a_A{}^{p-})^{1/p}$, the mean ionic activity of the salt; $\Delta n_C = n_{CM1} + n_{CM2} - n_{CM1M2}$, the number of moles of cations released at association of one mole of M_1 and one mole of M_2 macromolecules, and in the same way, Δn_A, Δn_W and Δn_L are the number of moles of anions, water molecules and ligands released, the γ terms are the activity coefficients of the macromolecular reference states, a_L is the ligand activity and p is the valency of the ion.

It should be emphasized that eqn. 1 is completely general and independent of any molecular model for the macromolecular binding process. Interpretation of experimental values of the derivative ($\mathrm{d}\ln K_{obs}/\mathrm{d}\ln a_{\pm}$) in terms of molecular quantities (number of cations, anions or water molecules released, number of ionic interactions, etc.) requires a molecular model. According to Record *et al.*[14], the association of ions with charged biopolymers can be modeled as either (a) mass action binding of ions to discrete classes of identical non-interacting sites on a macroion of arbitrary structure and charge distribution[7], or (b) condensation, the ionic strength-indepenent association of counterions with a linear polyion[17] (see details in Appendix I).

Based on Manning's condensation model[17], Record *et al.*[14] have derived a model from eqn. 1 to evaluate the stoichiometry of binding of cationic oligopeptides to nucleic acids (summary of their derivations are in Appendix II.).

The logarithm of the phenomenological equilibrium quotient derived by Record *et al.*[14] is

$$\ln K_{obs} = \ln K_T^0 - \zeta[1 - (2\xi)^{-1}] \ln m + \zeta\xi^{-1} \ln \delta\gamma_{\pm} \quad (2)$$

and

$$\left(\frac{\mathrm{d} \ln K_{obs}}{\mathrm{d} \ln m}\right)_{T,\ p,\ pH} = -\zeta\left[\psi - \xi^{-1} \frac{\mathrm{d} \ln \gamma_{\pm}}{\mathrm{d} \ln m}\right] \quad (3)$$

A series of experiments shows good agreement with data predicted by eqn. 3 (ref. 15, 18–21). It should be stressed, however, that this model neglects possible co-ion release and change in hydration during association.

The stoichiometric displacement model considers a pure ion-exchange process, where co-ion release and change in hydration are neglected, and estimates the number of counterions released at protein binding by d ln k'/d ln m, (k' = capacity factor). The thermodynamic model provides the same parameter (*cf.* eqn. 3). Basis of this similarity, the retention equation of the stoichiometric displacement model, at least formally, can be traced to Wyman's linkage theory[9].

In this paper, extension of this fundamental thermodynamic model to electrostatic interaction chromatography of proteins is studied. Using a stationary phase with known structure, polyethyleneimine (PEI), parameters of interaction of a large biopolymer, ovalbumin, were estimated at different mobile phase pH values.

EXPERIMENTAL

Ovalbumin (OVA), conalbumin (CON) and β-lactoglobulin A (B-LAC) were obtained from Calbiochem (La Jolla, CA, U.S.A.). Piperazine, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (bis-tris) and 1,3-bis[tris(hydroxymethyl)methylamino]propane (bistrispropane) were obtained from Aldrich (Milwaukee, WI, U.S.A.), tris(hydroxymethyl)aminomethane (Tris) from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and sodium chloride from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Experiments were carried out on a Model 1090 liquid chromatograph equipped with a filter-photometric detector (λ = 280 nm) from Hewlett-Packard (Palo Alto, CA, U.S.A.) and with a Rheodyne 7125 injection valve fitted with a 20-μl loop (Cotati, CA, U.S.A.). Chromatograms were recorded by using a Linear Series 1200 recorder (Reno, NV, U.S.A.).

A 50 × 4.6 mm I.D. column was packed with Synchropak Q300 strong anion-exchang support (Synchrom, Lafayette, IN, U.S.A.).

The mobile phase contained 20 mM piperazine · HCl at pH 4.8, 5.0 and 6.0, bis-tris · HCl at pH 6.5, bistrispropane · HCl at pH 7.0, Tris · HCl at pH 8.0 and various amounts of sodium chloride in order to vary the protein retention factor from 1 to 10 in isocratic elutions. Calculation of k' was performed according to $k' = (V_R - V_0)/V_0$, where V_R is the retention volume of the solute and V_0 is the hold-up volume which was determined according to the method described in ref. 22. Averages of three parallel determinations were used in the calculations, standard deviations of these measurements were always less than 2%. Mobile phase flow-rate was 1 ml/min and the temperature 25°C, in all cases.

