pH Dependence of Ion-Exchange Equilibrium of Proteins

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The adsorption equilibrium of bovine serum albumin on Q-sepharose, a strong anion exchanger, was studied with batch equilibrium experiments at pH between 4 and 9 and ionic strengths between 5 and 440 mmol/L. Dependence of the adsorption equilibrium on the ionic strength was modeled as an ion exchange reaction. A simplified mechanism of this ion exchange reaction also yielded an expression for the dependence of the equilibrium on the charge of the protein. This model describes the measurements well, using fitted constants with physically realistic values.

Introduction

Chromatographic separations account for a large share in the production costs of pharmaceutical proteins (Petrides et al., 1995). It is thus desirable to minimize these costs. Designing cost-effective separation processes requires knowledge and understanding of the equilibrium and kinetics of the adsorption. In this article we report theoretical and experimental results of the ion-exchange equilibrium of proteins.

The ion-exchange equilibrium of proteins has been described by various models. In the Langmuir model it is assumed that adsorption is a reaction between a protein and an adsorption site. This model can be used to describe adsorption isotherms at constant pH and ionic strength (Skidmore et al., 1990; Skidmore and Chase, 1990; Whitley et al., 1989). To describe the influence of the salt concentration on the equilibrium Hu et al. (1992) used the Langmuir model with an empirical relation between the equilibrium constant and the salt concentration and James and Do (1991) used the competitive Langmuir-Freundlich isotherm. In the steric mass action (SMA) model it is assumed that the adsorption is an exchange reaction between a free protein and a certain number of bound ions. This model is the most widely used model and incorporates the influence of the salt concentration (Kopaciewicz et al., 1983; Whitley et al., 1989; Velayudhan and Horvath, 1988; Brooks and Cramer, 1992; Bellot and Condoret, 1993; Li and Pinto, 1995). In the double-layer model (Ståhlberg et al., 1992; Roth and Lenhoff, 1993; Roth et al., 1996) it is assumed that no adsorption of proteins occurs at the ion exchanger surface but that the protein concentration is increased in the electric double layer of the ion exchanger surface. This model incorporates influences of ionic strength and protein charge. At present it can only be solved for high ionic strengths (> 50 mmol/L). There are no models that describe the influence of the protein charge, and thus of the pH, on the equilibrium at moderate ionic strengths. In this article we develop such a model.

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Theory

Steric mass action model

Usually protein binding to ion exchangers is described by the SMA model. In this approach it is assumed that the adsorption of a protein can be seen as an exchange reaction of the protein with a certain number of adsorbed ions

$$z_b\bar{S} + P \leftrightarrow z_b S + \bar{P} \tag{1}$$

Here S represents salt ions, P is the protein, overbars indicate the adsorbed state, and z_b is the binding charge. (For convenience we will develop the SMA model for exchange with monovalent ions, the extension to ions of higher valences is trivial.) The binding charge of the protein is the number of salt ions that exchange with the protein; it can be interpreted as the number of charges of the protein that bind to the ion exchanger. In general the binding charge of a pro-

tein is not equal to the net charge of the protein. If ideal behavior is assumed the equilibrium of this exchange reaction is given by

$$K_c \frac{c_P}{q_P} = \left(\frac{c_S}{q_S^*} \right)^{|z_b|} \tag{2}$$

Here c and q are the liquid and adsorbed phase concentration and the subscripts P and S refer to the protein and the salt ion, respectively; K_c is the equilibrium constant. The asterisk denotes adsorbed salt ions that are available for exchange. Not all adsorbed salt ions are available for exchange because previously adsorbed proteins will shield a number of them. Brooks and Cramer (1992) called this number (σ) the steric factor of the protein. For the adsorbed salt ion concentration available for exchange this yields

$$q_S^* = q_S - \sigma q_P \tag{3}$$

A rigorous expression for the equilibrium constant K_c is derived in the Appendix, and is given by

$$K_c = K_x \left(\frac{q_{\text{tot}}}{c_{\text{tot}}} \right)^{|z_b|-1} \tag{4}$$

with

$$q_{\text{tot}} = q_P + |z_b|(q_{\max,P} - q_P) \tag{5}$$

$$K_x = \exp\left(\frac{\Delta \mu_{\text{ex}}^0}{RT} \right) \tag{6}$$

Here K_x is the thermodynamic equilibrium constant, q_{tot} and c_{tot} are the sums of the molar concentrations of all species in the adsorbed and liquid phase respectively, $q_{\max,P}$ is the protein adsorption capacity of the ion exchanger, $\Delta \mu_{\text{ex}}^0$ is the chemical potential change of the exchange reaction, R is the gas constant (8.314 J/mol·K), and T is the absolute temperature.

To complete the equilibrium model the electroneutrality equation is required

$$q_S + |z_b| q_P = q_{\max,S} \tag{7}$$

where $q_{\max,S}$ is the adsorption capacity for salt ions. The adsorption capacity for the protein can be derived from Eqs. 3 and 7 (when $q_i^* = 0$ and thus $q_P = q_{\max,P}$) as

$$q_{\max,P} = q_{\max,S}/(|z_b| + \sigma) \tag{8}$$

This completes the conventional description of the adsorption equilibrium by the SMA model. The model is easily extended to more components. One has to be careful, however, because steric effects may be important. For example, large proteins may be excluded from areas where small proteins can enter (Skidmore and Chase, 1990); in that case, this model will fail.

Figure 1 shows some typical isotherms at varying ionic strengths. In this figure the adsorption capacity for the protein is unity, a larger equilibrium constant K_x will result in steeper initial slopes, while a larger binding charge z_b will result in larger distances between the isotherms at the various ionic strengths.

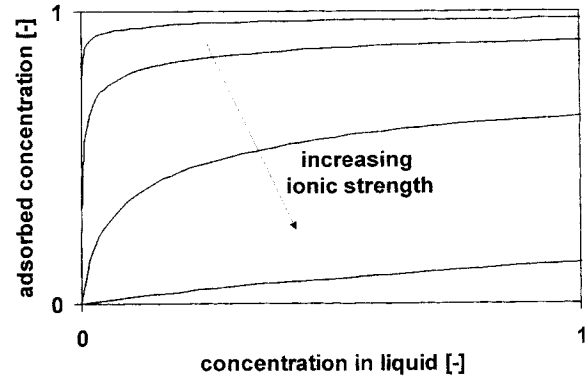

Figure 1. Typical SMA isotherm for proteins.

