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Interfacial thermodynamics of protein adsorption, ion co-adsorption and ion binding in solution

I. Phenomenological linkage relations for ion exchange in lysozyme chromatography and titration in solution

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In this paper we discuss the thermodynamics of ion binding in solution, protein adsorption and ion co-adsorption. The emphasis is on charge regulation effects. To this end, we introduce phenomenological linkage relations from which the ion binding can be calculated from the electrolyte dependency of proton titration curves and the co-adsorption from the electrolyte dependency of protein adsorption isotherms. The linkage relations are derived from classical interfacial thermodynamics, and thus offer an alternative approach as compared to the mass balance equations which are currently used in biotechnology, and Record et al.'s 1978 analysis of Wyman's Binding Polynomial for protein interactions. The co-adsorption theory is an extension of our previous analysis of ion binding in solution, which we include here for comparison of the ion co-adsorption with the ion binding in solution. The theory is applied to the chromatography of lysozyme on the strong cation exchanger 'mono S' and to the proton titration of lysozyme in solution. In the accompanying Part 2 of this paper the results are interpreted with a simple model.

Introduction

In this paper we discuss phenomenological relations for ion binding in protein titration and ion co-adsorption in protein adsorption. The relations are applied to protein ion exchange chromatography.

The topic is of some importance, in view of increasing demand in biotechnology for new purification methods; ion exchange chromatography of proteins is everyday practice in numerous biochemical and biophysical laboratories. Yet detailed studies of the underlying principles are very scarce: in only a few cases ion co-adsorption has been quantified directly in model experiments of protein adsorption on synthetic colloids [1–6]. The experiments indicate that in many instances electrostatic factors are not solely responsible for ion co-adsorption. For example, it is now well established that labile proteins may unfold upon adsorption, and that the unfolding may be accompanied by a shift in ion binding [7]. In other words, structural and electrostatic factors interplay.

In a slightly more general sense, the principle of charge adaption can be found in other types of two-phase systems as well; we will use the generic

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terms protein partition and ion co-partition to cover all these cases. In a previous paper [8] we discussed the ion co-partition when cytochrome c solubilizes in reverse micelles, using the same type of thermodynamic equations as we present here. In general, in biotechnology one can find many examples in which protein adsorption (e.g. chromatography) or protein partition (the solubilization just mentioned) depends on the electrolyte composition and pH of the aqueous protein solution (solubilization [8–11], chromatography [18–22]). Record et al. [12] reviewed charge adaption and the concomitant ion exchange in entirely different systems, e.g. protein–protein interactions and protein–nucleic acid complexation. Record et al. analysed the charge regulation with linkage relations from extension of Wyman's binding polynomial theory [12–16].

In our approach the linkage relations are given a classical interfacial thermodynamical foundation. As far as we know, the classical approach has not yet been pursued before, although Wyman already pointed out that the classical approach has "the authority of a completely general derivation" which complements the statistical approach which "provides an insight into the molecular basis of a phenomenon that comes from an statistical analysis" (citations from [13]). A thermodynamic approach is entirely phenomenological. Indeed, we do not need to define or consider quantities like binding constants, site binding etc.

The theory is an extension of our previous analysis of ion binding by proteins [17], part of which we include here for comparison of the ion co-adsorption with the ion binding in solution; the experimental data are taken from a paper by Tanford and Roxby [27].

The linkage relations for ion co-adsorption are applied to ion exchange chromatography of hen's egg-white lysozyme on a commercial cation exchanger, 'mono S' from Pharmacia. We present the experimental data as a 2D map of retention volumina versus salt activity and pH. Similar maps, although with small variations in medium conditions, have been published by several authors, using various proteins and column types [18–22]. In the accompanying paper [39] the phenomenological ion co-adsorption numbers are analysed with a model for the electrical double layer of an adsorbed lysozyme molecule. As far as we know, this is the first quantitative model which is in principle capable of predicting the retention by *a priori* assessment of a limited number of accessible parameters. In the biotechnology literature attempts are made to correlate 2D gel electrophoretic titration curves with ion exchange chromatograms [18,23,33]. Such correlations are purely qualitative, in the sense that the general prediction is that the higher the charge contrast is between protein and surface, the more the protein is retarded.

Hen egg-white Lysozyme (MW 14,600 Da) is a well studied protein (see Stryer's book [25] for general information). The structure has been determined some years ago by Blake et al. [24]; in crystal packing it has dimensions (hydrated) $4.5 \times 3.0 \times 3.0$ nm. The molecule is very stable in solution with a Gibbs energy of denaturation of about -4 J/gram [26]. Tanford and Roxby determined the proton titration curves in KCl solution [27] and Guanidine · HCl solution [28]; the isoionic point is at about pH 11.35, close to the isoelectric point (i.e.p.) at pH 11.0–11.3. A few reports of lysozyme adsorption on model substrates have appeared: adsorption on apatites measured by depletion [5], adsorption on hydrophilic silica and polystyrene-coated silica measured by reflectometry and streaming potential measurements [29], adsorption on polyoxymethylene, polystyrene and haematite by calorimetry and depletion measurements [30] and adsorption on alkylated silicon oxide by total internal reflection fluorescence measurements [31]. The studies show that lysozyme adsorbed on hydrophilic surface is probably in its native structure, with a monolayer packing corresponding reasonably well with crystal structure, and that adsorption on these surfaces is largely governed by electrostatic factors [5,29,30]. There are indications that on hydrophobic surfaces a fraction of the adsorbed molecules is unfolded [31].

Mono S is much in use as a chromatographic support, a literature survey gave more than 120 entries for publications in the past decade. Unfortunately, information on the physico-chemical properties is scarce. The support consists of rigid

monodisperse macroporous polymer particles with diameter 10 μm [32,34]. The resin is a hydrophilic 'polyether' [34], with charged groups $-CH_2-SO_3^-$ (ionic capacity 0.13–0.18 mmol/ml resin), pH stability range 2–12. References [33–37] are the reports from the manufacturer on properties, usage etc.

