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Thermodynamics of ion binding by proteins

Phenomenological linkage relations for binding of electrolyte and interpretation by double layer theory

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In this paper we report a new set of thermodynamic linkage relations for the binding of electrolyte by proteins. The relations are derived for protein solutions in membrane equilibrium with a reference solution, allowing a phenomenological definition of ion binding. This is an extension of Wyman's linkage theory. The theory is applied to the electrolyte dependence of proton titration curves for bovine serum albumin in KCl solution (C. Tanford, S.A. Swanson and W.S. Shore, J. Am. Chem. Soc. 77 (1955) 6414). The curves are re-analysed in terms of Esin-Markov coefficients. In addition, we discuss the interpretation of the phenomenological K^+ and Cl^- binding numbers in terms of a two-state binding model, in which part of the ions are thought to adsorb on specific sites at the protein surface and/or part in the diffuse layer. It is shown that the electrolyte binds largely in the diffuse layer, especially when the protein surface charge is high.

1. Introduction

In the present paper we discuss the capability of proteins to bind ions. More specifically, we are interested in the interfacial components of charge at the bovine serum albumin (BSA)-solution interface.

Several investigators have already made significant contributions to our knowledge of the ion binding by proteins. The works of Linderstrøm-Lang, Kirkwood, Tanford, Scatchard and Wyman are classical and well documented in the literature, including the textbook [1] and reviews [3–9]. Especially the Wyman theory is noteworthy because it integrates much of the other approaches. In essence, Wyman formulates the grand partition function, which he calls the binding polynomial, of the protein-solution interface in terms of distinct binding sites. Schellmann [5] clarified the statistical thermodynamic background of the binding polynomial. A special feature, introduced by Wyman, is the concept of linkage. In general, linkage means that if two types of ligands (bound ions or uncharged molecules) interact with each other, the binding constant of the first type becomes dependent on the bound amount of the second type and vice versa. These interdependences are summarised by the Maxwell relations from the differential of the binding polynomial. Wyman calls these Maxwell relations linkage relations.

An implicit but important assumption in Wyman's approach is that the ligands adsorb only on specific sites. For some ligands, e.g., uncharged

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molecules and protons in swamping electrolyte, this assumption is perfectly legitimate. However, in the (usual) case of charged proteins the ions may also bind in the diffuse layer. Recently, Stigter and Dill [23] have analysed the binding polynomial for the free energy of proton binding by inclusion of the electrostatic interactions through a Debye-Hückel interaction term.

Wyman's method resembles the site binding models which have recently become popular in proton adsorption studies of metal oxide colloids [10–12], especially with respect to the notion of distinct binding sites. The metal oxide studies have also shown the importance of the binding of electrolyte. At least two rules have emerged. The first is not very surprising, namely, that an increase of proton charge usually results in an increase in adsorption of counterions and a decrease in the adsorption of co-ions. The second rule is less obvious, namely, that a large body of data can be explained by a simple two-state model in which ions bind in two different loci, one locus being the surface itself, which may or may not contain distinct binding sites, the second locus being the diffuse part of the double layer. Depending on the system, binding in one locus is preferred over binding in the other. It may therefore very well be possible that certain experimental conditions favour site binding, while other conditions favour diffuse binding. A classical example of such a two-state approach is the Gouy-Chapman-Stern theory [22] in which the total electrical double layer is regarded as consisting of a diffuse layer and a surface-bound part (the Stern layer) in series.

In this article, we demonstrate the importance of binding of electrolyte in the diffuse ion atmosphere of protein by an analysis of the electrolyte dependency of proton adsorption by BSA in KCl solution. The experimental data — proton titration curves — are from an old paper from Tanford [15]. The titration curves were originally analysed by Tanford according to the Linderstrøm-Lang theory. Our analysis is in terms of a generalised version of Wyman's linkage relation for electrolyte co-adsorption. The important new point is that we derive the linkage relation from classical thermodynamics by purely phenomenological arguments. To this end, we introduce the concept of an external reference solution with which the protein solution is thought to be in membrane equilibrium. This phenomenological approach is completely general; we do not need to introduce concepts like site binding, binding constants, electrostatic interaction factors, etc.

The generalised linkage relation, when expressed in terms of a so-called Esin-Markov coefficient, is akin to the relation Lyklema introduced for the analysis of the ionic components of charge in a metal-oxide/electrolyte solution interface [13,14]. Lyklema derived the relation from the Gibbs adsorption equation, whereas here we prefer a derivation based on the Gibbs-Duhem relation for the protein solution. By doing so we automatically take into account any effects which might possibly stem from structural alterations of the protein due to ion binding.

The K^+ and Cl^- binding by BSA, as calculated by using the generalised linkage relations, is explained in terms of the two-state model. To this end, we identify the phenomenological ion binding numbers as sums of ensemble averages of diffuse and site binding numbers. This analysis is in the spirit of Stigter and Dill's [23] extension of Wyman's statistical analysis. In the model elaboration we use the full Poisson-Boltzmann profile around a prolate ellipsoid for the calculation of the diffuse charges.

2. Theory

Wyman pointed out two routes for the derivation of the linkage relations. The first is based on statistical thermodynamics as mentioned above. We shall return to it in section 5. This method is well known and in extensive use; in the words of Wyman [3] it "provides an insight into the molecular basis of a phenomenon that comes from a statistical analysis". The second method is based on classical chemical thermodynamics with "the authority of a completely general thermodynamic derivation". The starting point for the classical thermodynamic route is the Gibbs-Duhem relation for the protein solution, in which all the

extensive variables are written per mol protein (eq. 4a from ref. 4) in slightly different notation):

$$N_p\, d\mu_p = -S\, dT - \sum_{i \neq p} N_i\, d\mu_i + V\, dP \qquad (1)$$

where μ_i is the chemical potential of component i, S the entropy, N_p the total amount of protein, T the temperature, N_i the total amount of component i, V volume and P pressure. From the above Gibbs-Duhem relation, it is a straightforward matter to find the Maxwell (linkage) relations between the ratios N_i/N_p (= total amount of i per total amount of protein) and the chemical potentials. A typical example of such a relation is:

$$\left(\frac{\partial N_i/N_p}{\partial N_j/N_p}\right)_{\mu_{k\neq j},P,T} = -\left(\frac{\partial \mu_j}{\partial \mu_i}\right)_{N_j/N_p,\mu_{k\neq i},P,T} \qquad (2)$$

