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

II. Model interpretation of ion exchange in lysozyme chromatography

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In this paper we present a model for the ion exchange effects in protein adsorption. The model is applied to chromatography of lysozyme on strong cation exchanger 'mono S'. The experimental and general thermodynamic aspects have been discussed in Part 1, the preceding paper. The main modelling assumptions are (i) the charge regulation is confined to the small layer of contact between adsorbed protein and exchanger surface, (ii) the contact layer as a whole is electroneutral and (iii) the number of protein acid/base groups and exchanger surface acid groups which participate in the ion exchange is proportional to the area of the contact layer. The model is fitted to the experimental data by adjustment of only two or three parameters. The experimental co-adsorption numbers are very well reproduced. A few conspicuous features emerge: (i) the number of protein acid/base groups and exchanger surface acid groups in the contact layer varies with the medium conditions, such that the number is higher when the interaction between protein and exchanger surface is stronger. (ii) There is indirect evidence for structural alterations in the upper layers of the exchanger surface: the adsorbed protein is probably partly 'buried' in the surface.

Introduction

In this paper we present a quantitative model interpretation of ion exchange chromatography of proteins, applied to chromatography of lysozyme on cation exchanger Mono S. For the experimental and general thermodynamic considerations we refer to the accompanying paper [1]. The presented model is in principle capable of describing protein ion exchange chromatography with only a few parameters. As far as we know, the model is the first of its kind. However, models have already been presented for a few related processes. Recent polyelectrolyte adsorption theories [2–6], taking into account the full conformation statistics, have demonstrated the importance of coupling between pK and the local electrostatic potential. Modern computing techniques allow the calculation of electrostatic potentials around two interacting protein molecules, with almost full specification of the detailed molecular structure. Recent results point to the importance of the relative orientation of the charged groups [11]. Models for polymer solubilisation [8–10] in reverse micelles have shown that the interfacial layer of contact between protein and micelle is electroneutral. It is this constraint of electroneutrality which makes protein solubilisation highly dependent on pH and elec-

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trolyte concentration: when before encapsulation protein and micelle do not match in charge, ions have to be redistributed in order to make the protein–micelle complex neutral. A similar redistribution may be expected in protein ion exchange chromatography.

Our model for ion exchange in protein adsorption is an extension of the three-layer model of Norde and Lyklema (see review [7] and references therein). In this model, one layer is the solution side of the surface–protein complex, one layer contains the protein molecules, and the last layer is the layer of close 'atomic' contact between surface and protein. Experimental and theoretical analyses show that when the Debye length is smaller than the dimensions of the protein molecule, the solution layer titrates just as native protein in solution. Under these circumstances the protein layer shields the solution side completely from the surface and, reciprocally, the protein layer shields the contact layer completely from the solution. This implies that the contact layer is electroneutral. In bioelectrochemistry use is made of this effect by equating the relative decrease of the electric capacitance of the surface–protein complex to the degree of coverage of the surface with protein [12].

We assume that a similar type of shielding also applies to the isolated adsorbed lysozyme molecule (Fig. 1) on Mono S ion exchanger surface under the medium conditions reported in Part 1 of this article [1]: the size of the protein molecule is $4.5 \times 3.0 \times 3.0$ nm [16], whereas the Debye length is 0.3–0.6 nm (0.3–1 M NaCl).

The rationale behind the calculations is the following. From interfacial thermodynamics we

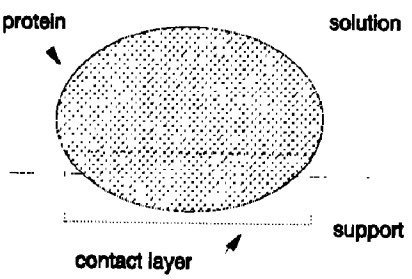

Fig. 1. Schematic view of side-on adsorbed lysozyme molecule. The contact layer is indicated.

deduced that the co-adsorption number of species i, Δr_i, is related to the differential of the Henry coefficient of adsorption η (~ capacity factor R) through the fundamental ion exchange linkage relation:

$$\Delta r_i = \left(\frac{\partial \ln(\eta)}{\partial \ln(a_i)} \right)_{a_{j \neq i}} \tag{1}$$

where a_i is the activity of i. In the preceding paper eq. (1) was used to calculate phenomenological co-adsorption numbers of acid/base, sodium ion and chloride ion from the experimental retention map. In the present paper we propose model expressions for the co-adsorption numbers, which are then fitted. The parameters are: a set of pK values for the proton titration in the contact layer, and a chloride expulsion parameter for the bare exchanger surface.