Equilibrium constant vs. net charge of the protein

To account for the influence of the pH on the equilibrium, the SMA model can be further explored by splitting the free energy of the exchange reaction into the electric enthalpy change and the nonelectric free energy change of the exchange reaction

$$\Delta \mu_{\text{ex}}^0 = \Delta h_{\text{ex}}^{\text{elec}} + \Delta \mu_{\text{ex}}^{0,n.e.} = \Delta h_{\text{ex}}^{\text{elec}} + \Delta h_{\text{ex}}^{0,n.e.} - T \Delta s_{\text{ex}}^0 \tag{9}$$

The electric enthalpy change or the electric work of the exchange can be calculated when an exchange process as depicted in Figure 2 is assumed. It is assumed that the binding charge of the protein is located at the adsorption area and that the rest of the net charge of the protein is located at the center of the protein. Furthermore, it is assumed that the charge of the Debye-Hückel atmosphere associated with the protein is also located at the center of the protein. The electric work (per mol of charges) W_{el} needed to bring two charges $z_1 e$ and $z_2 e$ from infinitely far apart to a distance d from each other can be calculated by an expression derived from the law of Coulomb:

$$W_{\text{el}} = \frac{N_{\text{av}} e^2}{4\pi \epsilon_0 \epsilon_r} \frac{z_1 z_2}{d} \tag{10}$$

Here N_{av} is Avogadro's number (6.022×10^{23} mol^{-1}), e is the electron charge, ϵ_r is the relative dielectric permittivity of the solvent (78.4) and ϵ_0 is the dielectric permittivity of vacuum (8.854×10^{-12} F/m).

Initially the protein is infinitely far away and the salt ions are adsorbed. First, the z_b salt ions are moved away from the ion exchanger. The electric work needed is

$$W_{\text{el}} = \frac{N_{\text{av}} e^2}{4\pi \epsilon_0 \epsilon_r} \frac{z_b^2}{r_S + r_I} \tag{11}$$

Here r_S is the radius of the salt ion and r_I is the distance

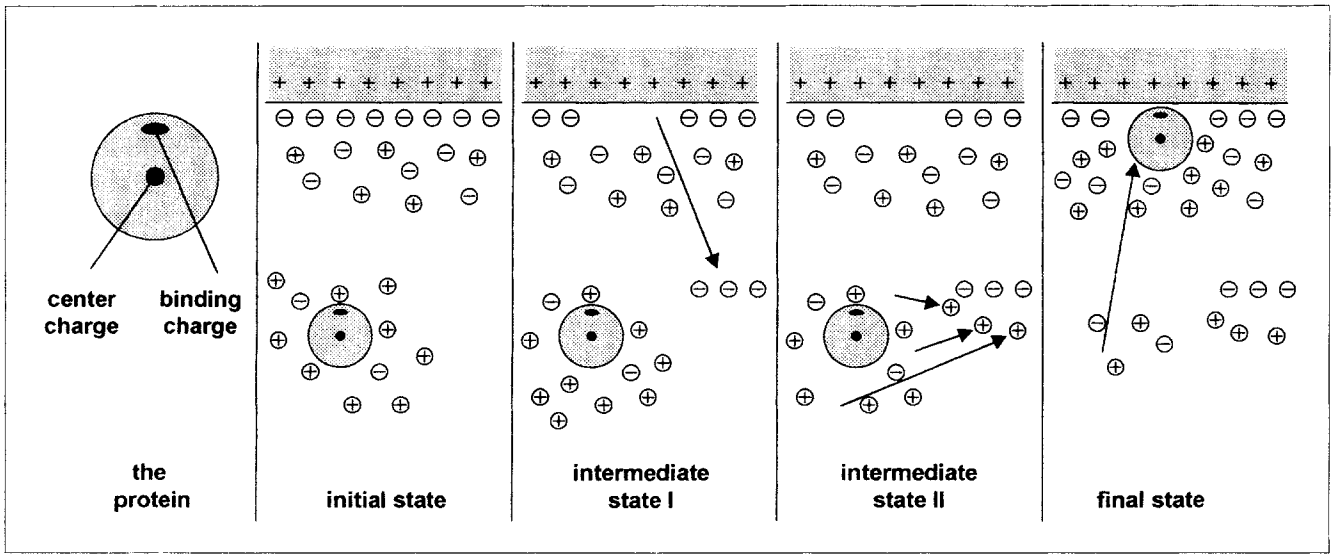

Figure 2. Mechanism of the protein ion exchange reaction.

between the surface and the charge of an ion exchange group. The counterions of the binding area of the protein then move from the protein to the salt ions coming from the ion exchanger. The electric work needed for this is neglected. Finally the protein is moved toward the ion exchanger. The work needed for this is the sum of the work of bringing the binding charge and the center charge to the surface of the ion exchanger:

$$W_{\text{el}} = \frac{N_{\text{av}} e^2}{4\pi\epsilon_0\epsilon_r}\left\{Z_{e.k.}(z_P - z_b)z_b\frac{\psi}{r_P + r_I} - \frac{z_b^2}{r_I + r_b}\right\} \quad (12)$$

Here z_P is the net charge of the protein, r_P is the radius of the protein, r_b is the distance between the surface of the protein and the binding charge and $Z_{\text{e.k.}}$ is the dimensionless electrokinetic charge, which is the correction factor which takes the charge of the Debye-Hückel atmosphere associated with the protein into account. Furthermore, a potential factor ψ is introduced in this equation to account for non-idealities. This potential factor will be discussed in the "Discussion" section.

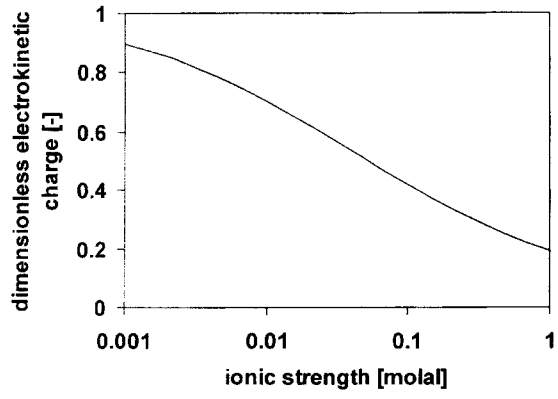

Figure 3. Dimensionless electrokinetic charge of a sphere with a radius of 2.7 nm (e.g., BSA).