A fourth order polynomial equation was fitted to the logarithm of retention

factor for ovalbumin *vs.* the logarithm of salt concentration. Linear regression analyses were used to fit eqn. 5 and 8 to retention data of ovalbumin.

RESULTS AND DISCUSSION

Extension of thermodynamic model to electrostatic interaction chromatography

As a first step, a quasi two-dimensional ion-exchange surface with N charges is considered instead of linear array charges on a polynucleotide. Therefore, in the equilibrium equation (eqn. A8) S represents the stationary phase and P is a symbol for peptides or proteins with ζ number of charges in their binding sites. For the sake of simplicity, a possible release of co-ions bound to the charged groups of the protein binding site and change in the protein's hydration state are neglected. This means that the reference state equilibrium and, consequently, the expression for the apparent equilibrium quotient (eqns. A8, 2, 3) remain unchanged.

The k' is expressed by the equation[22,23]

$$k' = K_{\text{obs}}\, \varphi \tag{4}$$

where φ is a chromatographic phase ratio that is independent of salt concentration. From eqn. 2 and 3

$$\ln k' = \ln K_T^0 + ln\, \varphi + \zeta\xi^{-1}\, ln\, \delta\gamma_{\pm} - \zeta\psi \ln m \tag{5}$$

and

$$\left(\frac{\mathrm{d} \ln k'}{\mathrm{d} \ln m}\right)_{T,\ p,\ \mathrm{pH}} = -\zeta\left[\psi - \xi^{-1}\frac{\mathrm{d} \ln \gamma_{\pm}}{\mathrm{d} \ln m}\right] \tag{6}$$

Eqn. 5 is considered as the retention equation of electrostatic interaction chromatography of proteins. According to eqn. 6, the slope of the plot ln k' *vs.* ln m is a function of the number of protein charges interacting with the stationary phase, ζ, the average charge spacing, b and the effect of mobile phase salt concentration on the mean ionic activity coefficient of the salt. For sodium chloride, d ln $\gamma_{\pm}$/d ln m is constant between 0.1 and 0.5 M concentration ($r = 0.9999$), and its value is -0.04 (ref. 24). Below 0.1 M, the absolute value of this slope decreases. Error arising from neglecting the second term in the right-hand side of eqn. 6 is expected to be approximately 3%.

Evaluation of the quaternized polyethyleneimine stationary phase

Distance between two nitrogen atoms in PEI was estimated by using atomic distance and angle values of glycine and trimethylamine[25]. Based on the significant repulsion forces between charged amine groups, it was assumed that two neighbouring N atoms are in the *trans* position. Therefore, the charge spacing, b, is approximately 3.9 Å. According to Rounds[26], the degree of quaternization of a well made quaternized PEI coating at maximum is 70%. At least 75–80% of tertiary amine groups left are ionized at pH 6.0 (ref. 27). At 65% quaternization of amine groups in PEI and 80% ionization of the residual tertiary amines, approximately 90% of the amino groups in

PEI are ionized. Therefore, the average charge spacing, b, is *ca.* 4.5 Å. Since PEI coatings can be several layers thick, the influence of layer thickness on the electrostatic potential must be considered. When the mobile phase salt concentration is over 0.1 M, the electrostatic field of the amine groups below the surface of the PEI coating is mostly shielded by mobile phase ions and their effect can be ignored.

Using the estimated b value (4.5 Å), the model provides values for the other parameters: $\xi = 1.59$ and $\psi = 0.686$. Assuming the quaternized PEI chain has an average radius about 4 Å (ref. 25), the thickness Δx of cylindrical shells of volume V around the polyelectrolyte chain (see eqn. A5), in the case of sodium chloride as electrolyte, is 12 Å which is equivalent to about four water layers.

Evaluation of elution data for ovalbumin

Retention of ovalbumin as a function of mobile phase salt concentration was measured at pH 5, 6, 7 and 8 (Fig. 1). The isocratic elution profiles were always single peaks. Eqn. 6 was used to determine ζ values (Table I). The slope of ln k' *vs.* ln m determined from polynomial equation, as a function of mobile phase salt concentration, m, is shown in Fig. 2. As expected, an increase in mobile phase pH increases the value of d ln k'/d ln m. The change in the derivative is most pronounced at pH 5 and decreases with increasing pH. The constant k' curves represent isoenergetic conditions. Increasing mobile phase pH and consequently the number of interacting charges decreases the free energy change of the interaction per unit ζ ($\approx$ d ln k'/d ln m); moreover, the smaller the k', the greater the change.