A prerequisite for the application of our model is that the adsorption is in equilibrium and in the Henry regime. This is not very common in protein adsorption in general. In case of adsorption on colloidal particles, proteins tend to adsorb irreversibly, especially when the proteins are labile and the surface hydrophobic [7]. This would seem to limit the applicability of the theory. However, modern separation techniques can be devised to optimise complete recovery of protein, with the native structure intact. For protein chromatography, this implies in many cases that the sorbent–protein interactions are chosen to be fairly weak (or can be made to be weak by adjustment of medium conditions) and that the adsorption is reversible. For the reported 2D retention map of lysozyme on Mono S [1] we have some, albeit indirect, experimental evidence that in this case the adsorption is indeed reversible and, moreover, in the Henry regime.

Theory

Ion exchange reactions in the contact layer

In our model we consider four types of ion exchange reactions. The reactions are chosen such that (i) apart from the electroneutrality of the

contact layer as a whole, also locally any net charge is directly compensated by the appropriate counter-ion, (ii) it is the irreducible set by which one could construct all the physically realistic reactions by recombination.

The first ion exchange reaction is that due to the interaction of the base groups (B) of the protein with the acid groups (S) on the exchanger surface. An example is:

$$[B\cdots H^+\cdots S^-]^c + [Na^+]^\alpha \rightleftharpoons [B\cdots Na^+\cdots S^-]^c + [H^+]^\alpha \quad (2)$$

The dotted line denotes to some kind of complex, e.g. saltbridge, 'α' is solution phase, 'c' is contact layer..

The second ion exchange reaction is that due to the compensation of titrated base by incorporated chloride ion:

$$[BHCl]^c \rightleftharpoons [B]^c + [H^+]^\alpha + [Cl^-]^\alpha \quad (3)$$

The third type of ion exchange reaction is that due to the neutralisation of the protein acid groups (A) by protons or sodium ions:

$$[AH]^c + [Na^+]^\alpha \rightleftharpoons [ANa]^c + [H^+]^\alpha \quad (4)$$

In this case the negative acid is compensated by either a proton or a sodium ion.

The fourth ion exchange equilibrium is that due to the titration, if any, of the exchanger surface acid groups in the contact layer. Some of these groups will already be compensated by the base groups of the protein (see above). The remainder must either be compensated by a proton or a sodium ion:

$$[SNa]^c + [H^+]^\alpha \rightleftharpoons [SH]^c + [Na^+]^\alpha \quad (5)$$

Binding numbers in the contact layer

The acid/base binding number in the contact layer r^c_{ab} is defined as the difference between the number of acid/base groups titrated in the contact layer and the number titrated on the bare ion exchanger surface. (For precise formulas see the Appendix to this paper). Combination of equilibria (2)–(5) gives for the acid/base binding number in the contact layer:

$$r^c_{ab} = \sum_{\text{bases}} N^c_b \alpha^c_b + \sum_{\text{acids}} N^c_a (\alpha^c_a - 1) + M^c (\alpha^c_s - \alpha^R_s) \quad (6)$$

where N^c_x is the number of protein acid/base groups of type x (b = base, a = acid) which are in the contact layer, α^c_x is the degree of titration, M^c the number of exchanger surface acids in the contact layer; α^c_s and α^R_s the degree of titration of the exchange surface acid in the contact layer (c) and the bare exchange surface (R) respectively. The difference in the last term in eq. (6) is just the excess in titration of the exchanger surface acids in the contact layer over that on the bare exchanger surface.