For small ions this line will shift more than two orders of magnitude to the right due to the smaller radius.

The charge of the Debye-Hückel atmosphere associated with a charged spherical object is a subject in books on electrophoresis (Radola, 1992) and surface chemistry (Hiemenz, 1977). When a protein moves in an electric field as in electrophoresis, its apparent or effective charge (the electrokinetic charge) will not be equal to its net charge due to the Debye-Hückel atmosphere of counterions around the protein which partly moves with the protein. The charge in the part of the ion atmosphere moving with the protein has to be subtracted from the total charge of the protein, yielding the electrokinetic charge. The dimensionless electrokinetic charge, the ratio of the apparent charge $z_{\text{e.k.}}$ and the net charge z_{net} of a sphere of radius r at a certain ionic strength is given by (Henry, 1933):

$$Z_{\text{e.k.}} = \frac{z_{\text{e.k.}}}{z_{\text{net}}} = \frac{f(r\kappa)}{1 + r\kappa} \quad (13)$$

$$\kappa^2 = \frac{\rho_w N_{\text{av}} e^2}{kT\epsilon_0\epsilon_r} I \quad (14)$$

$$I = \sum_{S = \text{all salt ions}} z_s^2 m_s \quad (15)$$

In these equations κ is the inverse Debye length, ρ_w is the density of the solvent, k is Boltzmann constant (1.38×10^{-23} J/K), I is the ionic strength, and m_S are the molalities of all the salt ions. The equation $f(r\kappa)$ is known as the Henry equation; it varies between the Smoluchowsky limit of 1 at low ionic strength and the Einstein limit of 1.5 at high ionic strength (Hiemenz, 1977). The dependence of the electrokinetic charge on the ionic strength for a sphere with a radius of 2.7 nm (which is about equal to the radius of a protein of 67 kDalton) is depicted in Figure 3.

If it is assumed that the distance between the surface and the charge of the salt ion is equal to the distance between the binding charge and the surface of the protein ($r_S = r_b$), then

Eqs. 11, 12 and 13 can be combined to give the total electric work of the exchange reaction

$$-\Delta h_{\mathrm{ex}}^{\mathrm{elec}} = W_{\mathrm{el}} = \frac{N_{\mathrm{av}} e^2}{4\pi\epsilon_0\epsilon_r}\psi\frac{f(\kappa r_P)}{1+\kappa r_P}(z_P - z_b)\frac{z_b}{r_P + r_I} \quad (16)$$

This completes the protein ion exchange model. If it is assumed that the binding charge of a protein, the non-electric free energy change of the exchange reaction, and the protein adsorption capacity of the ion exchanger are constants, and if the charge of a protein as a function of the pH is known, then this model can be used to describe the ion exchange equilibrium of proteins at varying pH and ionic strength.

Pore liquid

When adsorption equilibria are modeled it is always necessary to define the phases to which the concentrations under consideration apply. The phases in ion exchange are the liquid phase and the adsorbing or surface phase. The adsorbing phase consists of the interior surface of the ion exchanger and an adjacent layer of liquid with a certain thickness. The ratio of the thickness of this layer to the pore radius determines whether or not the liquid in the pores belongs (partly) to the liquid phase. (For convenience we will assume that there are pores in the interior of ion exchange particles, although many ion exchangers consist of a polymer network that contains no real pores.) Jansen et al. (1996) compared three approaches to describe the adsorption of small ions to ion exchangers: the SMA model with the pore liquid as part of the adsorbing phase; the SMA model with the pore liquid as part of the liquid phase; and the Donnan equilibrium with the pore liquid as part of the adsorbing phase.

The Donnan equilibrium is similar to the SMA model but assumes that the equilibrium constant is unity and that counterions and coions of the ion exchangers can enter the adsorbing phase. Equation 2 becomes

$$\left(\frac{c_i}{q_i}\right)^{1/z_i} = \left(\frac{c_j}{q_j}\right)^{1/z_j} \quad (17)$$

where i and j represent all combinations of ions.

The first approach does not allow penetration of coions into the ion exchanger. Jansen et al. (1996) showed that this is at variance with experiment. The second and the third approach do allow penetration of coions into the ion exchanger and are about equally good in describing the experimental results of Jansen et al. (1996). In this article we use the second approach.

Experimental Studies

Materials

Bovine serum albumin (BSA) was purchased from Boehringer Mannheim. Q-sepharose FF was purchased from Pharmacia Biotech. The other chemicals used were sodium chloride, acetic acid, acetate, tris and chloric acid. Some properties of BSA and Q-sepharose are found in Tables 1 and 2. In solution BSA is a prolate ellipse (cigar-shaped) with an axial ratio of about four (Peters, 1985). Q-sepharose FF consists of a network of strings of agarose with quaternary ammonium salts (strong anion exchange groups) attached.

Table 1. Some Properties of Q-sepharose FF

Average particle diameter	93 μm
Small ion capacity	0.2 mol/L sedimented bed
Exclusion limit	4×10^6 g/mol
Matrix holdup inside particle	6% vol. of sedimented bed
'Pore' diameter*	About 350 Å
Polymer string diameter**	About 56 Å

*According to the manufacturer, personal communication.
**Based on a cubic network with $d_{\mathrm{pore}} = 350$ Å and a particle fraction of 0.65 in the bed.

Experimental setup

Batch experiments were performed in 13-mL tubes; these were filled with 1 mL of a Q-sepharose FF suspension and the necessary amounts of freshly prepared solutions of protein, salt, and buffer and with milli-Q water (conductivity < 10 μS) up to total of 10 mL. The amount of Q-sepharose in the suspension was measured by filling some measuring cylinders with the suspension, and was about 0.7 mL sedimented bed per mL suspension. The tubes were sealed and their contents mixed overnight by rotation. After about 18 h the pH and the UV adsorption at 280 nm were measured.

The ionic strength was varied between 5 and 440 mM, the pH between 4 and 9, and the protein concentration between 0 and 20 g/L. Most experiments were performed at 25°C. Experiments with acetate buffer were performed at pH below 7, experiments with tris buffer were performed at pH above 6. Some experiments were performed at 5°C and at 37°C. The adsorbed protein concentration is calculated from the protein concentration in the liquid phase according to the mass balance

$$\epsilon_b q_P + \epsilon_l c_P = c_P^{\mathrm{tot}} \quad (18)$$

where ϵ_l is the liquid fraction in the tube, ϵ_b is the sedimented bed fraction in the tube and c_P^{tot} is the average concentration of protein in the tube.