Phenomenological linkage relations

Titration in solution

Binding numbers r_i of species i in solution phase α are defined for a protein solution L in membrane equilibrium with its dialysate R (Fig. 1a), according to:

$$r_i^{(w)\alpha} \equiv \left(c_i^{L\alpha} - c_i^{R\alpha} \cdot c_w^{L\alpha}/c_w^{R\alpha}\right)/c_p \qquad (1)$$

where 'c' is the concentration (in moles/unit volume), 'w' is water and 'p' is protein. By defining the binding number in this way as the excess in the protein solution over that in the reference solution, we avoid discussion of the binding mechanism itself: the definition is purely phenomenological. The binding numbers and chemical potentials are coupled by two Gibbs–Duhem relations, one for the protein solution and one for the reference solution. The coupling leads directly to the phenomenological linkage relations (see [17] and Appendix A), one integral linkage relation we will use is (eq. A3):

$$r_i^{(w)\alpha}(\mathrm{II}) = r_i^{(w)\alpha}(\mathrm{I}) + \int_{\mathrm{I}}^{\mathrm{II}} \left(\frac{\partial r_j^{(w)\alpha}}{\partial \mu_i}\right)_{\mu_{j\neq i}, c_p} \mathrm{d}\mu_j \qquad (2)$$

Equation (2) is our integral linkage relation which may be compared with Wyman's famous differential linkage relation (e.g. eq. (1.9) from Ref. [13]). The difference is that Wyman derived the relation from the Binding Polynomial, whereas we prefer here the classical thermodynamic approach. A detailed comparison is made in [17].

Co-adsorption

Extension of the above formalism to two-phase systems is straightforward. Co-adsorption numbers Δr_i are defined for a protein solution-surface two-phase system L, in membrane equilibrium with a two-phase reference solution-surface system R (Fig. 1b):

$$\Delta r_i^{(w)} = r_i^{(w)\sigma} - r_i^{(w)\alpha} \qquad (3)$$

where $r_i^{(w)\sigma}$ is the binding number in the surface phase:

$$r_i^{(w)\sigma} \equiv \left(\Gamma_i^{(w)L} - \Gamma_i^{(w)R}\right)/\Gamma_p^{(w)} \qquad (4)$$

where $\Gamma_i^{(w)}$ is the excess adsorption (in moles/unit area) with respect to water:

$$\Gamma_i^{(w)X} \equiv \Gamma_i^{\sigma X} - c_i^{\alpha X} \cdot \Gamma_w^{\sigma X}/c_w^{\alpha X} \qquad (5)$$

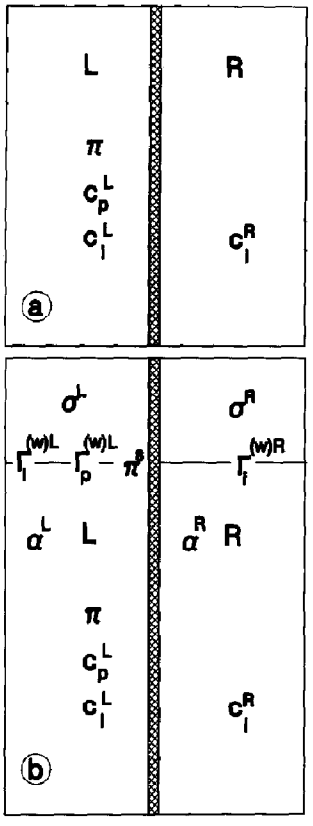

Fig. 1(a). Schematic of the model system. Protein solution L in membrane equilibrium with dialysate R. Symbols are defined in the text.

Fig. 1(b). Schematic of the solution–surface ($\alpha-\sigma$) two-phase model system. Symbols are defined in the text.

X = R or L. These definitions are, as stated before, completely phenomenological. No assumptions are made regarding the adsorption mechanism of the protein, binding mechanism of ions etc.

For the surface-solution double two-phase system there are four Gibbs–Duhem relations which are coupled by the membrane equilibrium, two for the surface phases and two for the solution phases. The linkage relation between protein adsorption and co-adsorption is derived in Appendix A. The result is a new integral equation (eq. A8):

$$\Delta r_i^{(w)} = \frac{1}{\Gamma_p^{(w)}} \int_0^{\pi} \left(\frac{\partial \Gamma_p^{(w)}/c_p}{\partial \mu_i} \right)_{\pi, \mu_{j \neq i}} d\pi \qquad (6)$$

where π is the osmotic pressure. In the present study the solution is dilute in protein, so that Van 't Hoff's law applies ($\pi = RTc_p$), the protein adsorption is in the Henry region (so that Δr is independent of surface coverage, denoted by the superscript ^{0}), and the molar fraction of water is approximately unity (so that we may omit the superscript $^{(w)}$).

The Henry coefficient η is defined as the initial slope of the adsorption isotherm:

$$\eta \equiv \lim_{c_p \to 0} \frac{A^L \Gamma_p}{V^L c_p} \qquad (7)$$

The co-adsorption number in the Henry regime is defined as:

$$\Delta r_i^0 \equiv \lim_{c_p \to 0} \Delta r_i \qquad (8)$$

Combination of eqs. [6–8] results in the limiting relation between the Henry constant and the co-adsorption number. We obtain the simple result:

$$\Delta r_i^0 = RT \left(\frac{\partial \ln(\eta)}{\partial \mu_i} \right)_{\mu_{j \neq i}} \qquad (9)$$

For convenience of notation we omit the superscript ^{0} in the following, so that Δr_i from now on stands for the limiting co-adsorption number as referred to in eqs. (7)–(9), unless otherwise stated.

Experimental

Hen egg-white lysozyme (Merck) was eluted isocratically on a Pharmacia mono S column, using a Pharmacia FPLC unit. The concentration of NaCl was variable (0.3–1 M), the buffer concentration 10 mM (formic acid pH 3.5–3.75), acetic acid (pH 4.00–5.75), N-morpholin ethane sulfonic acid (MES; pH 6.5). Initially, chromatograms were determined for various values of flow (0.2 ml/min–1 ml/min) and inject amount (2–20 μg). Lower and upper values gave similar results for the capacity factor (see below). The experiments reported here were with a flow of 1 ml/min and 10 μg injection. The shapes of the elution patterns were similar to those reported by the manufacturer: single sharp peaks with very little tailing. We did not check whether the injected amount was recovered completely. However, in a separate experiment we determined a complete retention map for cytochrome c, a small basic protein as lysozyme but intensely coloured, and found insignificant residual staining of the column. These observations demonstrate that under the selected operating conditions the protein adsorption is reversible and in the Henry region. This is of course a prerequisite for the use of the thermodynamic linkage relations.