Similarly, the binding number of chloride ions in the contact layer is the difference between the number of ions *in* the contact layer and the number of ions bound by the bare exchanger:

$$r^c_{Cl^-} = \sum_{\text{bases}} N^c_b \gamma^c_b - M^c \beta \quad (7)$$

where γ^c_b is the degree of binding of a chloride ion to a base, β is the degree of binding of a chloride ion to a surface acid on the bare ion exchanger surface. The degree of binding β will be a small negative number because chloride ion, as co-ion, is expelled from the electrical double layer of the bare ion exchanger surface.

Application to lysozyme adsorption on Mono S

In the specific case of lysozyme adsorption on mono S, of which the experimental results were reported in Part 1 of this paper [1], the model can be simplified somewhat in view of the limited range in pH (3.5–6.5) and salt concentration (0.3–1.0 M), so that the number of parameters can be reduced:

(i) We assume that the protein acid/base groups are distributed uniformly over the Lysozyme surface; the crystal structure [16] shows that this is a reasonable first approximation. Only a

fraction (f) of the groups is thought to be in the contact layer and hence under the influence of the exchanger surface. The remaining fraction ($1-f$) is thought to titrate as in solution. No assumptions are made regarding the dependency of f on the structural stability of the protein and the molecular structure of the exchanger surface. In fact, the main objective is the dependency of f on medium conditions, which will allow us, albeit indirectly, to draw some conclusions about the structural details. According to the definition of the fraction f, the number of acid/base groups in the contact layer N_x^c is simply the fraction f of the total number of acid/base groups N_x of the protein:

$$f \equiv N_x^c / N_x \tag{8}$$

where x = a or b.

(ii) Equilibrium [2] will probably lie far to the left because of the high pK's of the base groups. We assume therefore that all the bases in the contact layer are titrated, so that $\alpha_b^c = 1$.

(iii) In the previous paper we gave evidence that chloride ion does not interact specifically with lysozyme in solution, i.e. complexes of the kind depicted in eq. (3) do not occur in the solution phase. We assume here that this applies also to the contact layer, so that $\gamma_b^c = 0$. In other words, combination of (ii) and (iii) implies that we assume that every base in the contact layer is fully protonated, and that it is locally compensated by a deprotonated ion exchanger acid.

(iv) The effective equilibrium pK of reaction (4) is a difference between the pK_{H^+} of the proton/acid dissociation and the pK_{Na^+} of the sodium/acid complex: $pK \cong pK_{H^+} - pK_{Na^+}$. In principle the individual pK's could be corrected for the electrostatic potentials, according to $pK_x = pK_{\text{intrinsic},x} - 2.303\ F\psi_x/RT$, where ψ_x is the potential experienced by ion x (H^+ or Na^+) on the acid. It seems reasonable to suppose that the potential experienced by a sodium ion attached to the acid is roughly the same as that experienced by the proton on the acid; a consequence of the local charge compensation mechanism. This means that the difference of potentials in pK vanishes, so that we are left with the difference of the intrinsic pK's as the leading term. From mass balance we find:

$$\alpha_a^c = \frac{1}{[1 + 10^{\{-pK + pH + \log(a_s)\}}]} \tag{9}$$

where pK is a constant, independent of the local electrostatic potential.

(v) Considering the molecular structure of the exchanger surface acid, $-CH_2-SO_3^-$, the pK is probably < 2 [19]. This means that the acid groups on the bare exchanger surface must be completely titrated in the pH range 3.5–6.5, especially since a_s is high, hence $\alpha_s^R = 0$. We assume that also in the contact layer equilibrium (5) is completely to the left, so that $\alpha_s^c = 0$ and hence the last term in eq. (6) is zero: the excess titration of exchanger surface acids is neglected. This means that we assume that every surface acid in the contact layer which is not compensated by protein base, is compensated by an incorporated sodium ion.