Results

The adsorption model discussed in the theory section was put into a computer program. The following remarks have to be made about the model:

- The chloride acetate equilibrium constant for binding to the ion exchanger has been determined as unity; Eq. 17 is used to describe this equilibrium.

Table 2. Some Properties of BSA

Molecular mass	66,700 g/mol
Dimensions	40 Å×40 Å×140 Å
Radius based on sphere	26.9 Å
Density	1,360 kg/m^3
Isoelectric point	4.8

Table 3. Fitted Model Parameters

T [°C]	$q_{max,P}$ [g/L Bed]	σ	z_b	ψ	$\Delta\mu_{ex}^{0,n.e.}$ [kJ/mol]	$\Delta h_{ex}^{0,n.e.}$ [kJ/mol]	Δs_{ex}^{0}	# exp.
25	95	142	4.3	1.35	−59	—	—	339
5*	95	142	4.3	1.35	−54	−8	167	32
37*	95	142	4.3	1.35	−59			31
5	77	174	5.0	2.4	−79	−155	−288	32
37	83	161	4.6	2.0	−73			31

*Assuming that $q_{max,P}$, σ, z_b, and ψ are the same as at 25°C.

- Activities instead of concentrations of chloride and acetate in the bulk liquid phase are used in Eq. 2. The improvement in the fit due to this change is marginal.
- Seventy percent of the volume of the sedimented ion exchanger bed is taken as part of the liquid phase, as will be explained in the "Discussion" section.
- The binding charge, the adsorption capacity, the nonelectric free energy change of the adsorption, and the dielectric permittivity are all assumed to be independent of pH and ionic strength.
- The titration curve of BSA has been measured by Tanford et al. (1955) and yields an expression for the binding of hydrogen ions on BSA.
- Binding of small anions on BSA is assumed to be identical to that on HSA. For the binding of chloride ions and the effect of temperature on it, isotherms obtained by Scatchard and Yap (1964) are used. No acetate binding isotherms are known, therefore the chloride isotherm is used with the equilibrium constants increased by a fitted factor of 50. The same method with factors in the same order of magnitude is used by Scatchard and Yap (1964) and Scatchard et al. (1959) for the adsorption of iodide, thiocyanate, fluoride, and trichloroacetate to HSA.
- At the isoelectric point small cations hardly bind to HSA (Scatchard et al., 1950), although HSA has many negative groups. Since we have no other data on cation binding to albumin we assume that cations do not bind to BSA at all values of the pH. If this assumption is wrong then the previous assumption that acetate binds 50 times better than chloride is probably also wrong. However, we do expect that these assumptions give a good approximation of the difference between the charge of BSA in a tris buffer and in an acetate buffer.

The result is a model with four unknowns: the protein adsorption capacity of the ion exchanger, the binding charge, the potential factor and the nonelectric free energy change. These four parameters are fitted simultaneously for all experiments at a certain temperature. The best-fit parameters are shown in Table 3. Figure 4 is a parity plot for the experiments at 25°C. Figures 5 and 6 show some isotherms; Figure 7 shows the dependence of the adsorption on pH. The calculated lines are for one pH and ionic strength in the isotherm plots while the experimental points may vary 0.1 in pH units and 10% in ionic strength. Therefore the parity plot is the better measure for the goodness of the fit. The experiments in acetate buffer give better fits than the experiments in tris buffer, which will be discussed later.

Discussion

As can be seen from Figures 4 to 7, the results are quite good, certainly when compared to the change of six orders in the equilibrium constant over the pH range.

Pore liquid

Some experiments were performed at nonbinding conditions: low pH and high ionic strength. The binding is indeed close to zero if it is assumed that 70% of the volume of the sedimented bed is part of the liquid phase (Figure 8). This

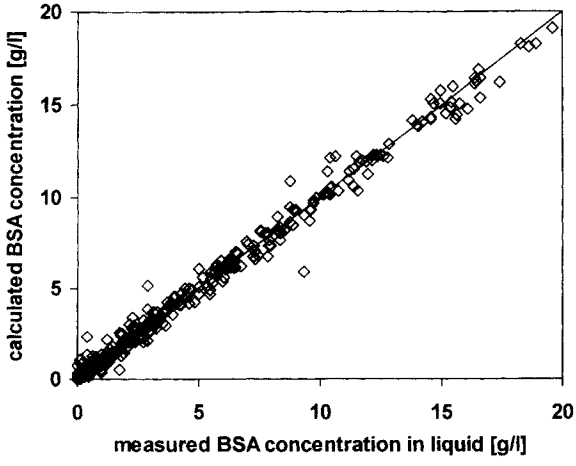

Figure 4. Parity plot of measured and calculated protein concentrations in the liquid phase at 25°C.

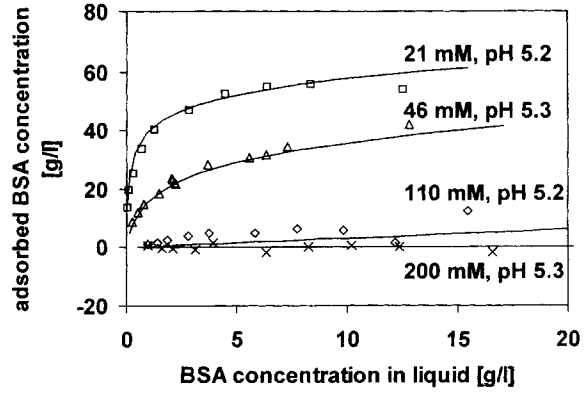

Figure 5. Measured and calculated adsorption isotherms of BSA on Q-sepharose FF.

At 25°C, pH about 5.2, and varying ionic strength.

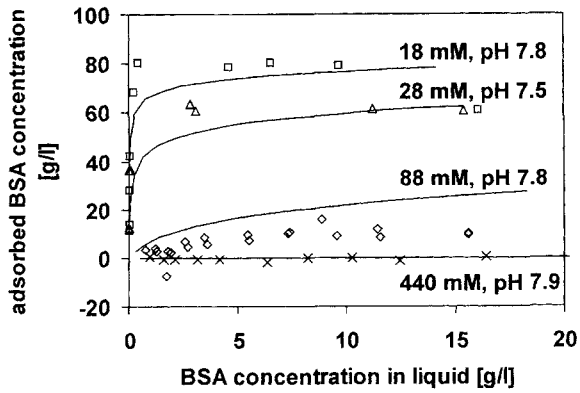

Figure 6. Measured and calculated adsorption isotherms of BSA on Q-sepharose FF.