Wyman [3,4] remarked already that the practical use of phenomenological relations such as eq. 2 is limited because no distinction is made between that fraction of i which is bound to the protein, and the rest which is not bound by the protein. However, phenomenological distinction between bound and unbound fractions is possible, provided that the protein is not regarded as an autonomous component, but in equilibrium with the dialysate in which it is embedded. In this way, a second Gibbs-Duhem relation can be formulated, and excess defined thermodynamically (see fig. 1). Consider an external reference solution (R) with which the protein solution (L) is in membrane equilibrium. The advantage of our choice of reference is that it is also applicable to concentrated protein solutions. We choose the membrane such that it is an ideal separator between the protein and the other species: the membrane is perfectly impermeable to the protein but fully permeable to all the other species. In this picture, all the differences in concentrations of the permeable species can be ascribed to some kind of interaction with the protein. It is therefore logical to define the bound amount of any component i as the excess with respect to water of the number of moles of i in the protein solution (L). In this definition of binding the actual mechanism of binding does not have to be specified; the definition is purely phenomenological, meaning that the excesses are measurable quantities. The relations between the bound amounts and the chemical potentials of the permeable components can easily be found from the Gibbs-Duhem relations of the protein and reference solution. For the protein solution we use eq. 1 again:

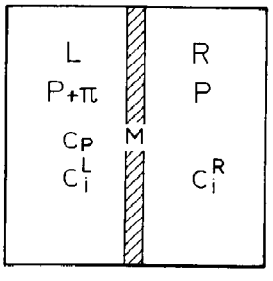

Fig. 1. A protein solution L in equilibrium with its dialysate R. P, pressure; π, osmotic pressure; c_i, c_w and c_p denote the concentrations of component i, water and protein, respectively.

$$N_p\, d\mu_p = -S^L\, dT + \sum_{i \neq p} N_i^L\, d\mu_i^L + V^L\, dP^L \qquad (3)$$

where the superscript L denotes the protein solution (L vessel). In this phenomenological approach the chemical potentials refer to electroneutral combinations of ions: they are mean chemical potentials rather than electrochemical potentials. In this picture, electroneutrality conditions and concepts like membrane potentials are superfluous, which facilitates the analysis considerably. For the reference vessel we have the supplementary relation:

$$N_w^R\, d\mu_w^R = -S^R\, dT - \sum_{i \neq w} N_i^R\, d\mu_i^R + V^R\, dP^R \qquad (4)$$

where we have expressed the chemical potential of water in the chemical potentials of the other permeable components. The superscript R corresponds to the reference vessel. In equilibrium the chemical potentials of all permeable components are the same everywhere, which gives $d\mu_i^L = d\mu_i^R = d\mu_i$, $i \neq p$. Combination of this condition with the two Gibbs-Duhem relations yields:

$$N_p\, d\mu_p = -\Delta S^{(w)}\, dT + V^L\, dP^L - \left(N_w^L/N_w^R\right) V^R\, dP^R - \sum_{i \neq w,p} \Delta N_i^{(w)}\, d\mu_i \qquad (5)$$

where the excess extensive variables $\Delta X^{(w)}$ ($X = S$, N_i and V below) are defined with respect to water:

$$\Delta X^{(w)} \equiv X^{L} - X^{R} \cdot N_w^{L}/N_w^{R} \tag{6}$$

It implies that $\Delta N_w^{(w)} = 0$ because all excesses are referred to that of water.

Two routes are open to analyse the two pressure differentials in eq. 5. One route would be to consider the pressure of the protein solution as the independent external pressure (external to both the protein solution and the reference solution), the other possibility would be to use the pressure of the reference solution for this purpose. In both cases the osmotic pressure π, defined as $\pi = P^{L} - P^{R}$, can be used as the second independent variable. The two corresponding formulas for the protein chemical potential are:

$$(P \equiv P^{L},\ \pi = P - P^{R})$$

$$N_p\, d\mu_p = -\Delta S^{(w)}\, dT + \Delta V^{(w)}\, dP - \sum_{i \neq w,p} \Delta N_i^{(w)}\, d\mu_i + (V^{L} - \Delta V^{(w)})\, d\pi \tag{7a}$$

$$(P \equiv P^{R},\ \pi' \equiv P^{L} - P)$$

$$N_p\, d\mu_p = -\Delta S^{(w)}\, dT + \Delta V^{(w)}\, dP - \sum_{i \neq w,p} \Delta N_i^{(w)}\, d\mu_i + V^{L}\, d\pi' \tag{7b}$$

It is a matter of taste rather than principle as to which equation is used for further analysis. One difference between the final equations is the coefficient of the osmotic pressure differential; for $P \equiv P^{R}$ this is the volume of the protein solution, while for $P \equiv P^{L}$ this is the volume of the protein solution minus the excess volume. In many practical cases $\Delta V^{(w)} \ll V^{L}$. We choose here eq. 7a, so that the protein solution has the external pressure P, and approximate $V^{L} - \Delta V^{(w)}$ by V^{L}.

For protein solutions Wyman defines so-called linkage or binding potentials Λ, which are obtained by Legendre transformations of the protein chemical potential as found by the Gibbs-Duhem relation (eq. 1). For example, one particular set of linkage potentials for a system containing two components is given by (from eqs 4 ref. 4):

$$\Lambda^{I} \equiv -\mu_p$$

$$\Lambda^{II} \equiv -\mu_p - (N_2/N_p)\mu_2$$

$$\Lambda^{III} \equiv -\mu_p - (N_1/N_p)\mu_1$$

$$\Lambda^{IV} \equiv -\mu_p - (N_1/N_p)\mu_1 - (N_2/N_p)\mu_2$$

Typically, in this picture, no reference solution is considered. However, similar linkage potentials can be defined for protein solutions in equilibrium with such references, namely, by simply replacing the totals N_i by the excess quantities $\Delta N_i^{(w)}$. Linkage potentials redefined in this way would have the osmotic pressure as an independent variable, so that the linkage relations are obtained for constant osmotic pressure. Linkage relations which apply for constant protein concentration, which is more practical, can easily be found after introduction of an additional type of linkage potential, which we shall call the reference-linkage or reference-binding potential Λ^{r}. It is defined as:

$$\Lambda^{r} = -\mu_p + \pi/c_p \tag{8}$$

In the limiting case where the protein concentration is vanishingly low, so that Van 't Hoff's law applies ($\pi = RTc_p$), the difference between Λ^{r} and Λ^{I} ($= -\mu_p$) is RT. Under these circumstances the bulk of the protein solution is a proper reference. The total differential of Λ^{r} reads

$$d\Lambda^{r} = s^{(w)}\, dT - v^{(w)}\, dP + \sum_{i \neq w,p} r_i^{(w)}\, d\mu_i - (\pi/c_p)\, d \ln c_p \tag{9}$$

where we have used the notation:

$$s^{(w)} \equiv \Delta S^{(w)}/N_p \tag{10a}$$

$$v^{(w)} \equiv \Delta V^{(w)}/N_p \tag{10b}$$

$$r_i^{(w)} \equiv \Delta N_i^{(w)}/N_p \tag{10c}$$

The number $r_i^{(w)}$ will be referred to as the binding number; it is the excess number of moles of i per mole of protein and is experimentally accessible.