(vi) We assume that the number of exchanger surface acids M^c in the contact layer is the area of the layer a_c times the (fixed) surface density of the acids σ_0: $M^c = a_c \cdot \sigma_0$. We assume furthermore that area a_c depends linearly on the fraction f of protein acid/base groups in the contact layer and the native protein surface area a_p, by a constant geometrical factor Ω: $a_c = a_p \cdot \Omega \cdot f$. For simple structures Ω could perhaps be calculated from geometry, e.g. suppose that the lower side of an adsorbed sphere with radius R is in the contact layer, then $f \cong 0.5$, $a_c \cong \pi R^2$ (the projection area of the sphere on the surface) and, because $a_p = 4\pi R^2$, Ω is about 0.5. Here, we do not need to impose a value for Ω.

(vii) On the bare surface the surface charge compensation will be partly due to sodium ion accumulation and partly to chloride expulsion from the surface ion atmosphere. In principle, we could estimate these contributions from an electrical double layer model, e.g. that of Gouy–Chapman–Stern. Such a model would introduce additional parameters, e.g. binding constants, Stern capacitances etc. Here, we assume simply that the surface charge density is so high that the chloride ion expulsion is very much smaller than the sodium ion accumulation. In other words, we assume that

the chloride ion expulsion per surface acid β is very small, so that $-\beta \ll 1$. Given this small value, and the relatively small variation in salt concentration we assume here that β is a constant. Combination of (vi) and (vii) gives for βM^c in eq. (7) $\beta M^c = C \cdot f$, where C, the expulsion parameter, is defined as:

$$C \equiv \beta \cdot a_p \cdot \Omega \cdot \sigma_0 \qquad (10)$$

which is a constant, to be determined by the fitting procedure.

(viii) The binding numbers is solution r_x^α are obtained from Esin–Markov analysis of Tanford's and Roxby's [14] experimental titration curves. This has been done in the previous article for 0.1 and 1 M KCl solutions [1]. The binding numbers in solutions of intermediate salt concentration are obtained from first order difference:

$$r_x^\alpha(\mathrm{pH}, a_s) \cong r_x^\alpha(\mathrm{pH}, a_s(0.1\ M)) + \delta_x \cdot \left[\log(a_s) - \log(a_s(0.1\ M))\right] \qquad (11)$$

where x is 'ab' or Cl^-, and δ_x is an Esin–Markov coefficient:

$$\delta_x \equiv \left(\frac{\partial r_x^\alpha}{\partial \log(a_s)}\right)_{\mathrm{pH}} \cong \frac{r_x^\alpha(1\ M) - r_x^\alpha(0.1\ M)}{\log(a_s(1\ M)) - \log(a_s(0.1\ M))} \qquad (12)$$

Results

Lysozyme appears to have an amount of acids, that may vary, depending on the isolation procedure. Tanford and Roxby reported that in their sample the protein contained seven aspartic acid residues (Asp), which is one less than calculated from the analytical composition. The Asp groups (Table 1) have a pK between 3–5. From the two glutamic acid (Glu) groups one has an anomalous pK of 6.4, whilst the other has a pK of 3.9. The α-COOH group has a pK of 4.3. In view of the limited pH range (3.5–6.5 experimentally, 3.75–5.75 in the fitting calculations, see Appendix), we conclude that three classes of acids could be involved in extra titration in the contact layer: the seven Asp groups, the one Glu group with the normal low pK and the single α-COOH group. The anomalous Glu group is assumed to be completely titrated, so that α_a^c (anomalous Glu) = 1 (fixed).

There is a minor problem regarding the choice of the number of acid groups. If our protein sample would contain the normal number of Asp, namely eight groups rather than the seven mentioned above, we would in principle make an error if we used Tanford's and Roxby's experimental titration curves. An *ad hoc* correction to the experimental proton titration curves seems undesirable, the more so because we do not know how many Asp groups our sample contained. However, the difference may be disregarded for arithmetic reasons. Suppose, for the sake of argument, that every acid would contribute equally to the net acid/base co-adsorption. The error in the model