At 25°C, pH about 7.8, and varying ionic strength.

means that 70% of the sedimented bed volume is accessible for BSA. From salt equilibrium experiments it is derived that about 92% of the bed volume is accessible for salt. It follows that about 22% of the void fraction in the bed is inaccessible for BSA, probably due to steric hindrance.

Protein adsorption capacity

The protein adsorption capacity is 95 g BSA per liter sedimented ion exchanger bed. The steric factor σ (the number of ion exchange groups per protein that do not participate in the binding of the protein) is calculated from this, giving a value of 142. This magnitude of the steric factor is caused by steric hindrance of two types. First, some groups are placed in such small pores that they are inaccessible for the protein. Second, some groups are covered by already bound protein but do not bind to it. Assuming that 35% of the sedimented bed consists of intraparticle volume accessible for BSA and that 57% of the sedimented bed consists of intraparticle volume accessible for small ions it can be estimated that 55 charged groups are always inaccessible for BSA and 83 groups are inaccessible through shielding by BSA.

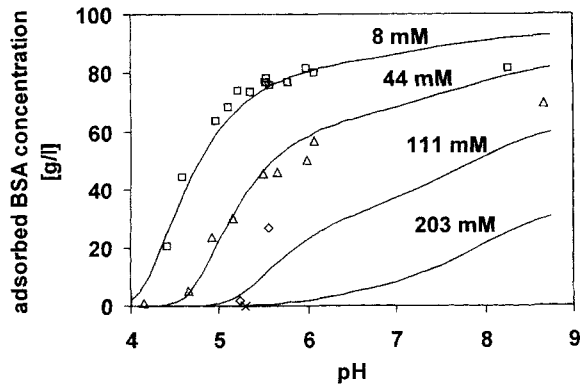

Figure 7. Measured and calculated adsorption isotherms of BSA on Q-sepharose FF.

At 25°C, 12 g/L total protein concentration (c_P^{tot} in Eq. 18), and varying ionic strength.

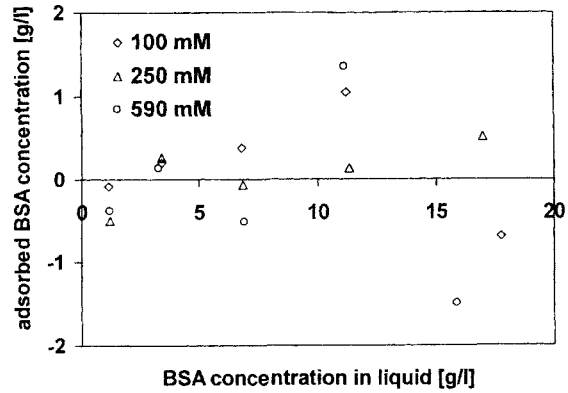

Figure 8. Adsorption isotherms of BSA on Q-sepharose FF at nonbinding conditions.

At 25°C, at pH 4.6, and varying ionic strength. The adsorbed BSA concentration was calculated assuming that 70% of the bed volume was part of the liquid phase. These experiments were performed with 5 mL of ion exchanger suspension instead of 1 mL in all other experiments.

The experiments at 25°C were split into groups according to the pH of the experiment and subsequently for each group the protein adsorption capacity was fitted with the other fit parameters taken as in Table 3. This yielded Figure 9 for the pH dependence of the protein adsorption capacity. The experiments at 25°C were also split into groups according to the ionic strength of the experiment and subsequently for each group the protein adsorption capacity was fitted with the other fit parameters taken as in Table 3. This yielded Figure 10 for the ionic strength dependence of the protein adsorption capacity.

The adsorption capacity does not depend on pH. Other researchers did find a dependence on pH. Van der Wiel (1989) found a maximum in the adsorption capacity close to the isoelectric point, and Norde and Lyklema (1978) had essentially the same finding. However, both of them used other ion exchangers that probably have a less hydrophilic support. This causes the protein to rearrange to a certain extent so that hydrophobic contact can be made between the ion exchanger support and hydrophobic groups of the protein. Rearranging is favored by a high net charge of the protein because the charges will tend to move apart. This is not the

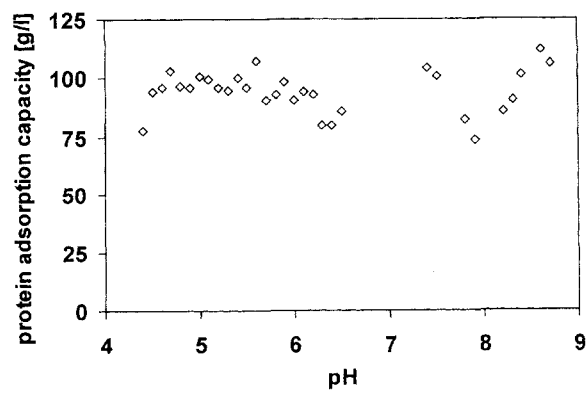

Figure 9. pH dependence of the BSA adsorption capacity of Q-sepharose FF at 25°C.

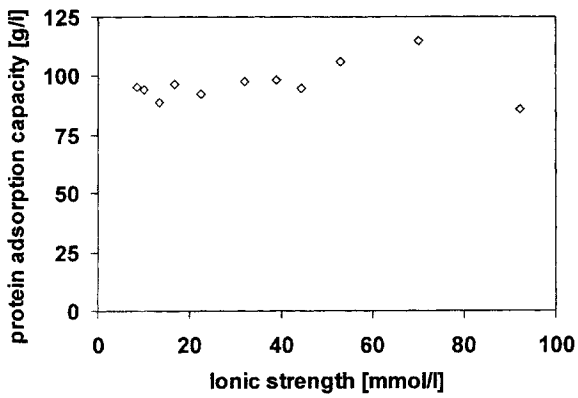

Figure 10. Ionic strength dependence of the BSA adsorption capacity of Q-sepharose FF at 25°C.

case at the isoelectric point and a higher capacity is possible. It can be concluded that hydrophobic interactions play essentially no role in the protein adsorption to Q-sepharose FF.

There is no measurable effect of the ionic strength on the protein adsorption capacity.