One particular useful linkage relation that can be derived from eq. 9 and that we shall use in the

following is obtained from the differential of $\Lambda^r - r_j^{(w)}\mu_j$. It is:

$$\left(\frac{\partial r_i^{(w)}}{\partial r_j^{(w)}}\right)_{\mu_{k\neq j},P,T,c_p} = -\left(\frac{\partial \mu_j}{\partial \mu_i}\right)_{r_j^{(w)},\mu_{k\neq i},P,T,c_p} \quad (11)$$

This is the required phenomenological linkage relation for protein solutions in membrane equilibrium with their dialysates. In comparison with Wyman's linkage relation (eq. 2) we note two important differences. Firstly, in eq. 11, the protein concentration is kept constant, while its differential is undetermined in eq. 2. Secondly, the left-hand side of eq. 11 contains partial differential coefficients of the binding numbers, while the left-hand side of eq. 2 contains partial differential coefficients of total amounts. In terms of information theory one might say that the information content of the partial differential coefficient of the chemical potentials increases by introduction of the reference, the extra information being the ability to calculate (excess) bound quantities rather than total amounts. The 'cost' of this additional information is the extra requirement that the protein concentration must be held constant.

3. Esin-Markov coefficient for Tanford's system

As stated in section 2, in phenomenological approaches all substances i are electroneutral components that are independent in the sense of the phase rule. The way in which they are related to individual ionic components of charge depends on the composition of the system. An elaboration will now be given for BSA in KCl, which is the system studied potentiometrically by Tanford [8,15] (two curves are reproduced in fig. 2). The approach we follow here is classical [13,14].

At constant pressure, temperature and BSA concentration, the differential of the reference-linkage potential for this system reads:

$$(\mathrm{d}\Lambda^r)_{P,T,c_{BSA}} = r_{HCl}^{(w)}\,\mathrm{d}\mu_{HCl} + r_{KOH}^{(w)}\,\mathrm{d}\mu_{KOH} + r_{KCl}^{(w)}\,\mathrm{d}\mu_{KCl} \quad (12)$$

In order to facilitate the notation we shall omit the superscript (w) in the remainder. As the BSA concentration is fairly low (0.6–4%) the ratio c_w^L/c_w^R is close to unity anyhow.

The acid-base dissociation equilibrium HCl + KOH $\leftrightarrows$ H_2O + KCl relates the mean chemical potentials of the electroneutral components HCl, KOH and KCl. The equation is

$$\mathrm{d}\mu_{HCl} + \mathrm{d}\mu_{KOH} = \mathrm{d}\mu_w + \mathrm{d}\mu_{KCl} \quad (13)$$

The differential $\mathrm{d}\mu_w$ is, as before, obtained from the Gibbs-Duhem relation in the reference vessel (isobaric and isothermic):

$$N_w^R\,\mathrm{d}\mu_w = -N_{HCl}^R\,\mathrm{d}\mu_{HCl} - N_{KOH}^R\,\mathrm{d}\mu_{KOH} - N_{KCl}^R\,\mathrm{d}\mu_{KCl} \quad (14)$$

So we find

$$\left(1 + c_{HCl}^R/c_w^R\right)\mathrm{d}\mu_{HCl} + \left(1 + c_{KOH}^R/c_w^R\right)\mathrm{d}\mu_{KOH} = \left(1 - c_{KCl}^R/c_w^R\right)\mathrm{d}\mu_{KCl} \quad (15)$$

In Tanford's experiments the electrolyte concentration is much lower than the water concentration, therefore, we can set to a good approximation $\mathrm{d}\mu_{HCl} + \mathrm{d}\mu_{KOH} = \mathrm{d}\mu_{KCl}$. Substitution in eq. 12 results in

$$(\mathrm{d}\Lambda)^r_{P,T,c_{BSA}} = (r_{HCl} - r_{KOH})\,\mathrm{d}\mu_{HCl} + (r_{KOH} + r_{KCl})\,\mathrm{d}\mu_{KCl} \quad (16)$$

Next we introduce the ionic binding numbers as follows

$$r_{H^+} - r_{OH^-} \equiv r_{HCl} - r_{KOH} \quad (17a)$$

$$r_{K^+} \equiv r_{KOH} + r_{KCl} \quad (17b)$$

$$r_{Cl^-} \equiv r_{HCl} + r_{KCl} \quad (17c)$$

The rationale of these definitions is simple: to obtain the binding number of a certain ion, the binding numbers of all electroneutral components containing the species must be added, if necessary multiplied by a valence factor. Our ionic binding numbers are identical to the ionic components of charge encountered in the theory for hydrophobic colloidal systems [13,14]. For notational convenience we write in the following $r_{a/b}$, the acid/base binding number, for the difference $(r_{H^+} - r_{OH^-})$. The acid/base binding number is positive when the acid adsorption is in excess over the base

adsorption; in the reverse case it is negative. It is noted that r_{H^+} and r_{OH^-} cannot be obtained independently because of the water dissociation equilibrium.

Combination of eqs 16 and 17 yields

$$(d\Lambda^r)_{P,T,c_{BSA}} = r_{a/b}\, d\mu_{HCl} + r_{K^+}\, d\mu_{KCl} \quad (18a)$$

Instead of eq. 18a we could have written

$$(d\Lambda^r)_{P,T,c_{BSA}} = -r_{a/b}\, d\mu_{KOH} + r_{Cl^-}\, d\mu_{KCl} \quad (18b)$$

Eqs 18a and 18b are fully equivalent.

Finally, we express the differentials $d\mu_{HCl}$ and $d\mu_{KCl}$ in terms of the experimentally accessible differentials $RT\, d \ln a_{H^+}$ and $RT\, d \ln a_s$, where a_{H^+} is the proton activity and a_s the activity of the salt ($a_s \equiv (a_+ a_-)^{1/2}$). In Tanford's system the salt concentration is much higher than the proton or hydroxyl concentration and we can write

$$d\mu_{HCl}/RT = d \ln a_{H^+} + d \ln a_s \quad (19a)$$

$$d\mu_{KCl}/RT = 2\, d \ln a_s \quad (19b)$$

Combining all, we find for the reference-linkage potential:

$$(d\Lambda^r)_{P,T,c_{BSA}}/RT$$

$$= r_{a/b}\, d \ln a_{H^+} + (r_{a/b} + 2r_{K^+})\, d \ln a_s \quad (20a)$$

$$= r_{a/b}\, d \ln a_{H^+} + (r_{K^+} + r_{Cl^-})\, d \ln a_s \quad (20b)$$

$$= r_{a/b}\, d \ln a_{H^+} + (2r_{Cl^-} - r_{a/b})\, d \ln a_s \quad (20c)$$

It is seen that only the electroneutral sum of the binding numbers of K^+ and Cl^- occurs in the expression, eq. 20b. This is the Donnan exclusion. Using the Maxwell relation (eq. 11), we find the relation between $r_{a/b}$ and r_{K^+} from eq. 20a and the relation between $r_{a/b}$ and r_{Cl^-} from eq. 20c:

$$\left(\frac{\partial r_{K^+}}{\partial r_{a/b}}\right)_{a_s, c_{BSA}} = 1/2(\beta - 1) \quad (21a)$$

$$\left(\frac{\partial r_{Cl^-}}{\partial r_{a/b}}\right)_{a_s, c_{BSA}} = 1/2(\beta + 1) \quad (21b)$$

where β is an example of a so-called Esin-Markov coefficient [13,14]. It is defined as

$$\beta = -\left(\frac{\partial \ln a_{H^+}}{\partial \ln a_s}\right)_{r_{a/b}, c_{BSA}} \quad (22)$$

Eqs 21 and 22 imply two important properties of the Esin-Markov coefficient. First, from eq. 21 we see that β is in essence a measure of the extent of ion exchange. A value of -1 for β means that for every extra bound H^+ one extra K^+ is expelled. Similarly, a value of $+1$ signifies that for every extra bound H^+ one extra Cl^- is taken up. When the charge compensation of bound protons is partly due to expulsion of co-ions and partly due to co-adsorption of counterions the Esin-Markov coefficient is between -1 and $+1$. The second important property of β, deduced from eq. 22, is that it is easily determined from the salt strength dependency of proton titration curves. This will be elaborated upon in section 4.

The integral representations of the differentials, eqs 21a and 21b, are the required binding isotherms of Cl^- and K^+:

$$r_{K^+} = r^{\#}_{K^+} + \int_{r^{\#}_{a/b}}^{r_{a/b}} 1/2(\beta - 1)\, dr_{a/b} \quad (23a)$$

$$r_{Cl^-} = r^{\#}_{Cl^-} + \int_{r^{\#}_{a/b}}^{r_{a/b}} 1/2(\beta + 1)\, dr_{a/b} \quad (23b)$$

Hence, if we can establish a reference point $^{\#}$ for which the potassium and chloride binding can be estimated, we can construct the counter- and co-ion binding isotherms absolutely. Such an estimate will be also be elaborated upon in section 4.

4. Results

In the application of the theory to Tanford's titration data we encounter two difficulties. First, a minor problem is that Tanford plotted the data in terms of the number (h) of hydrogen ions dissociated from the acid end point of the titration curve, rather than acid/base binding numbers. The acid/base binding number is thermodynamically defined as the excess of moles of acid bound over that of moles of base bound per mole of protein (eq. 17a). Alternatively, we can interpret the acid/base binding number as the number of hydrogen ions added to, or dissociated from, isoionic protein. The isoionic point pI is defined as the pH for which the acid binding cancels the base

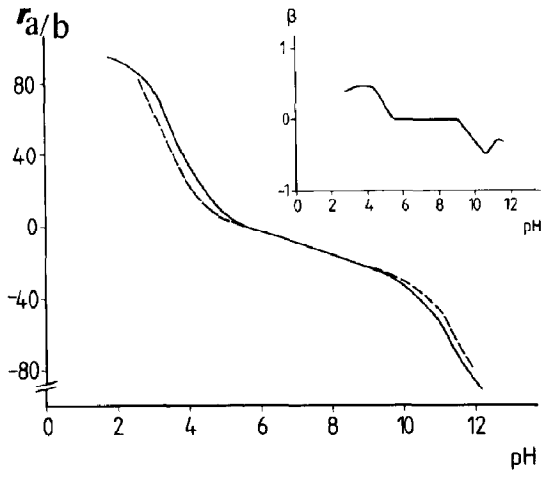

Fig. 2. Acid/base binding characteristics of bovine serum albumin. Data taken from Tanford et al. [15]. Titration curves for c_{KCl} 0.03 M (- - - - - -) and 0.15 M (———). The curves were obtained by Tanford, using an interpolation. (Inset) Esin-Markov coefficients for the mean titration curve in 0.07 M KCl.

binding, hence $r_{a/b}(pI) \equiv 0$. We redrew the original titration curves using Tanford's observation that h and $r_{a/b}$ are related via $r_{a/b} = 96 - h$. The isoionic points of the two curves in fig. 2 are slightly different; the pI is about 5.4 in 0.03 M KCl and about 5.6 in 0.15 M KCl. The original experimental points have been omitted; the drawn curves are copies of Tanford's interpolated curves.

The second, more serious difficulty is that we are not sure whether Tanford used a $-\log$(proton concentration) or $-\log$(proton activity) scale for the pH. We assume the latter.

The calculation of the potassium and chloride binding was carried out follows. First, we defined a mean titration curve on a mean pH scale, given by

$$\mathrm{pH}(r_{a/b};\mathrm{mean}) = \left\{\mathrm{pH}(r_{a/b};c_s = 0.03) + \mathrm{pH}(r_{a/b};c_s = 0.15)\right\}/2$$

The mean curve was calculated by digitizing the Tanford curves in intervals of 10 acid/base binding units. From the resulting mean curve the inverse relation $r_{a/b}$(pH;mean) immediately followed.

Next the mean Esin-Markov coefficient of the two curves was calculated from the finite difference ratio

$$\beta(r_{a/b}) \cong \left(\frac{\Delta \mathrm{pH}}{\Delta \log a_s}\right)_{r_{a/b}}$$

The activity coefficients of the salts were calculated from the tables from Parson's Electrochemical Handbook [16]. The pH values were directly read from the Tanford curves (if Tanford had used a $-\log$(proton concentration) scale, the calculated values would have been in error by a constant shift of a few hundredths of a unit of β). The Esin-Markov coefficient is plotted in fig. 2 (inset) as a function of pH, using the mean titration curve relation $r_{a/b}$ (pH;mean). The data show that β is zero over the wide pH range from about pH 5 to 9; outside this range β attains extreme values of about 0.5 and -0.5 for pH 4 and 11, respectively. These extreme values are consequences of the convergence of the titration curves near the two titration end points.