Table 1

pK values of lysozyme in KCl solution

Residue	[a] pK_{int}	[b] pK_{app}
α-COOH	4.3	3.1
Glu 7	3.9	2.6
Glu 35	6.4	6.1
Asp 48	5.2	4.3
Asp 52	4.5	3.4
Asp 66	3.1	1.6
Asp 101	5.4	4.5
Asp 18	3.4	2.0
Asp 87	3.5	2.1
Asp 119	3.8	2.5
His 15	6.6	5.8
α-NH_2	8.5	7.9
Lys 1	10.9	10.8
Lys 13	10.7	10.5
Lys 33	10.7	10.6
Lys 96	10.9	10.8
Lys 97	10.5	10.3
Lys 116	10.6	10.4
Tyr 20	10.6	10.3
Tyr 23	10.2	9.8
Tyr 53	11.7	12.1

[a] and [b] from Linderstrøm–Lang analysis by Kuramitsu and Hamaguchi [15] of experimental titration curves of Tanford and Roxby [14]. Theoretical analysis for 0.1 M KCl, 25 °C.

[a] pK_{int} is intrinsic pK from Linderstrøm–Lang analysis.

[b] pK_{app} is pH level where titration of the group is half.

[c] Groups with pK_{int} = 3.4, 3.5 and 3.8 could not be assigned to specific residues Asp 18, 87 or 119 separately.

co-adsorption number by a possible mismatch of one in the number of Asp is then about $\Delta r_{ab} \cdot 1/$ (total number of acids) $\cong 0.1\ \Delta r_{ab}$. This is an error of 10%, which is equal to, or less than the experimental error.

Initially, we tested the model with the three acid classes mentioned above. We found that the calculation procedure (Appendix) could not distinguish between the pK's of the α-COOH and the single normal Glu, so that we then tried a reduced two-class model, by combining the two groups into one class. We found a pK of 3.6 for the seven Asp groups, a pK of 3.1 for the couple Glu/α-COOH and $C = -1.0$ (the expulsion parameter defined in eq. 10). This gave a fit with a mean deviation 0.72 of the fitting function. (Corresponding to 0.1–0.2 deviation between experimental and theoretical co-adsorption numbers.) Further reduction by combining all acids in one class gave also a good fit, a pK of 3.5 for the collective nine acids and also $C = -1.0$, with a mean deviation of 0.73. Apparently, the variation in pH (3.75 to 5.75) and salt activity ($\log(a_s)$ from -0.38 to -0.75) in the fitting range is too small, and the experimental accuracy is too low, to distinguish properly between the pK's of the various acid groups.

Figures 2 (a) and (b) show the experimental co-adsorption numbers in comparison with the theoretical values from the two-class model. The dependency on pH, for various values of the capacity factor R, is very well reproduced. (In order to avoid overcrowding in the figures we only plotted the co-adsorption numbers for $\log(R) = -0.21$ and 1.00. The fit for the intermediate R values was equally good). The general appearance of the co-adsorption figures was already discussed in Part 1 [1].

In Fig. 3 we have plotted the fraction f of protein acid/base groups in the contact layer versus the capacity factor for different values of pH. Three striking features emerge. First, f is always between 0 and 1, the mean value being about 0.6. This is of course what we expect because f is defined as the *fraction* of protein acid/base groups which is in the contact layer. Note, however, that the calculation procedure (Appendix) does not impose the fraction f to lie between 0 and 1: the fact that we do find this supports the theory. The second feature is that f is smaller when the capacity factor is smaller, in other words: the weaker the protein–surface interaction, the smaller the number of acid/base groups in contact with the surface. The third feature is that f is larger, the larger the pH is. We do not have an explanation for the last effect. To a certain extent, it may be an artifact in view of the

