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MOLECULAR ORIENTATION OF IMMUNOGLOBULIN G AT HIGH CONCENTRATION ON AN ION-EXCHANGE SORBENT

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SUMMARY

The change of molecular orientation of IgG, bound on a strong-anion-exchange surface, was studied using a generalization of the stoichiometric displacement model, over the entire range of protein adsorption isotherms. The Z number was found to decrease with increasing stationary phase protein concentration, approaching a limiting value. The analogy of the multiple equilibria model within highly cooperative identical binding sites was suggested as a possible way to evaluate the observed change in Z number with the protein concentration.

INTRODUCTION

Development of preparative (non-linear) protein chromatographic theory has advanced in recent years. Of special importance are papers pertaining to the areas of thermodynamics and kinetics[1–3]. The mechanistic aspect of preparative protein chromatography is represented by a recent paper dealing with a theoretical study of the extension of the stoichiometric displacement model (SDM) to the non-linear portion of protein adsorption isotherms[4]. The study showed that the SDM may be valid, with certain restrictions, at high protein concentrations. The possibility of examining the displacement effect and molecular orientation of proteins toward the stationary phase surface as a function of solute conentration was briefly discussed as well.

In this paper, the change in molecular orientation of protein, bound to the stationary phase surface, is studied, using a model system of immunoglobulin (IgG) adsorption on an ion-exchange sorbent. Morrissey has also examined IgG adsorption on hydrophilic silica surface with transmission infrared spectroscopy[5], and he calculated the fraction of protein carbonyl groups involved in binding to the silica surface from the amide I adsorption band shift data. He found that IgG had a bound fraction of 0.2 at low solute concentrations but only of about 0.02 at high concentration. His parallel experiments with flat silica plates and ellipsometry, and with hydrophobic polystyrene lattices using quasi-elastic light scattering[6] strongly suggest that the orientation of adsorbed IgG can dramatically change with protein concentration. To avoid possible surface-mediated conformational changes or irreversible binding of IgG in our experiments, an anion-exchange stationary phase was used instead of silica or hydrophobic matrices. Ion-exchanger sorbents are widely used in IgG separation with nearly 100% recovery of activity[7].

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EXPERIMENTAL

Bovine immunoglobulin G (IgG) was obtained from Calbiochem Brand Biochemicals (La Jolla, CA, U.S.A.), 1,3-diaminopropane from Aldrich (Milwaukee, WI, U.S.A.) and sodium chloride from Mallinckrodt (Paris, KY, U.S.A.).

Frontal chromatography was performed on instrumentation consisting of a Model III Constametric precision solvent delivery system (LDC/Milton Roy, Riviera Beach, FL, U.S.A.), a Rheodyne 7161 injection valve (Rheodyne, Cotati, CA, U.S.A.), and a Model 153 UV detector with a low-volume preparative flow-cell (Altex Scientific, Berkeley, CA, U.S.A.). Chromatograms were obtained on a 1200 Series chart recorder (Linear Instruments, Reno, NV, U.S.A.). A "Rabbit" gradient HPLC system (Rainin, Woburn, MA, U.S.A.), controlled by an Apple IIe programmer (Apple, Rolling Meadows, IL, U.S.A.) was used for column regeneration.

Experiments were performed on a strong-anion-exchange column, derived from a non-porous polymer-based weak-anion-exchange column (Microanalyzer MA7P, Bio-Rad Labs., Richmond, CA, U.S.A.)[8].

Mobile phase conditions [0.100–0.108 *M* sodium chloride in 20 m*M* 1,3-diaminopropane (pH 10)] were set in such a manner that the first subclass of IgG (IgGl) was not bound to the stationary phase. The mass fractions of IgG, bound and unbound, in the sample were determined using a Bradford assay (Coomassie Protein Assay Reagent, Pierce, Rockford, IL, U.S.A.) of collected fractions of the subclasses after chromatographic separation. All calculations refer to the retained portion of the samples. Measurements were performed at 23°C. The column was regenerated after each frontal chromatographic experiment by using three regeneration cycles, consisting of a 1-ml injection of 0.1 *M* sodium hydroxide, followed by a 10-min gradient of sodium chloride from 0 to 0.5 *M* in 20 m*M* 1,3-diaminopropane (pH 10).