The isoenergetic curves are linear over 0.1 M salt concentration. The slope of these straight lines is equal to

$$-\left(\frac{\mathrm{d}}{\mathrm{d}\,m}\,\frac{\mathrm{d}\,\ln\,k'}{\mathrm{d}\,\ln\,m}\right)_{k',\ m\,>\,0.1\ M}$$

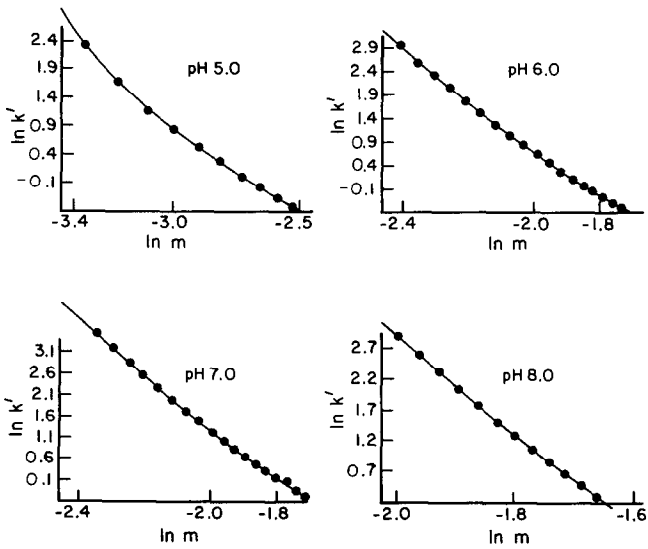

Fig. 1. Plot of ln k' *vs.* ln m for ovalbumin at pH 5, 6, 7, and 8. Solid lines represent the fit of a fourth order polynomial equation.

TABLE I

ζ VALUES OF OVALBUMIN DETERMINED BY EQN. 6

pH	ζ	*r*
5	4.82	0.984
6	7.48	0.997
7	8.53	0.998
8	11.06	0.999

which is depending on the change of stoichiometry, and decreases with an increase in binding energy ($\approx k'$) as it is shown in Fig. 3. According to this, it can be stated that this interaction is more sensitive to the change of ζ at low k' values than at higher ones. Below 0.1 *M* salt concentration, the isoenergetic curves deviate from a straight line. Plots of $(\mathrm{d} \ln k'/\mathrm{d} \ln m)_{\mathrm{pH}\ 5}$ *vs.* *m* are non-linear and shifted to higher values. At higher salt concentrations ($m > 0.05\ M$), however, the plot is straight and deviation of $[(\mathrm{d} \ln k'/\mathrm{d} \ln m)_{\mathrm{pH}\ 5,\ \mathrm{obs}} - (\mathrm{d} \ln k'/\mathrm{d} \ln m)_{\mathrm{pH}\ 5,\ \mathrm{lin}}]$ as a function of salt concentration approaches zero (Fig. 4). According to the thermodynamic model by Record *et al.*[14], $\mathrm{d} \ln k'/\mathrm{d} \ln m$ is constant. This prediction is more or less realized at the high salt concentration range (at pH 8). When decreasing mobile phase pH and concomitantly salt concentration required to elute OVA, the change in slope is more pronounced (Fig. 2). The slope of the isoenergetic curves at greater than 0.1 *M* salt concentration shows that the increase in $\mathrm{d} \ln k'/\mathrm{d} \ln m$ as a consequence of an increase in the ζ value (or in pH) is a linear function of the salt concentration required to elute OVA at constant free energy change.