The integration constants in the cation and the anion binding isotherms eqs 23a and 23b were estimated from a simple model. It is known that the isoelectric point of BSA (about pH 4.7) is somewhat lower than the isoionic point (pI 5.4–5.6); the difference is due to more favourable specific anion-protein interactions than specific cation-protein interactions [8,15,17]. We assume that the site binding by potassium is negligible below the pI, in agreement with the experimental finding that isotonic BSA binds very little sodium [17]. The consequence is that any co-adsorption of potassium below the pI must stem from its adsorption in the diffuse ion atmosphere. As the diffuse charge is zero at the isoelectric point, it follows immediately that the potassium binding number is also zero at this pH. Hence, the isoelectric point is a realistic point of reference in the integral equations [23], with

$$r^{\#}_{K^+} \cong 0$$

$$r^{\#}_{a/b} \cong r^{\#}_{Cl^-} \cong 10$$

The calculated potassium and chloride binding numbers are plotted in fig. 3 as functions of the

acid/base binding number. The points in the curves are not experimental points, but points from digitising the proton titration curves. The curves are almost, but not exactly, symmetrical. Note also the negative values of the potassium and chloride binding numbers which indicate expulsion of co-ions from the diffuse layer. Both to the left and right of the isoionic point the compensation of the proton charge is largely due to the positive binding of counter-ions rather than to expulsion of co-ions. To give some numbers, when an excess of 80 H^+ is bound, about 75% is compensated by the uptake of Cl^- and 25% by the exclusion of K^+. When there is a shortage of 60 H^+, about 67% is compensated by the adsorption of K^+ and 33% by the exclusion of Cl^-.

A comparison of our results with published experimental data on Cl^- and K^+ (or Na^+) binding by albumin is not very feasible because most of the literature on this subject is rather old. In the course of time, techniques and the preparation of materials have improved considerably.

As early as 1953 Carr [18] determined the binding of Cl^- by BSA in an NaCl solution of approx. 0.1 M by using equilibrium dialysis and a conductance method. Some binding numbers he found (we calculate them from one of his figures) were: about 60 for pH 3, 33 for pH 4 and 16 for pH 5. We find that the chloride binding number is about

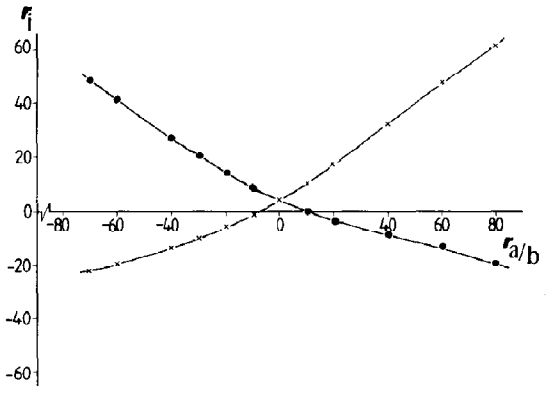

Fig. 3. Binding numbers of K^+ (●——●) and Cl^- (×——×) by bovine serum albumin according to the Esin-Markov analysis. $c_{KCl} = 0.07$ M. Points are from digitising fig. 2 with interval $10r_{a/b}$.

54 for pH 3, 25 for pH 4 and 8 for pH 5. The agreement is satisfactory. In a subsequent experiment, Carr [19] used potassium-selective membranes and found that for pH 10.8, 0.03 M KCl, the binding number of potassium is about 7. The potassium binding number we determined for pH 10.8 is 30, which is higher than the number of Carr by a factor of about 4. Perhaps the discrepancy is due to the low accuracy of the ion selective electrode technique Carr used.

Scatchard et al. [17] determined the binding of Cl^- and Na^+ by isoionic BSA, also by using ion selective electrodes. They found that in 0.07 M NaCl about seven Cl^- bind and that the binding of Na^+ is negligible (< 1). We find that isoionic BSA binds four Cl^- and four K^+. It appears to us that Scatchard's results violate the electronuetrality condition for the protein-solution interface.

5. Discussion

5.1. General remarks

So far we did not consider possible statistical thermodynamical model interpretations of the phenomenological relations. We already noted in the beginning of section 2 that Wyman's statistical thermodynamics formalism is much more in use than the classical approach we pursued here. However, we agree with Wyman that the phenomenological theory has more authority in the sense that it is completely general. In other words, any statistical thermodynamic ion binding model should be checked for its consistency with phenomenological linkage relations such as eq. 11. One such theory for which this check can readily be made is the original site binding theory of Wyman, when expressed in terms of the binding polynomial. Consider, for example, a solution dilute in protein, so that the bulk of the solution is identical to the dialysate, and assume that the protein may bind two types of ligands, A and B, which behave ideally in solution. We identify here the phenomenological excess binding numbers $r_A^{(w)}$ and $r_B^{(w)}$ as the ensemble averages of the numbers of molecules of A and B bound to the sites, $\langle s_A \rangle$ and $\langle s_B \rangle$, respectively. The statistical linkage rela-

tion for this case is well known (e.g. eq. 2.2 from ref. 3, it is:

$$\left(\frac{\partial\langle s_A\rangle}{\partial\langle s_B\rangle}\right)_{c_A} = -\left(\frac{\partial \ln c_B}{\partial \ln c_A}\right)_{\langle s_B\rangle} \tag{24}$$

which is morphologically similar to eq. 11.

As far as we know, linkage relations for more sophisticated ion binding models, like the Linderstrøm-Lang model (mean field) or the Tanford-Kirkwood model (electrostatic field dependent on configuration of ions over the sites) have not yet been derived explicitly. A general framework for such derivations could be the Macmillan-Mayer theory for electrolyte solutions (see ref. 21 for an introduction), which uses a reference solution similar to ours. Another route would be to follow Schellman's [5] interpretation of Wyman's binding polynomial and incorporate electrostatic interactions. This was done recently by Stigter and Dill [23], who used the binding polynomial for the calculation of the binding free energy. In appendix A we show — for the special case of the Gouy-Chapman-Stern model of a charged flat surface in an ideal dialysate — how this formalism leads to a linkage relation in which the phenomenological binding numbers are interpreted as the sums of ensemble averages of site binding $\langle s_i\rangle$ and diffuse binding $\langle d_i\rangle$: $\langle r_i\rangle = \langle s_i\rangle + \langle d_i\rangle$ (brackets denote ensemble averages). In the specific model we analysed, the protons were assumed to adsorb only on the sites, while the electrolyte ions were assumed to bind on sites and in the diffuse layer. This model gives (eq. A9):

$$\left(\frac{\partial(\langle s_+\rangle + \langle d_+\rangle + \langle s_-\rangle + \langle d_-\rangle}{\partial\langle s_{H^+}\rangle}\right)_{c_s} = -\left(\frac{\partial \ln c_{H^+}}{\partial \ln c_s}\right)_{\langle s_{H^+}\rangle} \tag{25}$$

where c_s and c_{H^+} are the salt and proton concentration, respectively, and $\langle d_+\rangle$ and $\langle d_-\rangle$ the ensemble averages of the diffuse bindings of the cations and anions, respectively. Comparison with the previous equation (eq. 24) shows immediately that interpretation of the linkage relation exclusively in terms of site binding for all ions may lead to a considerable error.