RESULTS AND DISCUSSION

The SDM has been established to characterize protein adsorption on ion-exchange columns[9] and generalized for multivalent counterions[10,11]. According to this general treatment, the equilibrium binding constant, K_b, is given by

$$K_b = \frac{[P_b]\,[D_0]^d\,[C]^c}{[P_0]\,[D_b]^d}\,\bar{\gamma} \tag{1}$$

where $\bar{\gamma} = (\gamma_{P_b}\gamma^d_{D_0}\gamma^c_C)/(\gamma_{P_0}\gamma^d_{D_b})$ and the symbol γ represents activity coefficients; $[P_b]$ is the protein concentration on the stationary phase; $[P_0]$ is the mobile-phase concentration of the protein associated with the number of co-ions (c) displaced by protein adsorption on the ion-exchange stationary phase surface; $[D_b]$ and $[D_0]$ are the counterion concentrations on the stationary phase and in the mobile phase, respectively; [C] is the concentration of the co-ion on the protein, and is negligible compared to the mobile phase concentration of the co-ion; d and c are the stoichiometic coefficients of the counterion and co-ion, respectively.

If Λ represents the total number of active sites (charges), the following mass balance exists

$$[\Lambda] = Z_d\,[D^0_b] = Z_d\,[D_b] + K_a Z_p\,[P_b] \tag{2}$$

where K_a is a solute accessibility coefficient characteristic of both the porous stationary phase and the protein, Z_p is the number of protein charges that interact with stationary phase active sites, Z_d is the valency of the salt counterion, and $[D_b^0]$ is the saturated concentration of counterion on the stationary phase when protein molecules are not present. Eqn. 2 may be rearranged to

$$[D_b] = [D_b^0]\left(1 - \frac{K_a Z_p [P_b]}{Z_d [D_b^0]}\right) \tag{3}$$

Substituting eqn. 3 into eqn. 1 and solving for $[P_b]$ yields

$$[P_b] = \frac{K_b[D_b^0]^d [P_o]\left(1 - \dfrac{K_a Z_p[P_b]}{Z_d[D_b^0]}\right)^d}{\bar{\gamma}\,[C]^c}[D_o]^{-d} \tag{4}$$

Eqn. 4 is equivalent to eqn. 5, derived for monovalent counterions in ref. 4.

Eqn. 4 has two possible chromatographic solutions, resulting in two different methods for measurement of Z. Combining eqn. 4 and the following equations, $[P_b] = K_d[P_o]$, $K_d = k'/\varphi$, $Z_d = 1$ ($d = Z_p$) and taking the logarithm, yields

$$\log k' = \log I - Z_p \log [D_o] \tag{5}$$

where $I = (\varphi K_b[D_b^0]^{Z_p})/(\bar{\gamma}[C]^c)$ = constant, φ is the phase ratio, and K_d is the distribution coefficient. Eqn. 5 is the retention equation of the SDM[9] which is valid at "infinitely low" protein concentrations (linear part of the isotherm) and in a narrow range of salt concentration. In this approach, small amounts of protein are isocratically eluted from columns at various salt concentrations to obtain k' values to solve eqn. 5 for Z. The other solution to eqn. 4 is

$$[P_b] = Q[D_o]^{-Z_p} \tag{6}$$

where

$$Q = \frac{K_b[P_o]\,[D_b^0]^d\left(1 - \dfrac{K_a Z_p[P_b]}{Z_d[D_b^0]}\right)^d}{\bar{\gamma}[C]^c} = \text{constant}$$

that is, $[P_o]$ and $[D_b^0]$ are constants. An assumption is made that activity coefficients are constant if $[P_o]$ is constant and the change in mobile phase salt concentration is very small. The charge density of a modern high-performance liquid chromatographic (HPLC) stationary phase is much higher than that of proteins, hence

$$\frac{K_a Z_p[P_b]}{Z_d[D_b^0]} \ll 1.0$$

Therefore, this factor is negligible. Other work[12] confirms that only a small error is introduced into calculation of Z when this assumption is used.