If ovalbumin has a mixed (electrostatic and hydrophobic) interaction with the stationary phase, the contribution of hydrophobic interaction to the overall interaction would be greater when the contact area of the protein is more hydrophobic (in other words, contains less charges). The closer the mobile phase pH is to the

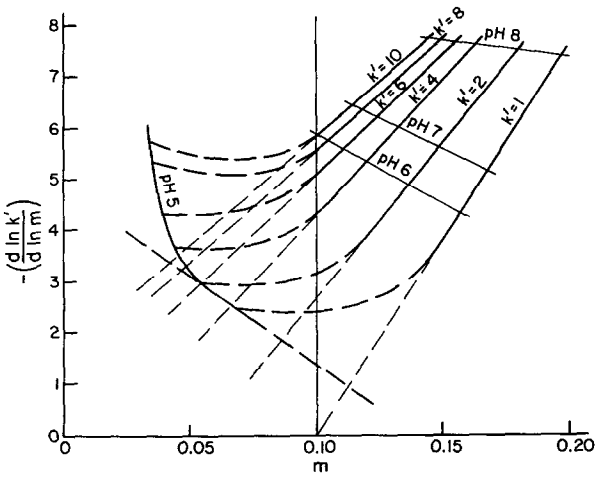

Fig. 2. $(\mathrm{d} \ln k'/\mathrm{d} \ln m)$ *vs.* *m* for OVA at different pHs.

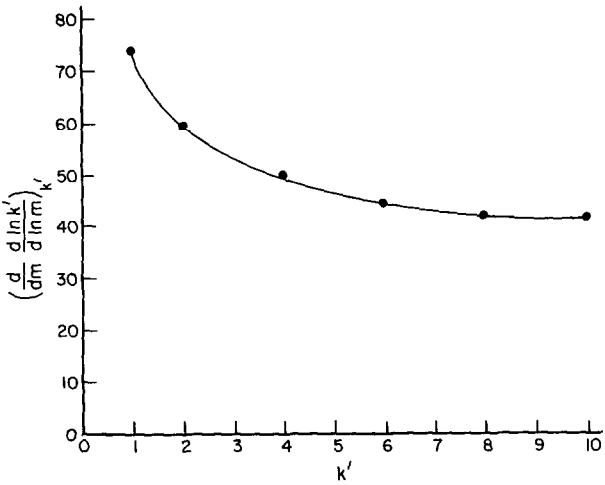

Fig. 3. Slope of the isoenergetic curves observed over 0.1 *M* salt concentration *vs.* retention factor.

isoenergetic point, the less charge there is on the protein surface. The isoelectric point of OVA is 4.7 (ref. 28); therefore, one would expect the binding site of OVA to be more hydrophobic at pH 5 than at higher pH values[29]. Thus, when evaluating the retention equation of OVA, both electrostatic and hydrophobic interaction should be considered[30].

The first term on the right-hand side of eqn. 1 derived by Tanford[10], considers hydrophobic interaction at high salt concentration. If we extend Tanford's equation to the low salt concentration range as proposed by Aune *et al.*[31], the slope of the retention equation can be expressed as follows:

$$\left(\frac{\mathrm{d}\ \ln\ k'}{\mathrm{d}\ \ln\ m}\right) = -\zeta\psi + \Delta(n_C + n_A - \frac{mp}{55.5} n_W)\left(1 + \frac{\ln\ \gamma_\pm}{\ln\ m}\right) \quad (7)$$

The number of moles of water molecules released at binding of 1 mole protein can be determined from the slope of the plot (d ln k'/d ln m) *vs.* m (Table II). According to eqn. 7, ζ and ($\Delta n_C + \Delta n_A$) cannot be determined independently. Other authors should have faced the same problem earlier[11,12,31]. If we assume that there is no ion release due to hydrophobic interaction, ζ can be estimated to be 6.92 at pH 5, 12.7 at pH 6, 13.4 at pH 7 and 12.9 at pH 8. ζ values show a jump between pH 5 and 6, but remain constant between pH 6 and 8. Therefore, ion release as a consequence of hydrophobic interaction cannot be ignored.

If ζ is set to be equal to that determined by eqn. 6, the total ion release due to hydrophobic interaction, $\Delta n_i \approx (\Delta n_A + \Delta n_C)$ can be estimated as well (Table II). At pH 6, 7, and 8, plots of d ln k'/d ln m are quasi linear and the slope of the plot [d/d m (d ln k'/d ln m)] decreases with increasing pH and concomitantly ζ values (*i.e.*, the contribution of change in hydration due to hydrophobic interaction). At pH 5, however, the plot is non-linear. The straight line relating to the slope of hydrophobic interaction (d ln k'/d ln m *vs.* m) at pH 5, was estimated using isoenergetic functions (Fig. 2). Parameters were determined fitting eqn. 7 to the estimated change in d ln k'/d