The elution volume V_e, the volume of the stationary phase V_s, the volume of the mobile phase V_m and the Henry coefficient η are related via:

$$V_e = V_m + V_s + \eta V_s = V_o + \eta V_s \qquad (10)$$

where V_o is defined as usual as:

$$V_o \equiv \lim_{\eta \to 0} V_e = V_m + V_s \qquad (11)$$

We determined V_o by eluting various proteins (lysozyme, bovine serum albumin, ribonuclease, cytochrome c, α-amylase and a flavodoxine) in appropriate elution media so that η was vanishingly low. We found $V_o = 1.01 \pm 0.02$ ml, depending only very slightly on the elution conditions, in agreement with the information from the manufacturer. The capacity factor R is defined as:

$$R \equiv (V_e - V_o)/V_o = \eta V_s / V_o \qquad (12)$$

The salt concentration c_s was converted to salt activity a_s by interpolation in the activity coefficient table for NaCl in Parson's Electrochemical Handbook [38].

Results

The retention map, log(R) against a_s for varying pH, is shown in Fig. 2. Two features are noteworthy, viz.(i) the lateral, almost parallel, displacement of the iso-pH log(R) vs. a_s curves for varying pH (also found by other authors [18–22] in related systems) and (ii) the distinct upward bending of the log(R) vs. a_s curves, especially when the retardation is small ($R < 1$). The latter effect is not immediately clear from the previously published retention maps [18–22] (increased retention in media of higher salt concentration has been attributed to salting-out, see for instance reports [21,22] regarding salting-out by $(NH_4)_2SO_4$ on columns with combined ion exchange and hydrophobic functionality) but we found the same phenomenon for all the proteins we investigated (see Experimental), including chromatography on the anion exchanger mono Q, also from Pharmacia.

The accuracy of log(R) is limited by the systematic error in V_o. For example, when V_e is 1.11 ml ($\log(R) = -1$), a systematic error of 0.02 in V_o implies a systematic error of about 0.1 in log(R). The error decreases rapidly for larger retardation; when $V_e = 1.41$ ml ($\log(R) = -0.4$) the error is only 0.02.

In view of the excess of salt with respect to buffer, we neglect the co-adsorption of the buffer. The ion co-adsorption numbers are then defined as [8]:

$$\Delta r_{ab} \equiv \Delta r_{HCl} - \Delta r_{NaOH} \tag{13a}$$

$$\Delta r_{Na^+} \equiv \Delta r_{NaCl} + \Delta r_{NaOH} \tag{13b}$$

$$\Delta r_{Cl^-} \equiv \Delta r_{NaCl} + \Delta r_{HCl} \tag{13c}$$

where Δr_{ab} is the excess co-adsorption of acid over that of base; in our case hydroxyl binding is probably negligible, we therefore may interpret Δr_{ab} here as a proton co-adsorption number. For the chemical potentials we use: $\mu_{NaCl} = \mu^0_{NaCl} + 2RT \ln(a_s)$, where a_s is the activity of the salt

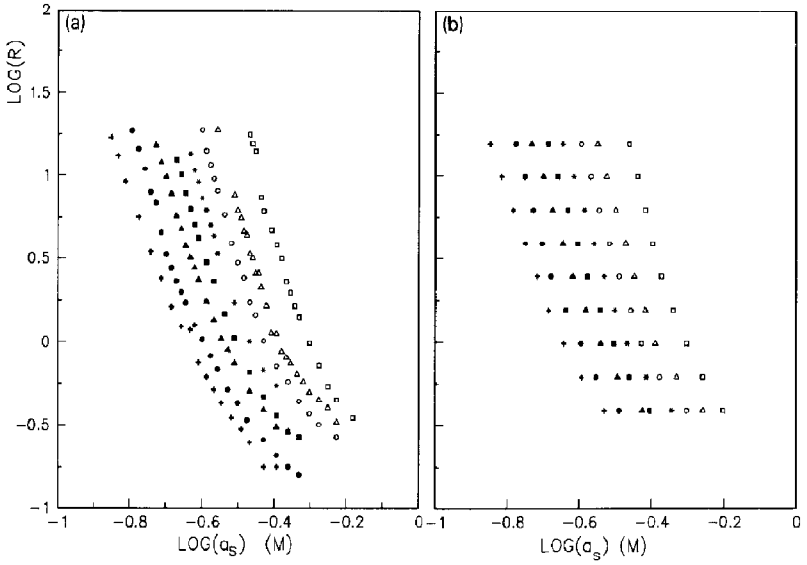

Fig. 2(a). Retention map of Lysozyme chromatography on mono S. Capacity factor R vs. salt activity a_s (M). The basis of the logarithms is 10. Buffers (10 mM): Formic acid (pH 3.5–3.75), Acetic acid (pH 4.00–5.75), *N*-Morpholin ethane sulfonic acid (pH 6.5). Flow 1 ml/min, 10 μg protein injected. Symbols: pH 3.5 (□), 3.75 (△), 4.00 (○), 4.25 (*), 4.60 (■), 5.00 (▲), 5.75 (●), and 6.50 (+).

Fig. 2(b). Interpolated (visual) smoothed net of 72 points in the retention map of Fig. 2a.

$(a_s = (a_+ \cdot a_-)^{1/2})$, $\mu_{HCl} = \mu^0_{HCl} + RT \ln(a_s) + RT \ln(a_{H^+})$, where a_{H^+} is the proton activity and $\mu_{NaOH} = \mu_{NaCl} - \mu_{HCl}(\mu_{H_2O}$ const.). More details can be found in [8,17].

The retention map is analysed in terms of the following partial derivatives (using pH $\equiv -\log(a_{H^+})$ and V_o and V_s are constant):

$$\phi \equiv \left(\frac{\partial \log(\eta)}{\partial \text{pH}}\right)_{a_s} = \left(\frac{\partial \log(R)}{\partial \text{pH}}\right)_{a_s} \quad (14a)$$

$$\epsilon \equiv \left(\frac{\partial \log(\eta)}{\partial \log(a_s)}\right)_{\text{pH}} = \left(\frac{\partial \log(R)}{\partial \log(a_s)}\right)_{\text{pH}} \quad (14b)$$

$$\rho \equiv -\frac{\epsilon}{\phi} = \left(\frac{\partial \text{pH}}{\partial \log(a_s)}\right)_{\eta} = \left(\frac{\partial \text{pH}}{\partial \log(a_s)}\right)_{R} \quad (14c)$$