Binding charge

The experiments at 25°C were split into groups according to the pH of the experiment. Subsequently, for each group the equilibrium constant was fitted while the binding charge and the protein adsorption capacity were taken as in Table 3; that is, only Eqs. 2–7 were used as the model and K_x in Eq. 6 was fitted. The results are plotted in Figure 11 together with the net charge of BSA in a buffer of 25 mM ionic strength. First, this figure strongly suggests a linear dependence of the log of the equilibrium constant on the net protein charge. Second, this figure is the main evidence that the assumption that the binding charge is independent of pH is good because then the dependence of the equilibrium constant on the protein charge can be explained by our model.

The question of the nature of the binding charge arises. Is there really an area on the protein with z_b charges that is

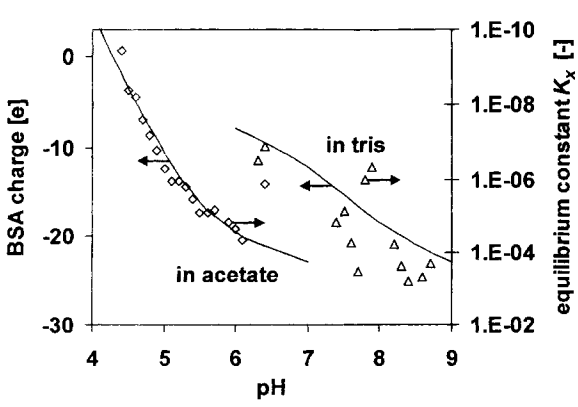

Figure 11. pH dependence of the fitted equilibrium coefficient and the net charge of BSA in acetate and tris buffers of 25-mM ionic strength.

Table 4. Values from Literature for Binding Charges of Various Proteins

Researchers	z_b Value
Li and Pinto (1995)	4.8
Whitley et al. (1989)	−0.2–3.6
Fausnaugh-Pollitt et al. (1988)	2.1–3.1
Kopaciewicz et al. (1983)	3.6–8.3
Raje and Pinto (1997)	4.8–6.0

responsible for the binding to the chromatographic contact area? Is there only one area or are there more? To shed some light on these questions some points will be put forward:

- Most researchers find values for the binding charge of a variety of proteins in the region between zero and eight; see Table 4. The procedure to determine the binding charge used by most researchers is based on taking the log of Eq. 2 at underload conditions, giving:

$$\log k' = \log \frac{q_P}{c_P} = C_1 - z_b \log c_S \tag{19}$$

where k' is the retention factor and C_1 is independent of the ionic strength. In the model presented in this article this equation would change to:

$$\log k' = \log \frac{q_P}{c_P} = C_2 - z_b\{C_3 z_{\text{e.k.}} + \log c_S\} \tag{20}$$

where C_2 and C_3 are independent of ionic strength. This procedure would now yield other values for the binding charge because the electrokinetic charge $z_{\text{e.k.}}$ depends on the ionic strength through both a change in the binding of small anions by BSA and a change in the dimensionless electrokinetic charge. The model predicts that the binding charge of BSA determined by Eq. 19 varies between 2.6 and 5.9. This partly explains the scatter in the binding charges measured by other researchers.

- The double-layer model of protein adsorption (Roth and Lenhoff, 1993) denies the existence of a binding charge. From this model, however, an apparent binding charge can be calculated with Eq. 19: the binding charge equals the differential of the log of the retention factor with respect to the log of the inverse of the ionic strength. The model predicts a strong dependence of the binding charge on the net protein charge. This does not agree with our results at moderate ionic strengths.

According to the double-layer model, diffusion of adsorbed proteins should be rapid. Our experimental evidence points in the opposite direction: surface diffusion of proteins on ion exchangers is very slow if it occurs at all.

The weak point of the double-layer model may be that the ion exchanger is assumed to have its charge dispersed over the surface instead of located at certain discrete points. Therefore the double-layer model cannot incorporate interaction of two discrete charges (one at the protein and one at the ion exchanger).

It can be concluded that the double layer adsorption model denies the existence of a chromatographic contact area and that there is no strong evidence for this denial, while there is evidence that such areas exist.

- Fausnaugh-Pollitt et al. (1988) were able to separate three variants of lysozyme varying by a single charged amino acid group on a cation exchanger. They concluded that although charged groups at different locations in a molecule are sensed by the sorbent, their contributions may not be equivalent. This indicates that interactions between discrete charges at the protein surface and the ion exchanger do play a role in the adsorption.

- For heparin, a flexible poly-ion, we measured binding charges in the range of 5 to 10, depending on the ion exchanger used. (These were propriatory measurements; we cannot provide further details.) This indicates that a more flexible molecule has a higher binding charge and that the geometry of the ion exchanger surface is important.

- It is important to figure out what determines the orientation of an adsorbed protein. In affinity chromatography it is clear that a certain active site on the protein determines its orientation (Regnier, 1987). In hydrophobic interaction chromatography there is also a certain active site, although it may be larger than in affinity chromatography, which determines the orientation (Regnier, 1987). In ion exchange chromatography there may be an area on the protein surface with an excess binding charge (an excess negative charge in the case of an anion exchanger). This then determines the orientation, but the adsorption forces work over a larger distance and the orientation of the adsorbed protein may also be determined by all the charges in the protein, that is, the dipole. As is shown by Roth and Lenhoff (1993), the electrostatic interaction between a protein and an ion exchanger does depend on the orientation of the protein. If the orientation is dipole dependent then the chromatographic contact area is not necessarily different from the rest of the protein surface.

Before we can draw a conclusion we have to (roughly) consider the geometry of the contact area. At the isoelectric point BSA has about 90 positive and 90 negative charges; we estimate that about 75% of the charged groups of the protein are located at the surface and about 10% of the protein surface is involved in binding; thus about 15 charged groups are located in the contact area of the protein. At the ion exchange surface about 60% of the 170 ion exchange groups that are available per protein are accessible for BSA; of those we guess that about 20% are located in the contact area of the ion exchanger; these are about 20 ion exchange groups per protein.

Upon adsorption these two surfaces make contact with each other. Now there are two possibilities. If the orientation of the protein is determined by the contact area, an excess of three to four charges in this area seems plausible. We would however expect this excess charge to depend on pH. If the orientation of the adsorbed protein is determined by the charge of the entire protein the contact area does not necessarily have an excess binding charge. In this case the value of the binding charge is determined by steric factors. When two irregular surfaces are brought into contact they usually have three contact points, or when the surfaces are slightly flexible they might have four or five contact points. Furthermore, if the pH changes then the ratio of positive to negative charges in the contact area of the protein will change; however if there are enough negative charges in this area, it is not expected that the binding charge will change.