Eqn. 6 shows the dependence of the stationary phase protein concentration on the salt concentration. The stationary phase concentration of proteins $[P_b]$ may be expressed by

$$[P_b] = \frac{m_b}{K_a A_s} \tag{7}$$

where m_b is the mass of protein bound, A_s is the stationary phase surface area, and $K_a A_s$ is constant.

By substituting eqn. 7 into eqn. 6, one can eliminate the need to measure the accessible stationary phase surface area

$$\log m_b = \log K^* - Z_p \log [D_0] \tag{8}$$

where

$$K^* = \frac{K_b K_a A_s [D_b^0]^d \, [P_0]}{\bar{\gamma}[C]^c} = \text{constant}$$

The second solution of eqn. 4 requires a series of frontal chromatograms to be obtained in a very narrow range of salt concentration and at constant mobile phase protein concentration. Therefore, eqn. 6 may be valid over the entire range of the protein adsorption isotherm, and in this way it is considered to be an "adsorption equation of SDM". Eqn. 8 was used in our experiments.

While saturating the column during the first step of the experiment, the high-energy binding sites of the stationary phase were irreversibly saturated with IgG. These high-energy binding sites represent about 5% of the total column capacity[12], and they have a very important role in analytical (linear) chromatography. However, in the case of preparative chromatography, they are much less important, and contribute to potential irreversible binding. A column with saturated irreversible binding sites has a ligand population with a quasi-homogenous energy distribution. Thus, we obtained a "new" column with properties different from the original, but which was easy to regenerate and allowed the collection of reproducible data. The same results of two consecutive parallel measurements at each set of conditions demonstrated satisfactory regeneration.

Protein adsorption isotherms were measured by frontal chromatography at five different sodium chloride concentrations within the total salt concentration range of 8 mM (Fig. 1). The reciprocal Langmuir isotherm equation was found to fit the data well. The Langmuirian behavior of the measured isotherms was checked by fitting to the exponential equation:

$$m_b = \frac{a[P_0]^n}{1 + b[P_0]^n} \tag{9}$$

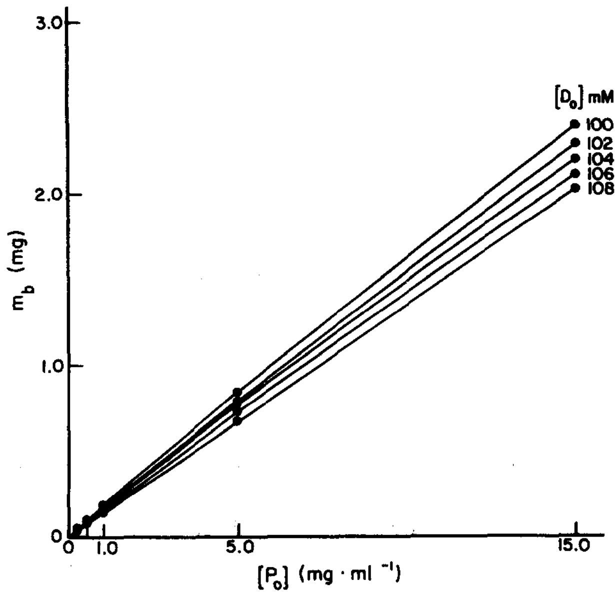

Fig. 1. Mass of bound IgG as a function of mobile phase IgG concentration. Conditions: 30 × 4.6 mm I.D. non-porous strong-anion-exchange column; mobile phase, 0.100–0.108 *M* sodium chloride in 20 m*M* 1,3-diaminopropane (pH 10); flow-rate, 0.2 ml/min; temperature 23°C.

as well. The value of exponent n was between 0.99 and 1.01 in each case. (The exponential isotherm equation shows a sigmoidal shape, and this behavior can frequently be found with regard to protein adsorption[3].) Preparative Z plots (log m_b *versus* log $[D_0]$) were constructed, using data of the horizontal sections of isotherms (at constant mobile phase protein concentrations, according to eqn. 8) (Fig. 2). Z plots show straight lines, proving that the activity coefficients of the components are truly