5.2. Application to titration of BSA

As noted, there is the general question as to whether the electrolyte binds in the diffuse part of the double layer and/or on binding sites on the protein. We recall that according to a statistical thermodynamical two-state model, the binding numbers can be written as $\langle r_i\rangle = \langle s_i\rangle + \langle d_i\rangle$, where $\langle s_i\rangle$ and $\langle d_i\rangle$ are the ensemble average numbers of site and diffusely bound ions of species i, respectively; the ensemble average binding number $\langle r_i\rangle$ may be identified with the phenomenological binding number as calculated by the Esin-Markov analysis. For further analysis, it is necessary to find an additional relation between the numbers of diffuse bound ions (to this end, we use diffuse double layer theory), from which we can estimate the number of site bound ions by comparison with the total binding numbers r_i.

The initial assumptions we make are: (i) the shape of the BSA molecule is constant; (ii) the different possible configurations of the ions on the sites have the same electrostatic free energy; (iii) fluctuations in surface charge density can be neglected; and (iv) the hydroxyl and proton binding in the diffuse layer can be neglected with respect to the proton binding on the sites. Assumption (i) implies that we neglect for the moment structural transitions, (ii) means that we use a mean field model for the calculation of the surface potentials, (iii) implies that for any given set of variables (pH, salt strength, etc.) one state of the surface charge is dominant; in statistical thermodynamical language one would say that we use the maximum term approximation for the grand partition function. Assumption (iv) is already satisfied for salt concentration $\gg c_{acid}, c_{base}$.

Let us denote using $d(+)$ the number of diffuse counter ions and with $d(-)$ the number of diffuse co-ions in the average configuration of the dominant state of the surface charge. When the potentials in the diffuse atmosphere are known for this particular state, these numbers can be calculated, from:

$$d(p) = c_s \int_V (e^{-z(p)F\psi/RT} - 1)\, dv \tag{26}$$

where z is the valency of the counter-ion ($p = +$) or co-ion ($p = -$), and v the volume element of integration. We solved the Poisson-Boltzmann equation for the potentials in the diffuse atmosphere around a prolate ellipsoide with dimensions ($4 \times 4 \times 4$ nm) in 0.07 M (1-1) electrolyte solution. The shape is that of a hydrated native BSA molecule [24]. The calculations were performed with the finite element package PDE/PROTRAN from IMSL. We modelled the ellipsoid surface as an equipotential plane (we return to this below); the calculations were repeated for various values of the surface potential. The results are shown in fig. 4. Plotted is the expulsion of the diffuse co-ions vs the accumulation of the diffuse counterions. The general shape is that of an hyperbola which flattens out gradually to a plateau. In fact, the analytical expression for a flat surface is a hyperbola: $d(-) = -d(+) \cdot d_m \cdot 1/[d_m + d(+)]$, where d_m is the maximum number of ions which can be expelled (this hyperbola relation is derived from analytical solution of the Poisson-Boltzmann equation; see below and appendix A). The curve shows that the theoretical maximum expulsion by native BSA is about 20 co-ions. A second noteworthy feature of the curve

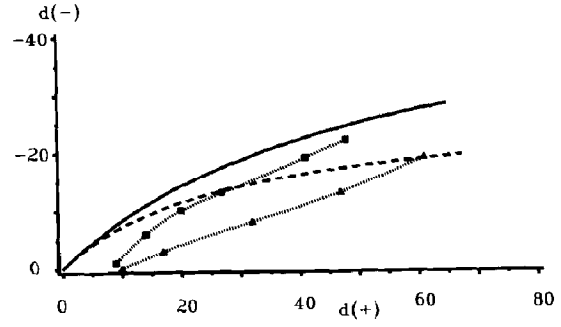

Fig. 4. Diffuse binding of counterions $d(+)$ and co-ions $d(-)$ from the solution of the Poisson-Boltzmann equation for a diffuse ion atmosphere around a prolate ellipsoid. The surface of the ellipsoid is an equipotential plane. $c_{KCl} = 0.07$ M. (———) Native structure ($4 \times 4 \times 14$ nm); (- - - - -) 50% expanded structure ($6 \times 6 \times 21$ nm). For comparison, electrolyte binding numbers from fig. 3 are included. Acid branch [(▲- - - - -▲); counterion: Cl^-, co-ion: K^+; $r_{a/b} > 10$] and base branch [(■- - - - -■): co-ion; Cl^-, counterion: K^+, $r_{a/b} < -10$]. Points are from digitization of fig. 2 in intervals of 10 acid/base binding unit.

is that the initial slope is -1. Why this is so can be understood by expansion of the integrand $[\exp(-(z(p)F\psi/RT) - 1]$ in eq. 26, which gives in first order $-z(p)F\psi/RT$ (the Debye-Hückel approximation) so that the integral $\int_V [\exp(-z(p)F\psi/RT) - 1]\, dv$ becomes linear in z and hence $d(+) \cong -d(-)$. The calculated curves show that the Debye-Hückel approximation is only reasonably good for counterion binding numbers less than about 10. In this regime the absolute charge on the surface ($\equiv |d(-) - d(+)|$) must be less than $2 \cdot d(+) \cong 20$. Norde [20] found experimentally that the zeta potential of BSA varies from 5.5 mV for pH 4 to -27.4 mV for pH 8 (measurements in 0.05 M veronal acetate/KNO_3 buffer). These are potentials which satisfy the Debye-Hückel criterion. Indeed, the corresponding variation in proton charge (fig. 2) is from about $+20$ (pH 4) to -15 (pH 8).

For comparison, we also include in fig. 4 some of the phenomenological electrolyte binding numbers from fig. 3. We took binding numbers from the acid branch of the titration curve ($r_{a/b} > 10$ corresponding to pH < pI) where the K^+ are the expelled co-ions and the Cl^- are the counterions, and we also took the binding numbers from the base branch ($r_{a/b} < -10$ corresponding to pH > 7) where the Cl^- are expelled and the K^+ are the counterions. The 'experimental' and theoretical curves differ with respect to some details, but we feel that the overall agreement is striking. The immediate conclusion must be that the simple model for the binding, which includes binding in the diffuse layer only, explains within a few ten percent the phenomenological findings. In other words: the overall picture is that the diffuse layer is the dominant locus for binding of ions other than H^+, OH^-, and not the surface.

There are a few mechanisms operative which may explain the small differences between the experimental and theoretical curves. These are:

(i) The phenomenological binding numbers are not pure diffuse binding numbers. The binding numbers from the base branch refer to the Cl^- as the co-ions and K^+ as the counter-ions. We know that chloride binds specifically so that the net negative chloride binding number may be the sum of a (small) positive site binding number and a

(large) negative diffuse binding number. Hence, the 'true' diffuse expulsion of chloride is possibly slightly larger than the phenomenological binding number. In addition, it seems reasonable to suppose that the contribution of chloride site binding decreases at a more negative surface potential. The phenomenological binding numbers of K^+ are also mixed quantities.