TABLE II

PARAMETERS DETERMINED BY FITTING EQN. 7

pH	Δn_w^a	Δn_i^b
5[c]	839	1.36
6	655	3.02
7	552	2.81
8	172	1.03

[a] Calculated for mid salt concentration value of measured range.
[b] Calculation based on $\zeta = \zeta_{eqn.6}$.
[c] Fit on $(\mathrm{d} \ln m'/\mathrm{d} \ln m)_{pH\ 5,\ lin}$ *vs.* m.

ln m as well (Table II). Non-linearity of the plot d ln k'/d ln m *vs.* m shows that the model does not take into account a phenomenon which has a significant role at low salt concentration. Change in Δn_i and Δn_W as a function of pH reflects to opposite effects on hydrophobic ion release. At low pH, close to protein isoelectric point, the binding site contains a relatively small amount of changes, *i.e.*, it is relatively hydrophobic (large $-\Delta n_W$ value) and the salt concentration necessary to elute the protein is low. This means that the population of ions at hydrophobic binding sites is low (low $-\Delta n_i$ value). When the mobile phase pH is increased and concomitantly salt concentration required for the elution of OVA also increases, the binding site of the protein contains an increasing number of charges, *i.e.*, its hydrophobicity decreases ($-\Delta n_W$ and number of hydrophobic binding sites decrease) but the occupation level of remaining hydrophobic binding sites increases. Consequently, $-\Delta n_i$ has a maximum.

The discrepancy between the model and measured data near the pI of a protein was found not only in the case of OVA but in that of conalbumin and β-lactoglobulin as well. Slopes (s), intercepts (I), correlation coefficients (r) of linear regression analyses were $s = -1.56$, $I = -5.41$, $r = 0.986$ for CON and $s = -1.81$, $I = -4.98$, $r = 0.988$ for B-LAC, respectively. This deviation may originate from neglecting possible change in co-ion release, effect of the unshielded amino groups below the surface of the PEI coating, change in hydration of ions, and in the worst case, aggregation or a possible conformational change in protein structure.

Relatively weak interactions of electrolyte ions with charged groups or polar

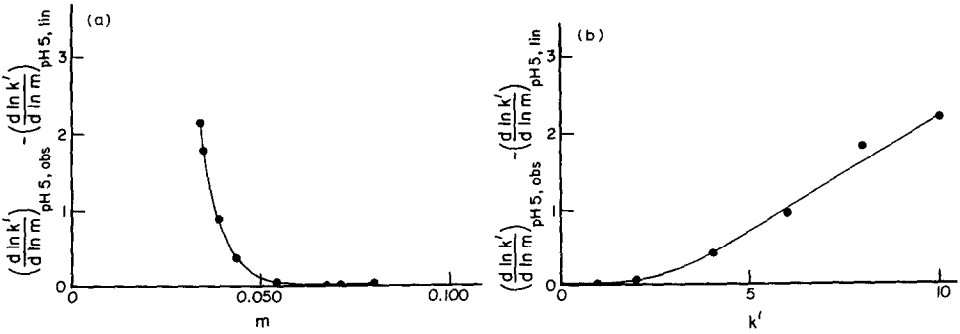

Fig. 4. Deviation of the change in the slope of ln k' *vs.* ln m at pH 5 from straight line observed over 50 mM, as a function of (a) salt concentration and (b) retention factor.

residues on the surface of a protein are important in determining its conformation, biological activity, solubility, aggregation and chromatographic behavior. There have been many investigations of salt effects on macromolecular equilibria started by Hofmeister[32] about one hundred years ago. The most detailed information currently available on ion–protein interaction can be obtained by using halide NMR[33]. Thermodynamic modeling of macromolecular binding has been used extensively[7,10,11,34]. In the case of electrostatic interaction chromatography of proteins, co-ion release at protein binding can be considered as an additional energy term to the total free energy change[12,34]:

$$\exp\left(-\frac{\Delta G_c^0}{RT}\right) = K_c = (1 + k_c a_c)^{-\Delta q_c} \tag{8}$$

where k_c and a_c are the intrinsic binding constant and activity of co-ion, respectively, and Δq_c is the number of co-ions released. Considering the fact that the isocratic measurements of the plot ln k' *vs.* ln m are usually performed at relatively low and in a narrow salt concentration range, changes of activity coefficient of salt can be neglected. Furthermore[14],

$$q_c = \zeta\theta_c \tag{9}$$

where $\theta_c = k_c m/(1 + k_c m)$.