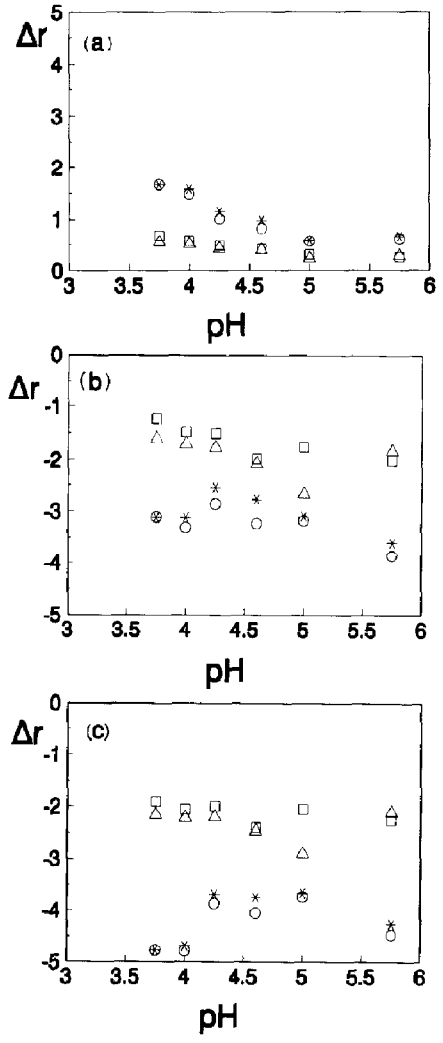

Fig. 2. Comparison of experimental (exp.) and model values (th.) of co-adsorption numbers. R is capacity factor (basis of logarithm is 10). Experimental values taken from [1]. Model with two acid classes. (a) acid/base, (b) chloride ion and (c) sodium ion. (□) exp. and (△) th. for $\log(R) = -0.21$, (○) exp. and (*) th. for $\log(R) = 1.00$.

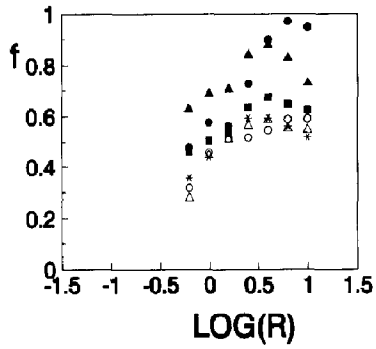

Fig. 3. Calculated values of fraction f of protein acid/base groups in the contact layer, for various values of pH and capacity factor R, model with two acid classes. pH: 3.75 (△), 4.00 (○), 4.25 (*), 4.60 (■), 5.00 (▲), and 5.75 (●).

smaller co-adsorption numbers for higher pH, which tends to rise the error in f.

Discussion

Evaluation of parameters

The small value of the expulsion parameter $C = -1.0$ is especially noteworthy. One of the expressions for the fraction f of protein/acid/base groups in the contact layer (eq. A11 of the Appendix) contains only C as a modelling parameter, namely in the denominator in the sum $(-C - r^{\alpha}_{Cl^-})$. The experimental values for the chloride binding in the solution phase, from the Esin–Markov analysis, are much larger than C: they range from about 5 to 7 (Fig. 4 in Part 1). This means that C determines the value of f for only about 10–20%. The implication is that in the present model the fraction f of protein acid/base groups in the contact layer has the character of a phenomenological variable, which hardly depends on the model parameters pK and C.

From the value for expulsion parameter C we can obtain a rough estimate of the degree of binding of chloride ion to a surface acid on the bare ion exchanger surface β. Equation (7) says that the total chloride ion expulsion on the bare ion exchanger surface, on an area of the contact layer size, is equal to βM^c. According to the assumptions (vi) and (vii) $\beta M^c = C \cdot f$. The average value of the fraction f of protein acid/base groups in the contact layer is about 0.6. This would imply that $-(f \cdot C) = 0.6$ chloride ions are expelled by the bare exchanger surface, on an area of size of the contact layer. According to the model interpretation, roughly 11 $(= f \cdot 19)$ protein base groups displace sodium ions from the exchanger surface acids. Because of the assumed local charge compensation of surface acids and protein bases (eq. 2) this implies that the total number of ion exchanger acids in the contact layer M^c is at least 11, so that $-\beta$ must be smaller than $(f \cdot C)/11 \cong 0.05$. This means that an acid on the bare ion exchanger surface is compensated by chloride ion expulsion by less than 5%, and by sodium ion accumulation by more than 95%. This is very different from what we calculated before for the ion atmosphere of lysozyme in solution [1], in which case co- and counter-ion compensate the protein charge about equally.