For the acid branch the situation is different. Here we have evidence that K^+ does not bind on the surface (see section 4) so that the phenomenological binding of K^+ should be entirely due to diffuse expulsion. In principle then, we could use the theoretical curve to calculate the diffuse accumulation of chloride and then, by comparison with the phenomenological binding number of chloride, the chloride site binding. Why this cannot be done in practice is shown below.

(ii) A remarkable difference between the experimental and theoretical curves is that near the plateau region the phenomenological binding numbers of the co-ions tend to be more negative than the maximally possible expulsion. A likely explanation is that the BSA molecule expands when it is highly charged. This was inferred already by Tanford from a Linderstrøm-Lang analysis of these same proton titration curves (fig. 2). Tanford also performed some viscosity measurements from which he concluded that the expansion in acid (pH < 4, 0.07 M) is about 50% [25]. Therefore, we also calculated the diffuse binding in the diffuse layer around a 50% expanded BSA molecule ($6 \times 6 \times 21$ nm) by the method outlined above. Comparison with the phenomenological curves (fig. 4) shows that for higher charges the phenomenological binding indeed gradually approaches that of binding in the diffuse layer around the expanded protein molecule. In retrospect, we can now understand more readily why the Esin-Markov coefficient tends to drop when the pH is very low or very high (inset, fig. 2). If BSA were a stable molecule, the diffuse expulsion would have saturated on a constant level, namely, the plateau of maximum expulsion. There, the Esin-Markov coefficient would have been -1 or $+1$ because additional accumulation of counterions would then have been the sole possibility for the compensation of the surface charge. Obviously, by expansion the level of maximum expulsion rises so that the Esin-Markov coefficient remains constant, or even drops.

Returning to the problem of calculation of the site binding (no *i* above), we here find that such a calculation is possible when accurate data on the pH dependence of the shape of the protein molecule are available.

(iii) A third issue is the distribution of the surface potentials. In our model calculation we assumed that the protein surface is an equipotential plane, whereas in reality the surface potential will be a function of some surface coordinate. It is impossible to estimate the potential variation and hence the error quantitatively without knowing the configuration of the sites and their interaction. This is what we tried to avoid in the first place. An argument in favour of the equipotential approach is that potential fluctuations over distances shorter than the Debye length are short-circuited by the diffuse layer. In the present case the Debye length is about 1.2 nm, which is smaller than the length scale of the protein.

(iv) Finally, there may be an effect due to the neglecting of the fluctuations in surface charge and in configurations. The model we used for the calculation of the diffuse binding numbers disregards fluctuations in surface charge, and uses a mean field model for the configurations. The neglecting of fluctuations, or for that matter, applying the maximum term method, is a very good approximation for large systems, which contain many sites. However, here we deal with a finite number of sites so that contributions from other than the maximum term may also be significant [21,23]. In addition, the mean field approximation for the configurations may possibly be in error because the average configuration will generate a potential profile different from the most probable configuration (see item iii above). Again, an estimate of these two types of errors requires a model for the sites. A rough estimate for the error in the maximum term approximation can be found as follows. Suppose, for the sake of argument, that the potential profile around a protein, containing Z surface-bound charges, is that of the potential profile above a flat surface with the same area as the protein. For this case, the binding number of

the diffuse counter- and co-ions can be calculated from the analytical formula (see appendix A, combine eqs A3, A4 and A7):

$$d(+) = d_m \cdot \left\{(\alpha^2+1)^{1/2} - \alpha - 1\right\} \quad (27a)$$

$$d(-) = d_m \cdot \left\{(\alpha^2+1)^{1/2} + \alpha - 1\right\} \quad (27b)$$

where α is defined as $\alpha \equiv Z/2d_m$ and $d_m \equiv 2Ac_s/\kappa$, A denotes surface area and κ the reciprocal double layer thickness. Elimination of α leads to the hyperbolic relation between $d(+)$ and $d(-)$ mentioned above. The question is now: does the same hyperbola functionality relate the ensemble averages of $d(+)$ and $d(-)$? The ensemble averages of the binding numbers of the diffuse counter- and co-ions are:

$$\langle d(+)\rangle = d_m\left\{\langle(\alpha^2+1)^{1/2}\rangle - \langle\alpha\rangle - 1\right\} \quad (28a)$$

$$\langle d(-)\rangle = d_m\left\{\langle(\alpha^2+1)^{1/2}\rangle + \langle\alpha\rangle - 1\right\} \quad (28b)$$

If we now want to find the relation between the ensemble averages of the numbers of diffuse counter- and co-ions we must determine an additional relation between the ensemble average $\langle(\alpha^2+1)^{1/2}\rangle$ and the ensemble average $\langle\alpha\rangle$. We define the function R as $R \equiv \langle(\alpha^2+1)^{1/2}\rangle - (\langle\alpha^2\rangle + 1)^{1/2}$; when R is zero the relation between the ensemble averages is again that of the hyperbola. The error in the diffuse charges is of the order $d_m \cdot R$. It is readily seen that R will be zero in the limit of the maximum term approximation because then only one state is sampled. In the general case R will be about largest when the average of α is close to unity. In other words, the relation between the ensemble averages will differ maximally from that between the maximum term numbers when the number of surface charges is approximately equal to $2d_m$. For $\langle\alpha\rangle \cong 1$, expansion of the square roots shows that then R is roughly $(\langle\alpha^2\rangle - \langle\alpha\rangle^2)/2$, or $\sigma^2/(8d_m^2)$, where σ^2 is the variance in the number of surface charges. The variance can be calculated from the proton titration curve (for the sake of argument, we identify Z here with the proton binding number), according to $\sigma^2 = \partial r_{H^+}/\partial \ln a_{H^+}$ (from the second derivative of the grand partition function [21]). From fig. 2, we find that σ^2 is about 17 in the acid region (pH 3–4), where the slope of the titration curve is maximal. The maximum expulsion d_m is about 20 (fig. 4), so that the error in the diffuse charges is maximally about 0.1. This is a small number. The conclusion must be that the ensemble averages of the numbers of the diffuse counter- and co-ions are very close to the corresponding data from the most probable surface charge.

6. Conclusion

It has been demonstrated that linkage relations can be defined for protein solutions in membrane equilibrium with a reference solution. These linkage relations enable the calculation of the binding of ions by proteins from acid-base titration data. The Cl^- and K^+ binding by BSA in 0.07 M KCl solution is to a large extent accounted for by binding in the diffuse layer.