Combining eqns. 8 and 9, the logarithm of the equilibrium quotient for ion-binding is

$$\ln K_c = -\zeta\, k_c m/(1 + k_c m) \ln (1 + k_c m) \tag{10}$$

In the case of a uni-univalent salt, interaction between a co-ion (with respect to the ion-exchange surface) and a protein charged group is relatively weak, *i.e.*, $k_c m \ll 1$, therefore, the additional term to the retention equation is

$$\ln K_c \approx -\zeta k_c m \ln (1 + k_c m) \tag{11}$$

or

$$\ln K_c \approx -\zeta\, (k_c m)^2$$

Eqn. 11 describes a convex curve which is the opposite curvature of the measured function. Therefore, based on the theory for site binding, co-ion release cannot be responsible for discrepancy observed at pH near pI of proteins.

The other possible explanation for the non-linear nature of the slope of (d ln k'/d ln m) *vs.* m is the effect of the unshielded amino groups below the surface of the PEI coating at low salt concentration in the eluent. In this case an apparent decrease of b number should be considered. Quantitative description of this effect needs further investigation.

At a pH near to pI, where the binding is relatively weak (low ζ value), elution salt

concentration is below 0.1 *M*. In this concentration region, the Debye–Hückel theory is valid, and the thickness of double layer around a charged group ($1/\kappa$) varies steeply with ionic strength of the mobile phase, while above 0.1 *M*, the change of $1/\kappa$ is relatively small. Deviation observed may be originated from the assumption that the change in macromolecular hydration can be neglected. In the Debye–Hückel region, this assumption is, probably, not valid. To consider change in ionic hydration, a model is needed to describe the binding of proteins to an ion-exchange stationary phase. At present a satisfactory model has not been developed for *large* biomolecules.

According to Eisenman[35], interaction of ionic species can be described as the sum of free energy change of electrostatic interaction and that of hydration.

$$\Delta G = \Delta G_{\text{e.s.}} + \Delta G_{\text{hydr.}} \tag{12}$$

In the case of electrostatic interaction of proteins and stationary phase, the influence of $\Delta G_{\text{hydr.}}$ on the overall process is a function of the nature of the interacting ions (cosmotrop, chaotrop) and the salt concentration of the bulk (thickness of the hydration shells).

According to Manning[36], though, the free energy of stabilization of the charged fraction due to the short-range effect accompanying dehydration, is expected to be fairly small compared to the non-specific long-range polyelectrolyte effect. Nevertheless, short-range effects due to dehydration of ions and polar groups may be the key to some of the most crucial problems in biology. If the affinity of a counterionic species for sites on the polyion contains a non-ionic component, the binding is more tight and localized resulting, probably, in an even greater change in ionic hydration. Since there is no quantitative description of this phenomenon available today, the possible contribution of dehydration of ions and polar groups cannot be evaluated.

At low salt concentration (below 0.1 *M*), the nature of the protein can be altered due to the unshielding effect of its charged groups[37]. The increased hydrophobicity of a protein due to the mobile phase pH set near to the p*I* and the alteration in protein structure at low salt concentration may result in protein aggregation.

Deviation of measured data from the model at the protein p*I* may be considered as a consequence of the combination of phenomena mentioned above. This founding warns us that care must be taken at designing a protein separation to obtain a desired purity and biological activity of the product.

CONCLUSION

The model investigated in this paper is an applied form of Wyman's linkage theory which is completely general. Consequently, it is valid not only for association, precipitation and conformational changes of proteins but for any kind or surface mediated chromatography of proteins (electrostatic interaction, hydrophobic interaction, reversed-phase, bioaffinity, and metal–chelate interaction) as well. Gradient elution chromatography of proteins can be characterised with a general relationship:

$$K_{\text{obs}} \approx \exp\left\{\left(\frac{\partial \Delta G}{\partial \mu_1}\right)_{T,\ p} \frac{\mu_1}{RT}\right\} \tag{13}$$

where $\mu_1 = -RT \ln a_1$, subscript 1 stands for ligand in general, and special solutions of eqn. 1 for different kind of interactions, provides expressions for $(\partial \Delta G/\partial \mu_1)_{T,p}$. Under practical separation conditions, the extended form of this thermodynamic model was successfully applied to the retention of a protein.