The binding charge is independent of pH and the experimentally determined value is close to the expected value based on steric factors. Therefore we suspect that the orientation of adsorbed proteins is determined by the charge distribution in the entire molecule and that steric factors are the most important factors determining the binding charge.

Overall it can be concluded that the orientation of an adsorbed protein is determined by the charge distribution in the entire protein; that a chromatographic contact area does exist, but that it is not necessarily different from the rest of the protein surface; and that the binding charge is expected to be determined by steric factors.

The question of the nature of the binding charge will probably remain until the (linearized or nonlinearized) Poisson-Boltzmann equation is solved for a system with discrete charges on the ion exchanger; even this may not yield decisive information because the scale on which these phenomena occur is so small that the assumption of the electrolyte as a continuum may not be valid.

Potential factor

It is found that the potential factor is 1.35. In the calculation it is assumed that the radius of BSA is 26.9 Å and the radius of the ion exchange group is 2 Å; furthermore the dielectric constant of water is used. For the dimensionless electrokinetic charge $Z_{e.k.}$ an average value of 0.42 is found; if this factor is put at unity the potential factor will be 0.55, which is a factor of 1.80 from unity. It has to be noticed that the fitted potential factor is based only on the charged amino acid groups in BSA that change their charge between pH 4 and 9.

Several effects may cause the potential factor to deviate from unity. It can be assumed that the potential factor is the product of several factors:

$$\psi = \psi_{ca}\psi_r\psi_s\psi_\epsilon\psi_{ia} = \psi_{ca}\frac{(r_P + r_I)_{\text{assumed}}}{(r_P + r_I)_{\text{actual}}}\psi_s\frac{(\epsilon_r)_{\text{assumed}}}{(\epsilon_r)_{\text{actual}}}\psi_{ia} \quad (21)$$

- ψ_{ca} is the charge asymmetry factor, which accounts for the charges not being exactly at the center of the protein. The calculations of Roth and Lenhoff (1993) indicate that this factor does not deviate much from unity. The reason for this may be that the potential change at the surface of the protein due to a change in the geometry of the charges inside the protein is dampened by the solution around the protein. The solvent has a higher dielectric constant than the protein interior and the solution contains an electrolyte.

- ψ_r is the radius factor, which accounts for a deviation of the actual radius from the assumed radius. The assumed radius is calculated from the molecular mass and the density of BSA. However BSA may have up to 20 g hydration water per mole (Ocon et al., 1987), in which case a value of 0.9 for the radius factor is found.

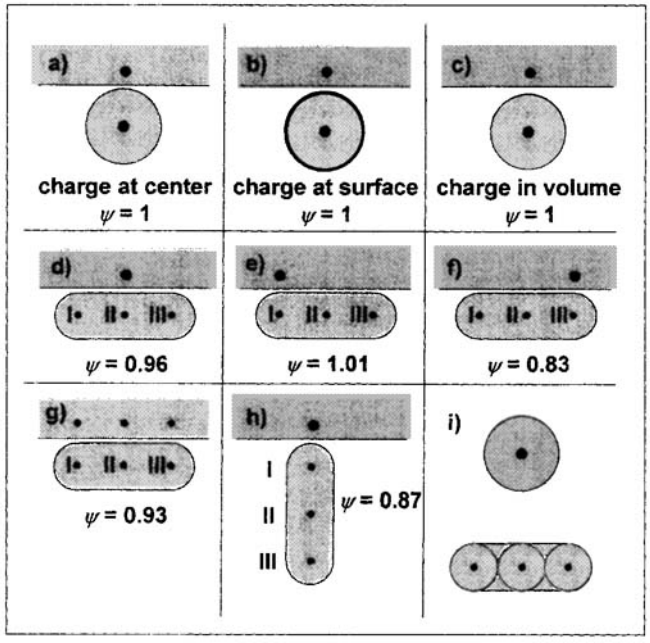

Figure 12. Theoretically calculated shape factors.

• ψ_s is the shape factor, which accounts for a deviation of the shape of the protein from a sphere. A better approximation for the shape of BSA is a kind of ellipsoid that is built up of three adjacent spheres (with a radius of 16.9 Å) with the holes filled up as in Figure 12i; each sphere represents one of the domains of BSA. Between pH 4 and 9 domain I changes 30 charges, domain II changes 26 charges, and domain III changes 21 charges. In Figure 12 some results are shown for the shape factor that can be calculated if a certain binding geometry is assumed. The shape factor might be somewhat smaller than unity; however, binding geometries with a higher shape factor are favored, because of the stronger binding and the shape factor is therefore probably close to unity. Another argument for this is that, as with the charge asymmetry factor, the potential change at the surface of the protein due to a change in the protein shape is damped by the solution around the protein.

• ψ_ϵ is the effective dielectric factor, which accounts for a deviation of the effective dielectric constant from the dielectric constant of water. The effective dielectric constant for the interaction of two charges inside a protein depends on the dielectric constant of water ($\cong 78$) and of the protein interior ($\cong 2$), the radius of the protein, the position of the charges, and the ionic strength in the water surrounding the protein (Hill, 1956; Gilson et al., 1985). At zero ionic strength and one charge in the center and the other at the surface of the protein the effective dielectric constant equals the dielectric constant of water; at higher ionic strengths the effective dielectric constant increases. If the protein is close to the ion exchanger the effective dielectric constant of the interaction of these two charges will probably be lower because the average dielectric constant around the protein is lowered; however it is unknown how much. Therefore a fairly large uncertainty is present in the effective dielectric factor.

• ψ_{ia} is the correction factor for the contribution of the ionic atmosphere of the protein. In the calculation of the electric work needed for adsorption it is assumed that a fraction $Z_{e.k.}$ of the ionic atmosphere of the protein is located far away and that the rest is located at the center of the protein. This is a rough assumption.

It can be concluded that bound water can have a small effect on the potential factor but that the uncertainty in the effective dielectric constant and the uncertainty in the location of the ionic atmosphere can account for a value of 1.3 or 1.4 of the potential factor.