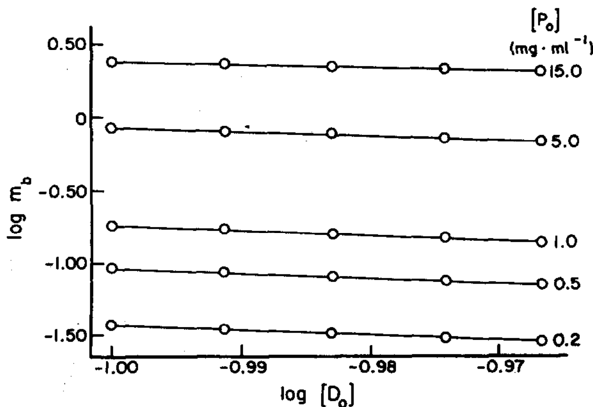

Fig. 2. "Preparative Z number plot" (log m_b *vs.* log$[D_0]$, eqn. 8) of IgG at different mobile phase protein concentrations. Conditions are the same as in Fig. 1.

constant. Dependence of logarithmic Langmuir parameters on the logarithmic salt concentration in the mobile phase was found to be linear

$$\log a = -4.37 - 3.64 \log [D_0] \tag{10}$$

and

$$\log b = -2.48 - 0.928 \log [D_0] \tag{11}$$

Huang and Horváth found similar relationships[13]. Combining the Langmuir equation and eqn. 6 one obtains eqn. 10, where

$$- \left.\frac{d \log a}{d \log [D_0]}\right|_{[P_0],b} = Z_{p,\text{lin}} \tag{12}$$

when $[P_0]$ is infinitely low, and $Z_{p,\text{lin}}$ is the Z number, measured in the linear range of the protein adsorption isotherm.

The IgG isotherms measured are characterized by the Langmuir equation. Jovanovic's equation[14] resulted in a significantly poorer fit (data not shown). Isotherms having the Langmuir form are often interpreted in the same manner as the gas adsorption analogues. It is recognized, however, that the premises on which the Langmuir isotherm is based often are not applicable to protein adsorption systems, and it is only coincidental that protein adsorption isotherms are sometimes described by the Langmuir model. The IgG isotherms were characterized by Langmuir form because of its excellent fit and for convenience.

Plots of Z *versus* protein mobile phase concentration show a sigmoid curve (Fig. 3a). The same picture is seen if Z is plotted *versus* mass of protein bound. There is a slight difference, in that more than one curve is obtained (in our case five). This "uncertainty" is an inherent attribute of the system. If the range of salt concentration goes to zero (Δ $[D_0] \rightarrow 0$), the five curves of the Z *versus* m_b plot form a single curve somewhere in the middle of the range. For the sake of convenience, we will use data measured at the central salt concentration, 0.104 m*M* (Fig. 3b). Fig. 3b shows that Z decreases with the stationary phase concentration of the protein and approaches a limiting value, *i.e.*, Z has a maximum value at infinitely low concentration and a minimum value at the saturation range of the protein adsorption isotherm. If the change in Z between two limiting values is a consequence of alteration in adsorption properties of IgG, this phenomenon can be treated as a change in orientation of adsorbed IgG, as suggested by Morrissey[5,6]. Thus, values of Z, measured as an actual population-weighted average of these two extremes, can be expressed by

$$Z_p = y_1 Z_1 + y_2 Z_2 \tag{13}$$

where y_1 and y_2 are the mole fractions of the two orientations, associated with the two limiting Z values. These mole fractions are functions of the stationary phase protein concentration; y_2 increases and y_1 decreases with protein concentration according to