The differential coefficients are related to the ion co-adsorption numbers through (Appendix B):

$$\Delta r_{Ab} = -\phi \quad (15a)$$

$$= \epsilon/\rho \quad (15a')$$

$$\Delta r_{Na^+} = 0.5(\epsilon + \phi) \quad (15b)$$

$$= 0.5\left(1 - \frac{1}{\rho}\right)\epsilon \quad (15b')$$

$$\Delta r_{Cl^-} = 0.5(\epsilon - \phi) \quad (15c)$$

$$= 0.5\left(1 + \frac{1}{\rho}\right)\epsilon \quad (15c')$$

The co-adsorption numbers were calculated as follows. First, we determined a smoothed net of 72 points in the retention map (Fig. 2b) by visual *inter*polation of nine points in each of the (eight) iso-pH log(R) vs. a_s curves. The points were chosen between and including log(R) = −0.4 to 1.2, with constant interval of 0.2 log(R) unit. From this net, we estimated ρ and ϵ for the inner $6 \times 7 = 42$ points (log(R) from −0.2 to 1, pH from 3.75 to 5.75) by a finite difference scheme according to a piecewise parabolic approximation; the boundary points were only used for the approximation of the second derivatives. Finally, the co-adsorption numbers were calculated from eqs. (15a′), (15b′) and (15c′). The accuracy is about 0.1–0.2 co-adsorption number units for Δr_{ab} and 0.2–0.5 for Δr_{Cl^-} and Δr_{Na^+}.

Note that the alternative procedure for calculation of the ion co-adsorption numbers, namely by use of eqs. (15a), (15b) and (15c), is possible by first estimating ϕ in every point of the net by application of eq. (14a). However, in the present context this would require extensive *extra*polation of the original retention map far beyond the boundaries in order to satisfy the condition of constant salt activity. This type of extrapolation

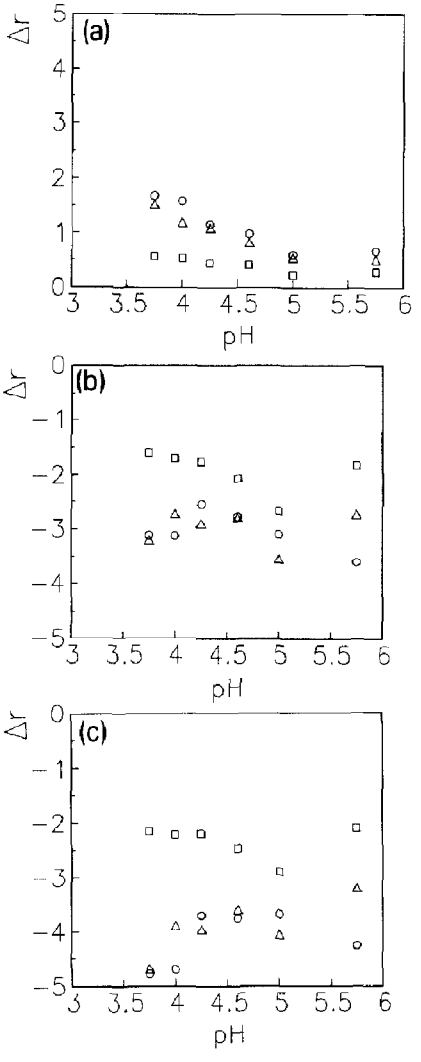

Fig. 3. Ion co-adsorption numbers for Lysozyme adsorption on mono S. vs. pH for various values of log(R). Calculated from Fig. 2(b). using piece-wise parabolic finite-difference scheme for the differential coefficients in eqs. (15a′)–(15c′). (a) Acid/base (≅ proton), (b) chloride ion, and (c) sodium ion. Log(R): −0.21 (□), 0.39 (△) and 1.00 (○). Note that acid/base coadsorption is always positive, whereas that for sodium and chloride is negative.

would be more inaccurate than the limited interpolation we have used here.

The results are shown in Fig. 3. It is noteworthy that the acid/base co-adsorption is always positive, indicative of some electrostatic induction effect. The sodium ions and chloride ion co-adsorption are negative, indicative of a compound ion exchange phenomenon: sodium ions are displaced from the ion exchanger surface, and chloride ions are displaced from the protein ion atmosphere. We will come back to these observations in some more detail in the discussion; in [39] we present a quantitative model which explains these results fairly well.

Titration in solution

In Fig. 4 Tanford's and Roxby's titration curves of lysozyme in KCl solution (0.1 and 1 M) are redrawn. The points are from digitising the published (interpolated) curves in intervals of 0.2–0.5 acid/base (proton) binding unit. The chloride ion binding was calculated from the integral equation eq. (2), re-written in terms of ion binding numbers, salt activity and pH according to the scheme outlined before [17]. The result is (Appendix C):

$$r^{\alpha}_{\mathrm{Cl}^-}(\mathrm{pH}) = r^{\alpha}_{\mathrm{Cl}^-}(\mathrm{p}I) + 0.5\left\{ r^{\alpha}_{\mathrm{ab}} - \int_{\mathrm{p}I}^{\mathrm{pH}} \delta_{\mathrm{ab}}\, \mathrm{d\ pH} \right\} \tag{16}$$

The coefficient δ_{ab} is an example of a so-called Esin–Markov coefficient (see [17] and references therein). It is a phenomenological quantity and defined as:

$$\delta_{\mathrm{ab}} \equiv \left(\frac{\partial r^{\alpha}_{\mathrm{ab}}}{\partial \log(a_{\mathrm{s}})} \right)_{\mathrm{pH}} \tag{17a}$$

where δ_{ab} can readily be calculated from the titration curves by some finite difference scheme. Here, we have only two curves, for 0.1 M and 1 M, so that we have to approximate δ_{ab} by first order finite difference:

$$\delta_{\mathrm{ab}} \cong \left(\frac{\left[r^{\alpha}_{\mathrm{ab}}(1\ M) - r^{\alpha}_{\mathrm{ab}}(0.1\ M) \right]}{\left[\log(a_{\mathrm{s}}(1\ M)) - \log(a_{\mathrm{s}}(0.1\ M)) \right]} \right)_{\mathrm{pH}} \tag{17b}$$

In this approximation δ_{ab} is of course independent of the saltstrength.

Note that eq. (16) is derived from phenomenological principles only, no assumptions are made regarding the mechanism of binding. In the introduction we already mentioned that the isoelectric point is near pH 11.0–11.3, very close to the isoionic point pI 11.35. This indicates to very small specific binding of the salt in the isoionic point. Here, we assume that the chloride binding in the isoionic point is zero, by definition the acid/base binding number is also zero in the isoionic point. The sodium ion binding follows directly from the calculated chloride ion binding and the electroneutrality condition—we neglect here the difference between binding characteristics of sodium ion and potassium ion.