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APPENDIX I

In the case of site binding, the association of a single ligand L with a class of N identical, independent sites on a macromolecule M is considered. Ignoring hydration effects and using the unligated macromolecule as the reference state, the binding polynomial[18] is

$$\Sigma_M = (1 + k_{L,M}a_L)^N \tag{A1}$$

where a_L is activity of the ligand and $k_{L,M}$ the intrinsic binding constant of L to a site on M. Applying Wyman's linkage theory[9], the derivative of eqn. A1 is

$$\left(\frac{d \ln \Sigma_M}{d \ln a_L}\right)_{a_W,\ a_C,\ a_A} = n_{L,M} = \frac{Nk_{L,M}a_L}{1+k_{L,M}a_L} = N\theta_{L,M} \tag{A2}$$

where $\theta_{L,M} = n_{L,M}/N$ is the binding density.

According to Manning's condensation theory[17], if the macromolecule can be modeled as a linear array of N univalent charges of average spacing b, then the extent of association of monovalent counterions with the polyion can be determined by a dimensionless charge density parameter,

$$\xi = e^2/bDkT \tag{A3}$$

where D is the bulk dielectric constant, k is the Boltzman factor and T is the absolute temperature.

Assuming that the interaction of bound and free ions with the solvent is the same, and the "bound" ions may translate freely within the volume V centered along the axis of the polyelectrolyte, charging the ionic groups of a polyelectrolyte against their mutual electrostatic repulsion and mixing free cations, bound cations and solvent molecules, were found to result in an equilibrium state where the number of associated counterions per fixed charge, θ_D, has a value between 0 and 1. Furthermore, θ_D does not tend to zero with m but remains positive[38]. For a 1:1 salt, at the free energy

minimum, in the limit $m \rightarrow 0$, the fraction of a counterion condensed on a infinite polyion per structural polyion charge is

$$\theta_D = 1 - \xi^{-1} \tag{A4}$$

The quantity of θ_D has been determined for native DNA using ^{23}Na NMR, and it was found to be independent of sodium chloride concentration in the range of 0.005 to 0.5 *M* (ref. 33). The volume of the region surrounding the polyelectrolyte within which cations are "bound", *V*, is described by eqn. 6 (ref. 38):

$$V = 10^3 e v^{-1} Z^{-1} (1 - Z^{-1}\xi^{-1}) (\kappa b)^2 m^{-1} \tag{A5}$$

where v is the number of *Z*-valent ions per formula salt, and κ is the Debye–Hückel parameter. *V* is independent of *m*, since the ratio κ^2/m is independent of *m*.

The thermodynamic binding parameter, ψ, contains two important quantities characterizing the interaction of the polyion with the counterions: the extent of condensation, θ_D, and the screening effect of low-molecular-weight ions on the interactions of these residual polyelectrolyte charges, which is thermodynamically equivalent to the binding of an additional fraction $(2\xi)^{-1}$ of a counterion per polyion structural charge[15]

$$\psi = \theta_D + (2\xi)^{-1} = 1 - (2\xi)^{-1} \tag{A6}$$

APPENDIX II

The equilibrium where a nucleic acid, S, consisting of *N* nucleotides interacts with an oligopeptide, P, with ξ positive charges in its binding site for S, and forms a complex, SP, is described as:

$$S + P \overset{K_{obs}}{\rightleftharpoons} SP \tag{A7}$$

and the phenomenological equilibrium quotient, K_{obs}, is

$$K_{obs} = \frac{[SP]}{[S]\ [P]} \tag{A8}$$

This equilibrium is established in a dilute aqueous solution of the oligopeptide which contains an excess of salt $C_{p+}A_{p-}$. (Effect of pH on K_{obs} is not considered here.) K_{obs} is, in general, a function of the activities of the participant species, as a result of indirect effect of low-molecular-weight ions on the macromolecular activity coefficients. If the change in macromolecular hydration during associaton is negligible, the reference state equilibrium where the polypeptide is in hydrated but unligated form and the nucleic acid is hydrated and with its complement of condensed counterions is

$$P^0 + S^0 \overset{K^0}{\rightleftharpoons} SP^0 + \Delta r^0 D \tag{A9}$$

where D is the counterion,

$$\Delta r^0 = r_S^0 - r_{SP}^0 \tag{A10}$$

the number of moles of counterion released at the binding of 1 mole of oligopeptide, and

$$K^0 = \frac{[SP^0]}{[S^0]\,[P^0]}\, a_D{}^{\Delta r} \tag{A11a}$$

$$= K_T^0 \frac{\gamma_S{}^0 \gamma_P{}^0}{\gamma_{SP}{}^0} \tag{A11b}$$

where K_T^0 is the thermodynamic equilibrium constant and γ terms are the activity coefficients at the reference state. The phenomenological equilibrium ratio[9,10,34] is