$$\frac{[P_2]}{[P_1]} = k[P_b]^n \tag{14}$$

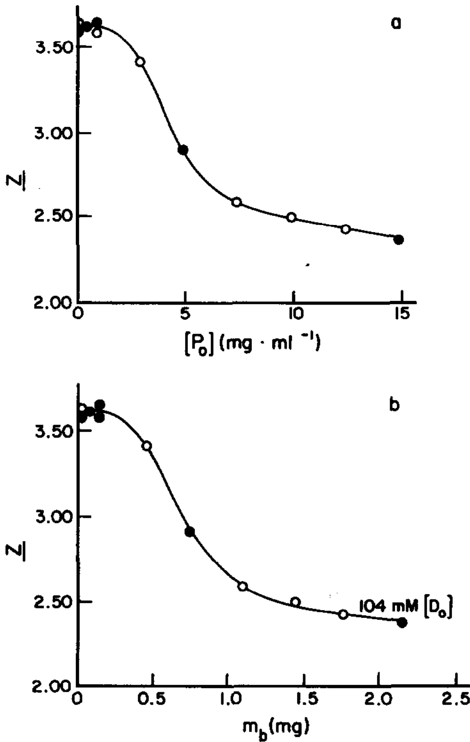

Fig. 3. (a) Z as a function of mobile phase protein concentration. Conditions are the same as in Fig. 1. (b) Z as a function of mass of IgG bound at 0.104 M sodium chloride mobile phase concentration. Conditions are otherwise the same as in Fig. 1. Solid circles show Z values measured, open circles represent Z values calculated from adsorption isotherm equations (Fig. 1). Non-linear least-squares fitting is shown by solid line.

where $[P_1]$ and $[P_2]$ are the concentrations of the first and second orientation, k and n are constants, and

$$[P_b] = [P_1] + [P_2] \tag{15}$$

and

$$y_2 = \frac{[P_2]}{[P_b]} \tag{16}$$

Combining eqns. 14, 15 and 16, one obtains y_2 as a function of stationary phase protein concentration

$$y_2 = \frac{k[P_b]^n}{1 + k[P_b]^n} \tag{17}$$

Combining eqns. 7, 13, and 17, and expressing Z_p, yields

$$Z_p = Z_1 - \frac{(Z_1 - Z_2)Km_b^n}{1 + Km_b^n} \quad (18)$$

where

$$K = \frac{k}{(K_a A_s)^n}$$

Eqn. 18 shows the relationship between Z_p and mass of protein bound, and thus, it should fit the measured data. Non-linear least squares regression analysis was used to fit the data[15] (Fig. 3b, solid line). Fitting was done on a series of Z numbers, calculated from protein adsorption isotherm equations as well. The parameters are as follows: $Z_1 = 3.61$, $Z_2 = 2.36$, $K = 3.28$, $n = 3.49$, residual sums of squares = 0.00469 and the approximate standard error from squared residuals = 0.0306.

Eqn. 17 is formally equivalent to the multiple equilibria model with n highly cooperative identical binding sites, where k is the equilibrium constant and n is the Hill coefficient, the number of interacting binding sites[16]. Since the n value is greater than 1.0 ($n = 3.49$), the equilibrium between the two orientations has a positive cooperativity. In other words, when the surface protein concentration is increased, a change in the orientation of bound protein molecules allows more protein molecules to gain access to the surface area. If this analogy is appropriate, it means that three or four protein molecules can interact with each other and result in an equilibrium between the two orientations of adsorbed IgG at a given stationary phase protein concentration.

Another possible cause of alteration in Z is the multilayer adsorption of IgG. In this case, Z_f would relate to the adsorption of the first layer and the lower limiting value of Z_p, now labelled Z_m, to the adsorption of all the succeeding layers of IgG. Z_p would be calculated according to eqn. 13 also (see details in Appendix). Non-linear least-squares regression analysis was used[17] to fit eqn. A16 to a series of values calculated from protein adsorption isotherm equations. This model does not fit. The calculated parameters are as follows: $Z_f = 3.53$, $Z_m = -11.3$, $[P_0^0] = 156$, $n = 103$, and residual sums of squares 0.494. This suggests that the change of Z as a function of mobile phase protein concentration is not a consequence of multilayer adsorption of IgG.

The fact that molecular orientation of IgG at the surface of an ion-exchange sorbent is a function of surface concentration may have broader implications. The first is that molecular orientation of other asymmetrically shaped molecules may also change with protein concentration with concomitant changes in affinity. This leads to the possibility that selectivity will be a function of feed in a mixture of symmetrical and asymmetric molecules. When a column is operated in the "non-linear mode", it should be optimized under the same conditions. Another implication is the possibility that crowding by other molecular species may also change the IgG orientation at surfaces. This would mean that the chromatographic behavior of IgG could be dependent on the concentration, and this should be reflected in the shape of the competitive adsorption isotherms of feed components. Finally, the influence of surface fouling must be

considered. As non-eluted proteins build up on the stationary phase surface, a permanent crowding factor is built into the system. Consequently, changes in molecular orientation and resultant chromatographic behavior would occur at lower concentrations of proteins.