The results are shown in Fig. 4. It is evident

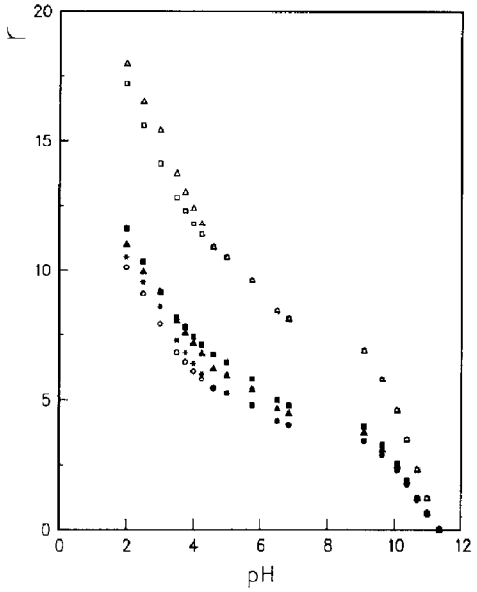

Fig. 4. Ion binding by lysozyme in 0.1 and 1 M KCl solution, respectively. Proton binding data from experimental titration curves of Tanford and Roxby [27], interpolated and digitised every 0.2–0.5 proton binding unit. Proton binding number interpreted as acid/base binding number for thermodynamic calculations. Symbols (0.1 M, 1 M): Acid/base (□, △), Cl^- (Esin–Markov) (○, ∗), and Cl^- (Poisson–Boltzmann) (■, ▲). (EM) from Esin–Markov analysis (eq. 16); (PB) from solution of Poisson–Boltzmann equation for diffuse charges around prolate ellipsoid 4.5 × 3.0 × 3.0 nm. (eq. 18).

that the chloride binding is about half the proton binding; this is a consequence of the overlap of the $c_s = 0.1$ M and $c_s = 1$ M curves which makes δ_{ab} zero for pH larger than about 4.5, so that in eq. (16) the term with the acid/base binding number is dominant. It follows that because of the electroneutrality condition the other half of the proton binding must be compensated by expulsion of sodium (potassium) ions.

Experimental data on the binding of salt by lysozyme is not available. Tanford and Roxby [27] analysed the titration curves of Fig. 4 with the Tanford–Kirkwood theory, realising that the proton binding cannot be described by a mean field theory; today it is well known that the active site is negatively charged under physiological conditions, whereas the overall charge is positive [24]. Nevertheless, Kuramitsu and Hamaguchi [40] reanalysed the Tanford and Roxby curves with the Linderstrøm–Lang model because "the use of the Tanford–Kirkwood theory has no clear advantage over the use of the simple empirical formula of Linderstrøm–Lang... while the latter well explains the ionization behaviour of... the catalytic groups in hen lysozyme". Neither Kuramitsu and Hamaguchi nor Tanford and Roxby did mention any evidence for site-bound chloride ion or potassium ion.

For comparison, we included in Fig. 4 chloride ion binding numbers from a simple model we devised ourselves, in which chloride ions are supposed to adsorb only in the diffuse part of the electrical double layer around a charged prolate ellipsoid ($4.5 \times 3.0 \times 3.0$ nm, the dimensions of native lysozyme [25]). In the calculation procedure use is made of a finite element method to solve the Poisson–Boltzmann equation, with the Stern potential as one of the boundary conditions. More details can be found in [17], where we performed a similar analysis of the ion binding by bovine serum albumin. For given Stern potential the calculations yield the total protein charge and the co-ion and counter-ion binding in the diffuse layer. The total protein charge is identified with the proton charge, so that by combination with the experimental proton titration curves (from Tanford and Roxby) a plot can be constructed of the counter-ion binding (= chloride ion binding) vs. the pH.

The single assumption is that the Helmholtz plane—the Stern layer surface—of the protein can be modelled as an equipotential plane. This may be a bad approximation for the titration of the surface groups because of the spatial distribution of surface potentials (note, however, the remark of Kuramitsu and Hamaguchi cited above), but it may be more accurate for modelling the diffuse atmosphere because of the short-circuiting of potential fluctuations over a Debye length scale (0.3–1 nm in the present case) in the diffuse part of the electrical double layer.

We find that the agreement between model and phenomenological calculation is fairly good (Fig. 4). This is a further indication that the neglect of specific salt binding is correct.

The model offers a simple explanation for the finding that the chloride binding is about half that of the proton binding. The binding number r_x of counter-ion ($x = Cl^-$) and co-ion ($x = Na^+$) in the diffuse atmosphere is calculated from:

$$r_x = c_s \int_V (e^{-z(x)F\psi/RT} - 1)\, dv \qquad (18)$$

where z is the valency, ψ is the electrostatic potential and dv the volume element of integration over the space V around the prolate ellipsoid. Expansion of the integrand, retaining only the linear terms (the Debye–Hückel approximation), gives $-z(x)F\psi/RT$ so that the integral becomes linear in z and hence $r_{Cl^-} \cong -r_{Na^+}$. By consequence of the electroneutrality condition this implies that the proton binding is equally compensated by the accumulation of chloride ion and the expulsion of sodium ion. Apparently, the Stern potential is for pH > 4.5, where $\delta_{ab} = 0$, smaller than about 25 mV. We have no direct experimental evidence to substantiate this. Norde [7] found for a related small basic protein, ribonuclease, zeta-potential variation from + 20 mV (pH ≅ 4) to 0 mV (pH = 9 ≅ i.e.p.) in veronal–acetate–KNO_3 buffer, ionic strength 0.05 M. In media of higher ionic strength these values will even be smaller.

Close inspection of the chloride ion binding curves shows that for pH < 4.5 the phenomenological binding number in 1 M is larger than in 0.1 M, whereas the Poisson–Boltzmann calculation predicts the opposite. The reason for this

small discrepancy is perhaps more related to inaccuracies in the procedure of calculation rather than intrinsic inadequacies of the theory. Namely, the calculation of the phenomenological ion binding numbers may be in error because of the first order approximation of δ_{ab}, thereby neglecting second-order effects due to the difference in saltstrength. It is possible that the binding numbers from the Poisson–Boltzmann calculation are more accurate. The model ion binding numbers are calculated using the accurate experimental proton binding numbers as input for the protein charge. This introduces two opposing effects: (i) when the experimental proton binding number is higher, the theoretical chloride binding is forced to be higher by virtue of the experimentally fixed boundary condition and (ii) given the protein charge, in 1 M the expulsion of co-ion is higher than in 0.1 M (maximum expulsion scales as $\sim A \cdot D \cdot c_s \sim A(c_s)^{1/2}$ where A is surface area of protein and D the Debye length) so that, because of the electroneutrality condition, the accumulation of counter-ion is smaller in 1 M than in 0.1 M. Apparently, in the present case effect (ii) wins, although near pH 4 (i) and (ii) are of equal importance and balance.