The thermodynamic method is very general. Only such simplifications were made as conform to usual experimental conditions. Hence, the method is also applicable to other systems and can be readily extended to describe more complicated processes, such as the co-partitioning of low molecular weight ions with proteins or polyelectrolytes in two-phase systems. In subsequent papers we intend to present elaborations [26].

Appendix A

A1. A model elaboration: Linkage relation for coupled diffuse and site binding (eq. 25)

Consider a single, rigid, flat surface of area A in dilute (1-1) electrolyte solution. We assume that the surface contains n proton binding sites, m cation binding sites and l anion binding sites. We assume furthermore that the dialysate behaves ideally. The surface grand partition function Ξ [21], or binding polynomial [3–5] for this case is (see eqs 12 and 13 of ref. 23 for the case of proton site binding only):

$$\Xi = \sum_{i=0}^{n}\sum_{j=0}^{m}\sum_{k=0}^{l} c_H^i \cdot c_s^{j+k} Q_{ijk}^0 e^{-G_{el}(i,j,k)/RT} \quad (A1)$$

where Q^0_{ijk} is the canonical partition function of the chemical interactions, including the degeneracy factors of type $\binom{n}{i}\binom{m}{j}\binom{l}{k}$ (or more complex expressions, the precise functionality is unimportant here), c_{H^+} the proton concentration, c_s the salt concentration and $G_{el}(i,j,k)$ the electrical free energy of state ijk. The numbers of site-adsorbed ions are: i (protons), j (cations) and k (anions). The surface charge density $\sigma_0(i,j,k)$ is $F\cdot(i+j-k)/A$, where A is the surface area. The electrical free energy is obtained by a convenient reversible charging process [23]:

$$G_{el}(i,j,k) = \int_0^{\sigma_0(i,j,k)} A\psi_0 \, d\sigma_0 \tag{A2}$$

In the present case we use the Gouy-Chapman-Stern double layer model of a flat surface. In this picture, $\psi_0(\sigma_0)$ is the mean electrostatic surface potential for a certain state of charging σ_0. The model leads to an analytical expression for the surface potential in terms of the surface charge:

$$\psi_0 = \frac{\sigma_0}{K_s} + \frac{2}{\beta}\operatorname{arcsinh}\left(\frac{\beta\sigma_0}{2K}\right) \tag{A3}$$

where β is F/RT, K is $\epsilon\kappa$, where ϵ denotes the dielectric constant of the solution and κ the reciprocal double thickness ($\kappa \equiv (2\beta F c_s/\epsilon)^{1/2}$), and K_s is the capacitance of the Stern layer; K and K_s are per unit area. We also introduce here the Stern potential ψ_s, which is related to the surface potential and surface charge through:

$$\sigma_0 = K_s(\psi_0 - \psi_s) \tag{A4}$$

Combination of eqs A3 and A4 gives the relation between the surface charge and the Stern potential: $\sigma_0 = 2K/\beta \cdot \sinh(\beta\psi_s/2)$. Next, we determine the derivative of $\ln \Xi$ with respect to $\ln(c_s)$ and $\ln(c_{H^+})$. The derivative with respect to $\ln(c_{H^+})$ is readily determined; it is (we replace here the compound sum by the single sign Σ):

$$\left(\frac{\partial \ln \Xi}{\partial \ln c_{H^+}}\right)_{c_s} = \frac{1}{\Xi}\sum_{ijk} i c^i_{H^+} c_s^{j+k} Q^0_{ijk} e^{-G_{el}(i,j,k)/RT}$$
$$= \langle i \rangle = \langle s_{H^+} \rangle \tag{A5}$$

where the brackets denote ensemble averages; $\langle s_{H^+} \rangle$ is the average number of protons bound to the sites. The differential with respect to $\ln(c_s)$ we find by combination of eqs A1–A4 and application of the chain rule:

$$\left(\frac{\partial \ln \Xi}{\partial \ln c_s}\right)_{c_{H^+}}$$
$$= \frac{-A}{\Xi RT}\sum_{ijk} c^i_{H^+} c_s^{j+k} Q^0_i e^{-G_{el}(i,j,k)/RT} \int_0^{\sigma_0(i,j,k)}$$
$$\times \left(\frac{\partial \psi_0}{\partial \ln(c_s)}\right) d\sigma_0$$
$$+ \frac{1}{\Xi}\sum_{ijk} c^i_{H^+}(j+k)$$
$$\times c_s^{j+k} Q^0_{ijk} e^{-G_{el}(i,j,k)/RT}$$
$$= -A\beta/F\left\langle \int_0^{\sigma_0(i,j,k)} \left(\frac{\partial \psi_0}{\partial \ln(c_s)}\right) d\sigma_0 \right\rangle$$
$$+ \langle j+k \rangle$$
$$= \frac{2AK}{F\beta}\left\langle \left(\left(\frac{\beta\sigma_0(i,j,k)}{2K}\right)^2 + 1\right)^{1/2} - 1 \right\rangle$$
$$+ \langle s_+ + s_- \rangle$$
$$= 2d_m\langle \cosh(\beta\psi_s/2) - 1 \rangle + \langle s_+ + s_- \rangle \tag{A6}$$

where d_m is defined as $2Ac_s/\kappa$, and s_+ and s_- are the site binding numbers of the cations and anions, respectively. From the Poisson-Boltzmann profile we also have analytical formulas for the expulsion of the cations (diffuse binding number d_+) and accumulation of the anions (d_-) in the diffuse layer. These are:

$$d_+ = d_m(e^{-\beta\psi_s/2} - 1) \tag{A7a}$$

$$d_- = d_m(e^{\beta\psi_s/2} - 1) \tag{A7b}$$

Elimination of ψ_s gives the hyperbola relation $d_- = -d_+ \cdot d_m \cdot /(d_m + d_+)$ referred to in section 5. Comparison of eqs A7a and b with eq. A6 immediately leads to the conclusion that the differential coefficient is identical to the sum of the ensemble averages of the diffuse and site binding numbers. Combination of eqs A5–A7 gives:

$$d\ln \Xi = \langle s_{H^+} \rangle \, d\ln(c_{H^+}) + (\langle s_+ \rangle + \langle d_+ \rangle + \langle s_- \rangle + \langle d_- \rangle)\, d\ln(c_s) \tag{A8}$$

From which we can construct a linkage relation between the proton and electrolyte binding, from the differential of $[\ln \Xi - \langle s_{H^+} \rangle \ln(c_{H^+})]$. It is:

$$\left(\frac{\partial \langle s_+ \rangle + \langle d_+ \rangle + \langle s_- \rangle + \langle d_- \rangle}{\partial \langle s_{H^+} \rangle} \right)_{c_s} = -\left(\frac{\partial \ln c_{H^+}}{\partial \ln c_s} \right)_{\langle s_{H^+} \rangle} \quad \text{(A9)}$$

which is eq. 25.

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