CONCLUSIONS

The change of molecular orientation of IgG, bound to an ion-exchange surface, was studied, using an extension of the stoichiometric displacement model over the entire range of protein adsorption isotherms. Z was found to decrease with an increase in stationary phase protein concentration. These results seem to verify Morrissey's finding[5,6].

The method of Z measurement over the entire range of the protein isotherm may allow studies of protein adsorption states at different feed sizes and concentrations, including protein–protein interaction on the stationary phase, protein denaturation on the surface, associations between protein molecules in the mobile phase, multilayer formation, etc.

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APPENDIX

A treatment of multilayer adsorption of proteins at a surface based on an analogy to the BET model[18] will be presented below. This treatment will be restricted to the number of protein layers formed (n) instead of an infinite number of layers.

In the BET model, adsorption and desorption can occur only at exposed surfaces. Adsorption of the first layer is characterized by Z_f, the Z number and Q_f, the adsorption energy, and for all succeeding layers, Z_m and Q_m, respectively. The condition for equilibrium is taken to be that the amount of each type of layered surface reaches a steady state value relative to the next deeper layer. S_0 is the uncovered surface fraction, S_1 is the surface fraction covered by monolayer, S_2 is covered by bilayer, and S_n is covered by n layers. For S_0 the equilibrium

$$k_{\mathrm{fON}}\,[\mathrm{P_0}]\,S_0 = k_{\mathrm{fOFF}}\,S_1\,\mathrm{e}^{-Q_f/RT} \tag{A1}$$

exists, where k_{fON} and k_{fOFF} are the rate constants. For all succeeding surfaces:

$$k_{\mathrm{mON}}[\mathrm{P_0}]\,S_{i-1} = k_{\mathrm{mOFF}}S_i\,\mathrm{e}^{-Q_m/RT} \tag{A2}$$

It then follows that

$$S_1 = yS_0, \tag{A3}$$

$$S_2 = xS_1, \text{ and} \tag{A4}$$

$$S_i = x^{i-1}S_1 = yx^{i-1}S_0 = qx^iS_0, \tag{A5}$$

where

$$x = \frac{k_{\text{fON}}}{k_{\text{fOFF}}} [\text{P}_0]\, \text{e}^{Q_f/RT} = \frac{[\text{P}_0]}{[\text{P}_0^0]} \tag{A7}$$

$[\text{P}_0^0]$ is the protein solubility, and

$$q = \frac{y}{x} = \frac{k_{\text{fON}}\, k_{\text{mOFF}}}{k_{\text{fOFF}}\, k_{\text{mON}}}\, \text{e}^{(Q_f - Q_m)/RT} \tag{A8}$$

Then for n layers:

$$\frac{m_{\text{tot}}}{m_1^0} = \frac{\sum_{i=1}^{n} iS_i}{\sum_{i=0}^{n} S_i} = \frac{qS_0 \sum_{i=1}^{n} ix^i}{S_0 + qS_0 \sum_{i=1}^{n} x^i} \tag{A9}$$

where m_{tot} and m_1^0 are the total mass of protein adsorbed and the mass of protein adsorbed at the monolayer point, respectively. Insertion of the algebraic equivalents into the sums yields

$$m_{\text{tot}} = \frac{m_1^0 \dfrac{qx}{1 - x} [1 - (n + 1)\, x^n + nx^{n+1}]}{1 + (q - 1)\, x - qx^{n+1}} \tag{A10}$$

Eqn. A10 is considered to be the BET equation for n adsorbed layers.

The apparent Z value measured, Z_p, at given mobile phase solute concentration, $[\text{P}_0]$, is the mass-weight average of Z_f and Z_m

$$Z_p = \frac{Z_f m_f + Z_m m_m}{m_{\text{tot}}} \tag{A11}$$

where m_f and m_m are the mass of protein adsorbed in the first and all succeeding layers, respectively. If the ratio of masses in the layers is equal to the ratio of surfaces covered by these layers, m_f can be expressed as

$$\frac{m_f}{m_1^0} = \frac{\sum_{i=1}^{n} S_i}{\sum_{i=0}^{n} S_i} = \frac{A_0 - S_0}{A_0} \tag{A12}$$

where A_0 is the total surface area $\left(A_0 = \sum_{i=0}^{n} S_i\right)$.