Discussion

Comparison of ion binding in solution and ion co-adsorption

The results demonstrate that it is possible to analyse ion binding in solution and ion co-adsorption with interfacial thermodynamics. Obviously, the method is purely phenomenological and thus does not offer mechanistic explanations. This is especially unfortunate for the adsorbed state for which there are no quantitative models available [39]. However, a few qualitative interpretations of the ion co-adsorption can be made:

(i) We noticed that the acid/base co-adsorption is positive. The reason may be that the negative cation exchanger surface, containing acid groups that are strong in aqueous media become weaker, and/or they may induce extra proton binding by the acid/base groups of the protein. In a simple mean field model [8] the shift in degree of titration $\Delta\alpha$ would be $\Delta\alpha \cong 2.3\alpha(1-\alpha)\,\Delta\mathrm{pK}$ (a_s and pH const.) where $\Delta\alpha$ is the shift in degree of titration α and ΔpK the shift in effective pK. The model implies that $\Delta\alpha$ will be largest when $\alpha = 0.5$, this is when the pH is equal to the pK in solution, or, in other words, when the acid/base groups buffer maximally. Lysozyme contains about 10 acid groups which can be titrated in the region pH 2–5 [39], whereas only one acid and one base is titrated between pH 5–9. We find that the increase in the acid/base co-adsorption number with lower pH is indeed concomitant with the rise in proton buffering capacity of the protein, which corroborates the above interpretation.

(ii) The negative co-adsorptions of the sodium and chloride ions can be understood as a compound displacement effect. Basically, sodium ion is displaced as counter ion from the ion exchanger surface and chloride ion is displaced as counter ion from the ion atmosphere around the protein. In addition there is displacement of the co-ions. We have demonstrated that the expulsion of sodium ion from the protein ion atmosphere in solution is considerable: approximately half of the proton charge is balanced by a deficit of sodium ion, this is 5–7 ions in the region pH 3–6 (Fig. 4). In the adsorbed state this deficit is probably smaller because a part of the protein ion atmosphere will be replaced by counter charges in the layer of close contact between protein and surface. The decrease in deficit, something one might term *negative* displacement, adds a positive contribution to the net co-adsorption number.

General remarks: comparison with other methods

Mass balance equation

As far as we know, this is the first phenomenological analysis of these types of systems where the individual ion co-adsorption numbers are identified. An alternative approach would be to use the mass balance equations of the adsorption (partition) equilibrium. The method, especially popular in the literature on ion exchange chromatography [18–20], originates from an old study by Boardman and Partridge [20] of the elution behaviour of cytochrome c and haemoglobulins on an Am-

berlite resin column. Typically, an adsorption equation is devised in which co-adsorbing ions participate as reactants. A study of the shift of the equilibrium upon a change in concentration of one of the reactants then reveals the co-adsorption. A specific example of such a reaction (with slight modification in notation taken from Ref. [18]) is that of a positive protein P^{Z+}, carrying Z chloride ions as the counter-ions, adsorbing on a surface (protein adsorbing-)site containing Z negative surface groups S^- each with a bound sodium ion, thereby liberating the Z chloride ions and the Z sodium ions:

$$\left[P^{Z+}\cdot Cl_Z^-\right]^{\alpha} + Z[S^-\cdot Na^+]^{\sigma}$$

$$\rightleftharpoons \left[P^{Z+}\cdot S_Z^-\right]^{\sigma} + Z[Na^+]^{\alpha} + Z[Cl^-]^{\alpha}$$

where α is solution and σ surface. Further analysis, leading to a Langmuir type protein adsorption isotherm, yields for the capacity factor R of the column ($\sim$ Henry coefficient of adsorption) a power law of the type $R \sim c^{-2Z}$, where c is the salt concentration (note that the linkage relation in this case would be $Z = -0.5\ \partial\ \log(R)/\partial\ \log(c)$). In our terminology, the thermodynamic equivalent is $\Delta r_{Na^+} + \Delta r_{Cl^-} = \partial\ \log(R)/\partial\ \log(a_s)$ (combine eqs. 15b and 15c), so that apart from the trivial correction for the activity coefficient, $Z = -0.5(\Delta r_{Na^+} + \Delta r_{Cl^-})$. Inspection of the mass balance equation immediately reveals that the redistributions of the co-ions are completely neglected. This may lead to considerable errors. For example, we have demonstrated that the shift in expulsion of sodium ion from the protein ion atmosphere may be significant. In principle, this applies also to the redistribution of chloride ion as co-ion of the ion exchanger surface, but in [39] we give evidence that this is probably a minor effect.

A second approximation is that the acid/base co-adsorption number is assumed zero. It follows that $Z = -\Delta r_{Na^+} = -\Delta r_{Cl^-}$ because of electroneutrality. A popular interpretation of Z from the older work, "(Z is) the number of sodium ions displaced when one poly-ion is adsorbed" [20], seems to refer to this special case, where it is tacitly assumed that displacement is from the ion exchanger surface only.

The method of Record et al. [12]

Record et al. also discussed a theoretical analysis of charge adaptation, in terms of the binding polynomial analysis of Wyman [13,14] (see also the lucid reviews of Schellman [15] and Weber [16]). In essence, Record et al. formulate grand canonical partition functions of the interacting species (macromolecule A, B and complex AB), assuming the presence of distinct binding sites. These binding sites are thought to release or take up extra ions upon complexation of A with B to form AB. The linkage relations are of the type $\Delta n_L = -\partial\ \mathrm{Log}(K)/\partial\ \log(a_L)$ (eq. 2.20 from [12]), where $\Delta n_L \equiv n_{L,A} + n_{L,B} - n_{L,AB}$ ($n_{L,X}$ molecules L bound to macromolecule X), K is the complexation constant and a_L the activity of the ligand.