$$K_{obs} = K^0\, \Sigma_{SP}^0 / \Sigma_S^0\, \Sigma_P^0 \tag{A12}$$

where Σ terms are the binding polynomials[34] reflecting activity coefficient effects and interrelationhips among "ligand" activities. Combining eqns. A11 and A12 leads to

$$\ln K_{obs} = \ln K_T^0 + \ln \frac{\gamma_S{}^0 \gamma_P{}^0}{\gamma_{SP}{}^0} + \ln \Sigma_{SP}^0 - \ln \Sigma_S^0 - \ln \Sigma_P^0 \tag{A13}$$

where the binding polynomials

$$\Sigma_P^0 = 1 \tag{A14}$$

$$\Sigma_S^0 = a_D{}^{r_S^0} \tag{A15}$$

$$\Sigma_{SP}^0 = a_D{}^{r_{SP}^0} \tag{A16}$$

From eqns. A9, and A13–A16, it follows that

$$\ln K_{obs} = \ln K_T^0 + \ln \frac{\gamma_P{}^0 \gamma_S{}^0}{\gamma_{SP}{}^0} - \Delta r^0 \ln a_D \tag{A17}$$

According to the condensation model[38]

$$r_S^0 = N(1 - \xi_S{}^{-1}) \tag{A18}$$

and

$$r_{SP}^0 = (N - \zeta)\,(1 - \xi^{-1}) \tag{A19}$$

Assuming that $\xi_S^{-1} = \xi_{SP}^{-1} = \xi^{-1}$, then from eqns. A9, A18, and A19 it follows that

$$\Delta r^0 = \zeta (1 - \xi^{-1}) \tag{A20}$$

Note that the following approximations are made to obtain the activity coefficients in eqn. A17: (a) only electrostatic contributions to the activity coefficients are considered; (b) since ln γ is an excess electrostatic free energy, contributions to ln γ from different regions on a macroion may be additive.
Extending this derivation further,

$$\ln \gamma_{P^0} = \ln \gamma_{p^0,\text{site}} + \ln \gamma_{P^0,\text{remainder}} \tag{A21}$$

Assuming that

$$\ln \gamma_{P^0,\text{site}} = \zeta \ln \gamma_D \tag{A22}$$

and

$$\ln \gamma_{SP^0} = \frac{N - \zeta}{N} \ln \gamma_{S^0} + \ln \gamma_{P^0,\text{remainder}} \tag{A23}$$

and moreover, for the polynucleotide[38]

$$\ln \gamma_{S^0} = \frac{NG_{el}}{RT} = -N\xi^{-1} \ln \kappa b = -0.5N\xi^{-1} \ln [D] - N\xi^{-1} \ln \delta \tag{A24}$$

where G_{el} is the excess electrostatic free energy per mole of structural charges, κ is the Debye–Hückel screening parameter and $\delta = 0.33b$ in aqueous solution near 25°C. Therefore,

$$\ln \frac{\gamma_{P^0}\gamma_{S^0}}{\gamma_{SP^0}} = \zeta\xi^{-1} (0.5 \ln [D] + \ln \delta) + \zeta \ln \gamma_D \tag{A25}$$

For uni-univalent electrolyte, $[D]=[C] = m$, and assuming that $\gamma_D=\gamma_C=\gamma_\pm$

$$\ln K_{obs} = \ln K_T^0 - \zeta[1-(2\xi)^{-1}] \ln m + \zeta\xi^{-1} \ln \delta\gamma_\pm \tag{A26}$$

and

$$\left(\frac{\mathrm{d} \ln K_{obs}}{\mathrm{d} \ln m}\right)_{T,\ p,\ \text{pH}} = -\zeta\left[\psi - \xi^{-1} \frac{\mathrm{d} \ln \gamma_\pm}{\mathrm{d} \ln m}\right] \tag{A27}$$

NOTE ADDED IN PROOF

During the review of this paper, Melander *et al.*[39] have published an article in which they have also used Manning's condensation theory to treat retention data of

proteins obtained on different ion-exchange columns. This article has provided a different perspective of the phenomena involved in ion-exchange chromatography of proteins. The retention equation developed by Melander *et al.* which combines both electrostatic and hydrophobic theory has allowed a comprehensive analysis of retention data of proteins.

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