Combining eqns. A5, A8, and A12, and inserting the algebraic equivalents into the sums yields the expression

$$m_f = \frac{qx(1 - x^n)m_1^0}{1 + (q - 1)\,x - qx^{n+1}}\,. \tag{A13}$$

The mass of adsorbed protein in the second and all succeeding layes, m_m

$$m_m = m_{tot} - m_f, \tag{A14}$$

i.e.,

$$m_m = \frac{m_1^0 qx}{1 + (q - 1)\,x - qx^{n+1}} \left\{\frac{x - x^n\,[n(1 - x) + x]}{1 - x}\right\} \tag{A15}$$

Combining eqns. A11, A13 and A15

$$Z_p = \frac{Z_f(1 - x^n)\,(1 - x) + Z_m x[1 - nx^{n-1} + x^n(n - 1)]}{1 - x^n - nx^n} \tag{A16}$$

where $x = [P_0]/[P_0^0]$ (see eqn. A7).

Eqn. A16 expresses the relationship between Z_p and $[P_0]$.

LIST OF SYMBOLS

A_0	stationary phase area in eqn. A12
A_s	stationary-phase surface area
a	first Langmuir isotherm parameter
b	second Langmuir isotherm parameter
c	stoichiometric coefficient of co-ion in eqn. 1
[C]	concentration of the co-ion related to the protein
d	stoichiometric coefficient of counterion in eqn. 1
$[D_0]$	mobile phase counterion concentation
$[D_b]$	stationary phase counterion concentration
$[D_b^0]$	saturated-stationary-phase counterion concentration

i	running index at multilayer adsorption in eqn. A5
I	constant in eqn. 5
K	constant in eqn. 18. [$K = k/(K_a A_s)^n$]
K^*	constant in eqn. 8
K_a	accessibility coefficient[19]
K_b	equilibrium constant in eqn. 1
K_d	distribution coefficient
k	constant in eqn. 14
k'	retention factor
k_{fON}	adsorption rate constant in eqn. A1
k_{fOFF}	desorption rate constant in eqn. A1
k_{mON}	adsorption rate constant in eqn. A2
k_{mOFF}	desorption rate constant in eqn. A2
m_1^0	mass of protein adsorbed at the monolayer point in eqn. A9
m_f	mass of protein adsorbed in the first layer at multilayer adsorption in eqn. A11
m_m	mass of protein adsorbed in the second and all succeeding layers at multilayer adsorption in eqn. A11
m_{tot}	total mass of protein adsorbed in eqn. A9
n	number of layers at multilayer adsorption in eqn. A9
n	constant in eqn. 14
$[P_1]$	concentration of protein bound with first orientation
$[P_2]$	concentration of protein bound with second orientation
$[P_b]$	stationary phase protein concentration
$[P_0]$	mobile phase protein concentration
$[P_0^0]$	protein solubility in the mobile phase in eqn. A7
Q_f	adsorption energy for the first layer in eqn. A1
Q_m	adsorption energy for the second and succeeding layers in eqn. A2
q	parameter in eqn. A8
R	universal gas constant
S_0	uncovered fraction of the stationary phase surface in eqn. A1
S_i	fraction of surface covered by i protein layers in eqn. A2
T	absolute temperature
x	parameter in eqn. A7
y	parameter in eqn. A6
y_1	mole fraction of the first orientation
y_2	mole fraction of the second orientation
Z_1	Z number associated with the first orientation
Z_2	Z number associated with the second orientation
Z_d	valency of the counterion
$Z_{p,lin}$	Z_p measured at "infinite low" protein concentration
Z_p	number of monovalent counterions displaced from the stationary phase when one molecule of protein is bound
Z_f	Z number associated with the first adsorbed layer in eqn. A11
Z_m	Z number associated with the second and all succeeding adsorbed layers in eqn. A11
$\bar{\gamma}$	overall activity coefficient in eqn. 1

γ_i activity coefficient of the *i*-th component in eqn. 1
Λ total number of active sites (charges on the stationary phase)
φ phase ratio

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