In principle, Record et al.'s statistical method must yield the same relations as the classical thermodynamic method we use. The classical approach has the advantage that the ion binding and co-adsorption are treated purely phenomenologically. For example, it is not immediately clear how the redistribution of the diffuse charges—which we have shown to be of importance—can be incorporated in the formalism of Wyman's Binding Polynomial. Recently [17] we have shown for a special case, namely ion adsorption on a flat rigid surface, how this should be done in the study of ion binding in solution.

The integral linkage relation eq. (6) is, as far as we know, new. In this paper we used it only in the limit of low adsorption. However, an interesting feature is that it is also applicable to situations where the surface coverage is high. For these systems the statistical method is not very well suited because additional complex modelling assumptions have to be made regarding the protein-protein interactions. In a following publication we will come back to these considerations.

Conclusion

We have shown that by classical thermodynamic reasoning the ion co-adsorption in protein ion-exchange chromatography and ion binding in solution can be analysed phenomenologically. No

assumption shave to be made regarding the mechanism of ion binding, ion co-adsorption or adsorption mechanism of the protein. The results indicate that ion binding in solution can be explained by binding in the diffuse ion atmosphere. A quantitative model for the ion co-adsorptions is presented in Part II of this paper [39].

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Appendix A

Phenomenological integral linkage relations

Titration in solution

We briefly recall here our previous analysis of ion binding in solution [17]. The differential for the protein chemical potential is found from combination of the two Gibbs–Duhem relations of the two solution phases (Fig. 1a). The result is (eq. (7a) from (17) slightly rewritten and simplified to isothermic and isobaric conditions):

$$\mathrm{d}\mu_\mathrm{p} = -\sum_{i\neq \mathrm{w,p}} \Delta \mathrm{r}_i^{(\mathrm{w})\alpha}\, \mathrm{d}\mu_i + \frac{1}{c_\mathrm{p}}\mathrm{d}\pi \qquad \text{(A1)}$$

where π is the osmotic pressure. It is assumed that the protein solution has the external pressure P and that the excess volume $\Delta V^{(\mathrm{w})}$ is much smaller than V^L (or $c_\mathrm{w}^\mathrm{L} \cong c_\mathrm{w}^\mathrm{R}$). No other assumptions are made.

The differential of the reference-linkage potential $\Lambda^\mathrm{r} \equiv -\mu_\mathrm{p} + \pi/c_\mathrm{p}$ is:

$$\mathrm{d}\Lambda^\mathrm{r} = \sum_{i\neq \mathrm{w,p}} r_i^{(\mathrm{w})\alpha}\, \mathrm{d}\mu_i - \frac{\pi}{c_\mathrm{p}}\mathrm{d}\ln(c_\mathrm{p}) \qquad \text{(A2)}$$

Cross-differentiation of the binding numbers (c_p const.) and subsequent integration over one of the chemical potentials gives:

$$r_i^{(\mathrm{w})\alpha}(\mathrm{II}) = r_i^{(\mathrm{w})\alpha}(\mathrm{I}) + \int_\mathrm{I}^\mathrm{II} \left(\frac{\partial r_j^{(\mathrm{w})\alpha}}{\partial \mu_i}\right)_{\mu_{j\neq i}, c_\mathrm{p}} \mathrm{d}\mu_j \qquad \text{(A3)}$$

which is eq. (2).

Co-adsorption

Consider the aqueous protein solution L of volume V^L in equilibrium with a surface of area A^L (Fig. 1b). The surface Gibbs–Duhem relation is:

$$A^\mathrm{L}\,\mathrm{d}\gamma^\mathrm{L} = -\sum_{i\neq \mathrm{w,p}} N_i^{\sigma\mathrm{L}}\,\mathrm{d}\mu_i - N_\mathrm{w}^{\sigma\mathrm{L}}\,\mathrm{d}\mu_\mathrm{w} - N_\mathrm{p}^{\sigma\mathrm{L}}\,\mathrm{d}\mu_\mathrm{p} \qquad \text{(A4)}$$

where γ the is surface tension; N_i^L is total number of moles of i in L, the superscript $^\sigma$ denotes the surface phase, $^\alpha$ the liquid phase. The balance reads $N_i^\mathrm{L} = N_i^{\alpha\mathrm{L}} + N_i^{\sigma\mathrm{L}}$.

In addition to the Gibbs–Duhem relation for the surface phase there is the corresponding Gibbs–Duhem relation for the liquid phase:

$$0 = -\sum_{i\neq \mathrm{w,p}} N_i^{\alpha\mathrm{L}}\,\mathrm{d}\mu_i - N_\mathrm{w}^{\alpha\mathrm{L}}\,\mathrm{d}\mu_\mathrm{w} - N_\mathrm{p}^{\alpha\mathrm{L}}\,\mathrm{d}\mu_\mathrm{p} \qquad \text{(A5)}$$

Combination of eqs. (A4) and (A5) gives the Gibbs adsorption equation. We prefer here the elimination of the water chemical potential, so that the adsorptions ($\Gamma \equiv N^\sigma/A$) are given in excess with respect to water:

$$\mathrm{d}\gamma^\mathrm{L} = -\sum_{i\neq \mathrm{w,p}} \Gamma_i^{(\mathrm{w})\mathrm{L}}\,\mathrm{d}\mu_i - \Gamma_\mathrm{p}^{(\mathrm{w})\mathrm{L}}\,\mathrm{d}\mu_\mathrm{p} \qquad \text{(A6)}$$

So far the relations are classical. Now, consider the problem of co-adsorption. We realise that the adsorptions of all species may be coupled. For example, suppose the protein is positively charged, then it is likely that the adsorption of acid on the surface is inhibited, possibly through some electrostatic interaction. Also, the strength of this electrostatic interaction will depend on the salt adsorption through screening. How are we now going to define co-adsorption?. One approach would be to make the *a priori* assumption that the excess adsorption of any of the species i refers to adsorption on the surface itself. In this picture, the species i and the protein are for example thought to compete for surface binding sites. However, it is likely that the adsorbing protein itself also adjusts its charge so that a part of the excess adsorption of i will refer to this species as it is bound to adsorbed protein. In other words, by using the *a priori* assumption of adsorption on the surface

only, we could make a substantial error by neglecting the adsorption of *i on* adsorbed protein. It follows that we should really treat the system as a black box and consider only the phenomenological effects, without a possibly too limited assumption regarding the mechanism of binding.

If we then approach the problem purely phenomenologically we must find some kind of thermodynamic reference which allows us to separate the total excess adsorption of any species i in a part which is not affected by the adsorbed protein and a part which is. By saying so, we have already identified a natural reference for the case where the protein adsorption is low, namely the bare surface 'far' in between adsorbed protein molecules, so that in this 'bulk' of the surface phase locally the adsorptions are not affected by the protein adsorption.