The temperature dependence of the exchange reaction

At 5°C the adsorption of BSA on Q-sepharose is slightly lower than at 25°C; the equilibrium constant K_x changes by a factor of 2.2. At 37°C the adsorption is slightly higher than at 25°C; the equilibrium constant K_x changes by a factor of 1.8. These equilibrium constants are calculated assuming that $q_{\max,p}$, σ, z_b, ψ, and anion binding on BSA at 37°C are the same as at 25°C. From these values nothing can be deduced about the entropy and the enthalpy change of the exchange reaction because the amount of small anions adsorbed on the protein is temperature-dependent.

From the fitted values of the nonelectric free energy change of the adsorption, $\Delta\mu_{ex}^{0,n.e.}$, the nonelectric enthalpy change and the entropy change of the adsorption are calculated with Eq. 9. The results are in Table 3. The nonelectric free energy change of adsorption is unfavorable for adsorption mainly because of enthalpy differences. There is a fair uncertainty, however, in the enthalpy and entropy change of the adsorption. The calculated enthalpy change also includes all electrostatic contributions that are independent of pH between pH 4 and 9. For instance, if tris binds better and acetate binds worse on BSA than we assumed, then a different nonelectric free energy change of adsorption will result.

The overall nonelectric free energy change is unfavorable for adsorption. Therefore BSA needs a sufficiently high net negative charge to adsorb and it would not be expected to adsorb at a pH below the isoelectric point. The isoelectric point is generally assumed to be about 4.8; however, in an acetate buffer the acetate will adsorb to the BSA and the isoelectric point will shift to a lower value. Based on our experiments, it is estimated that it shifts to about 4.3.

Conclusions

The adsorption of BSA to Q-sepharose at different pH values and ionic strengths can be well described by the SMA model, extended with a dependence of the equilibrium constant on the pH via the electrokinetic charge of the protein. The model gives a better understanding of the adsorption of proteins to ion exchangers than was previously possible. For example, it is shown that the binding of buffer ions to a protein can change the equilibrium.

It remains uncertain whether the model is suitable to describe the adsorption of other proteins on ion exchangers. The model is probably suitable when the following conditions are fulfilled:

• The hydrophobic interactions between the protein and the ion exchanger play a negligible role.

• The binding charge of the protein is independent of pH.

The model uses the electrokinetic charge of protein, a nonequilibrium property, to describe an equilibrium. This concept seems to be useful in other areas of research, such as in the ultrafiltration of protein solutions.

The physical meanings of two of the fit constants in the model, the potential factor and the binding charge, remain uncertain. This uncertainty will probably remain until the (linearized or nonlinearized) Poisson-Boltzmann equation is solved for a system with discrete charged groups at the ion exchanger.

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Notation

$\Delta G_{\text{reaction}}$ = free energy of reaction, J/mol
Δh_{ex} = enthalpy change of the exchange reaction, J/mol
Δs_{ex} = entropy change of the exchange reaction, J/mol·K
ϵ_b = volume fraction of sedimented bed
ϵ_l = volume fraction of the liquid phase
C = constant, independent of ionic strength
e = charge of one electron, 1.602×10^{-19} C
h = enthalpy, J/mol
q_S^* = adsorbed small ion concentration available for exchange with a protein, mol/L

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Appendix: Derivation of the Steric Mass Action (SMA) Model

In this appendix we derive an equilibrium relation for the exchange reaction defined in Eq. 1. For the chemical potentials of proteins and salt in both phases we have

$$\mu_i = \mu_i^0 + RT \ln(\gamma_i x_i) \tag{A1}$$

$$\overline{\mu_i} = \overline{\mu_i^0} + RT \ln(\overline{\gamma_i}\,\overline{x_i}) \tag{A2}$$

where μ_i is the chemical potential, x_i is the mole fraction, γ_i is the activity coefficient, subscript i is the protein or the salt ion, 0 indicates the standard state and overbars indicate the adsorbed state. At equilibrium the free energy of the reaction will be zero and an equilibrium relation can be derived.

$$\Delta G_{\text{exchange}} = 0$$

$$= |z_b|(\overline{\mu_S} - \mu_S) + (\mu_P - \overline{\mu_P})$$

$$= |z_b|\left\{\overline{\mu_S^0} + RT\ln(\overline{\gamma_S x_S}) - \mu_S^0 - RT\ln(\gamma_S x_S)\right\}$$

$$+ \left\{\mu_P^0 + RT\ln(\gamma_P x_P) - \overline{\mu_P^0} - RT\ln(\overline{\gamma_P x_P})\right\}$$

$$= \Delta\mu_{\text{ex}}^0 + RT\ln\left\{\frac{\gamma_P x_P}{\overline{\gamma_P x_P}}\left(\frac{\overline{\gamma_S x_S}}{\gamma_S x_S}\right)^{|z_b|}\right\} \Rightarrow$$

$$\exp\left(-\frac{\Delta\mu_{\text{ex}}^0}{RT}\right) = \frac{\gamma_P x_P}{\overline{\gamma_P x_P}}\left(\frac{\overline{\gamma_S x_S}}{\gamma_S x_S}\right)^{|z_b|} \tag{A3}$$

This can also be written as

$$K_x \frac{\gamma_P x_P}{\overline{\gamma_P x_P}} = \left(\frac{\gamma_S x_S}{\overline{\gamma_S x_S}}\right)^{|z_b|} \tag{A4}$$

where the equilibrium constant K_x is defined as

$$K_x = \exp\left(\frac{\Delta\mu_{\text{ex}}^0}{RT}\right) = \exp\left(\frac{|z_b|\Delta\mu_{\text{ex},S}^0 - \Delta\mu_{\text{ex},P}^0}{RT}\right) \tag{A5}$$

This result is known as the SMA model or the stoichiometric displacement model. The x in K_x makes clear that this relation is expressed in mole fractions. When it is expressed in concentrations, that is,

$$K_c \frac{c_P}{q_P} = \left(\frac{c_S}{q_S^*}\right)^{|z_b|} \tag{A6}$$

and when the activity coefficients are unity, the relation between K_c and K_x is:

$$K_c = K_x\left(\frac{q_{\text{tot}}}{c_{\text{tot}}}\right)^{|z_b|-1} \tag{A7}$$

where q_{tot} and c_{tot} are defined as

$$q_{\text{tot}} = q_P + |z_b|(q_{\max,P} - q_P) \tag{A8}$$

$$c_{\text{tot}} = c_P + c_S + c_{\text{water}} \tag{A9}$$

Note that q_S^* in Eq. A6 is the concentration of adsorbed salt ions that can participate in the exchange process.

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