However, cases of low coverage with protein do not occur very often. It is therefore better to introduce the external reference with which the current two-phase system is in membrane equilibrium (Fig. 1b). As before, we choose the membrane such that it acts as an ideal separator of the protein, we add here the property that it extends over both phases.

By virtue of the equilibrium condition, we may couple the two Gibbs–Duhem relations in the protein solution-surface system to the two additional Gibbs–Duhem relations in the reference (these are similar to eqs. (A4–A6) except that protein is not included and ^{L} is replaced by ^{R}). The coupling leads to a differential for the surface pressure π^{s}, defined as $\pi^{s} \equiv \gamma^{R} - \gamma^{L}$:

$$\mathrm{d}\pi^{s} = \sum_{i \neq w,p} \Gamma_{p}^{(w)} \, \Delta r_{i}^{(w)} \, \mathrm{d}\mu_{i} + \frac{\Gamma_{p}^{(w)}}{c_{p}} \mathrm{d}\pi \qquad \text{(A7)}$$

Cross-differentiation and integration over π yields the integral linkage relation between the protein adsorption, the protein concentration and the co-adsorption:

$$\Delta r_{i}^{(w)} = \frac{1}{\Gamma_{p}^{(w)}} \int_{0}^{\pi} \left(\frac{\partial \Gamma_{p}^{(w)} / c_{p}}{\partial \mu_{i}} \right)_{\pi, \mu_{j \neq i}} \mathrm{d}\pi \qquad \text{(A8)}$$

where it is assumed that the total co-adsorption $\Delta r_{i}^{(w)} \cdot \Gamma_{p}^{(w)}$ is zero in the limit of zero osmotic pressure. Eq. (A8) is the desired eq. (6).

Appendix B

Linkage relations for limiting ion co-adsorption numbers

The differential of $\ln(\eta)$ expressed in terms of the mean chemical potentials of NaCl, HCl and NaOH is:

$$RT \, \mathrm{d} \ln(\eta) = \Delta r_{HCl} \, \mathrm{d}\mu_{HCl} + \Delta r_{NaOH} \, \mathrm{d}\mu_{NaOH} + \Delta r_{NaCl} \, \mathrm{d}\mu_{NaCl} \qquad \text{(B1)}$$

The chemical potentials are related via the electrolyte dissociation equilibrium, in good approximation $\mathrm{d}\mu_{H_2O} = 0$ for not too concentrated systems [17] so that $\mathrm{d}\mu_{HCl} + \mathrm{d}\mu_{NaOH} = \mathrm{d}\mu_{NaCl}$. In combination with the expressions $\mu_{NaCl} = \mu^{0}_{NaCl} + 2RT \ln(a_s)$, where a_s is the activity of the salt ($a_s = (a_+ a_-)^{1/2}$) and $\mu_{HCl} = \mu^{0}_{HCl} + RT \ln(a_s) + RT \ln(a_{H^+})$, where a_{H^+} is the proton activity this gives in $^{10}\log$, $\mathrm{pH} \equiv -\log(a_{H^+})$):

$$\mathrm{d} \log(\eta) = -\Delta r_{ab} \, \mathrm{d}\,\mathrm{pH} + (\Delta r_{Na^+} + \Delta r_{Cl^-}) \, \mathrm{d} \log(a_s) \qquad \text{(B2)}$$

For the partial derivatives (eqs. 14) we find:

$$\phi = -\Delta r_{ab} \qquad \text{(B3a)}$$

$$\epsilon = \Delta r_{Na^+} + \Delta r_{Cl^-} \qquad \text{(B3b)}$$

Combination with the electroneutrality condition $\Delta r_{ab} + \Delta r_{Na^+} = \Delta r_{Cl^-}$ gives (also using $\rho \equiv -\epsilon/\phi$):

$$\Delta r_{ab} = -\phi \qquad \text{(B4a)}$$

$$= \epsilon/\rho \qquad \text{(B4a')}$$

$$\Delta r_{Na^+} = 0.5(\epsilon + \phi) \qquad \text{(B4b)}$$

$$= 0.5\left(1 - \frac{1}{\rho}\right)\epsilon \qquad \text{(B4b')}$$

$$\Delta r_{Cl^-} = 0.5(\epsilon - \phi) \qquad \text{(B4c)}$$

$$= 0.5\left(1 + \frac{1}{\rho}\right)\epsilon \qquad \text{(B4c')}$$

which are the desired eqs. (15a–c).

Appendix C

Esin–Markov analysis of ion binding in solution

The differential of the reference-linkage potential for constant protein concentration is (eq. 20c in Ref. [17]):

$$(\mathrm{d}\Lambda^{\mathrm{r}})_{c_p}/RT = r^{\alpha}_{\mathrm{ab}}\,\mathrm{d}\ln(a_{\mathrm{H}^+}) + (2r^{\alpha}_{\mathrm{Cl}^-} - r^{\alpha}_{\mathrm{ab}})\,\mathrm{d}\ln(a_{\mathrm{s}}) \qquad \text{(C1)}$$

Cross-differentiation yields (in $^{10}\log$, $\mathrm{pH} = -\log(a_{\mathrm{H}^+})$):

$$\left(\frac{\partial r^{\alpha}_{\mathrm{Cl}^-}}{\partial \mathrm{pH}}\right)_{a_{\mathrm{s}}} = 0.5\left[-\left(\frac{\partial r^{\alpha}_{\mathrm{ab}}}{\partial \log(a_{\mathrm{s}})}\right)_{\mathrm{pH}} + \left(\frac{\partial r^{\alpha}_{\mathrm{ab}}}{\partial \mathrm{pH}}\right)_{a_{\mathrm{s}}}\right] \qquad \text{(C2)}$$

Integration from pI (where $r^{\alpha}_{\mathrm{ab}} \equiv 0$) to pH gives:

$$r^{\alpha}_{\mathrm{Cl}^-}(\mathrm{pH}) = r^{\alpha}_{\mathrm{Cl}^-}(\mathrm{p}I) + 0.5\left\{r^{\alpha}_{\mathrm{ab}} - \int_{\mathrm{p}I}^{\mathrm{pH}} \delta_{\mathrm{ab}}\,\mathrm{d\,pH}\right\} \qquad \text{(C3)}$$

which is the desired eq. (16).

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