There is an independent rough check of the number of ion exchanger acids in the contact layer. The manufacturer of the column [19] states that the maximum load of protein is several tens of mg protein per ml ion exchange resin. The density of ion exchange acids is 0.13–0.18 mmol/ml resin, so that the maximum number of ion exchanger acids per protein molecule is several tens (with 50 mg load the number would be about 40). Our (lower bound) estimate of M^c falls within this range.

Comparison of the pK values in the contact layer with the pK's in solution (Table 1) shows that their shifts are small. A precise estimate of the magnitude of the shifts is not very well possible because we can only distinguish between maximally two classes of acids groups, whereas the Linderstrøm–Lang analysis can distinguish between almost every separate acid group. However, a shift of only a few tenths of a pK unit downward seems a prudent estimate.

We remarked already in the theory section that an acid pK in the contact layer must be regarded as the difference in pK's of the binding of protons and sodium ions. For example, suppose that the pK_{H^+} in the contact layer is the same as that in solution. By consequence, the shift in pK must then come from a small positive value of a few

tenths for pK_{Na^+}. Every pK unit corresponds to 2.3 units kT interaction energy/ion, so that the small pK_{Na^+} would correspond to a weak interaction. Other combinations of shifts of pK_{H^+} and pK_{Na^+} are of course also possible. It would be too speculative to decide which combination is the most likely one, further (experimental) information is clearly needed. For the moment it seems reasonable to suppose that pK_{Na^+} is not very positive, because there is no specific interaction of sodium ion with the protein in solution [1], and there is no *a priori* reason why this should be different in the contact layer.

The fraction f of the protein acid/base groups in contact with the surface is an interesting quantity because it is closely connected to the structural state of the adsorbed protein. The simple finding that f is *not* constant implies already that the structural state varies with the medium conditions. In this respect, we note that at least three mechanisms for structural alteration may interplay:

(a) Adjustment of the internal protein structure. This occurs commonly in the case of adsorption of structurally unstable proteins. It is then even possible that (partial) unfolding on the surface is a favourable process [7]. However, lysozyme is a rigid, stable molecule [17], and several studies [20–22] have shown that lysozyme is probably in its native structure when adsorbed on a hydrophilic surface. We expect therefore that also on Mono S surface lysozyme will have the native structure.

(b) Adjustment of orientation of a rigid protein. Recently, we determined the orientation of cytochrome c on SnO_2, as a function of surface potential and medium conditions [18]. It was shown that under some circumstances one orientation may be favoured over the other. In the case of cytochrome c the preference of orientation is probably determined by interaction of the large electrostatic dipole and the electric field of the surface. For lysozyme adsorption purely geometrical effects may also be important because the shape of the protein is that of a prolate ellipsoid [16], so that end-on and side-on adsorption may have different Gibbs energies of interaction. Both orientations (and intermediate states) will have a different area of the contact layer, so that an adjustment of orientation will be reflected by a change in the fraction f of protein acid/base groups in contact with the surface.

(c) Adjustment of adsorbent surface structure. We do not know how the Mono S surface looks like. The manufacturer indicates that 'polyethers' [19] are present. This may imply that the upper layers of the surface are somewhat flexible, so that the surface can adjust its structure in response to the adsorbing protein. In the case of a thick flexible layer one may even envisage complete encapsulation of the protein. The higher values of f (> 0.5) seem to indicate that the protein molecule is indeed partly 'buried' in the surface, and the more so, the stronger the protein–surface interaction is.

Possibilities for further modelling

We limited the model to ion exchange effects only. It is well known that in solutions of high salt concentration (e.g. $> 1\ M$) proteins may be forced to adsorb by way of the salting-out effect, especially when the surface is not too polar. It is easy to see that salting-out implies a concomitant positive phenomenological co-adsorption because then the differential on the right hand side of eq. (1) is positive. Salting-out phenomena are sometimes also found with ion exchange columns, apparently because the surface combines ion exchange and hydrophobic functionality and/or electrolyte is used which is known to induce salting-out (e.g. $(NH_4)_2SO_4$ [13]). The support we used, Mono S, is hydrophilic [19] and NaCl is a poor salting-out agent [23], and not effective in the limited concentration range we used.

It should not be so difficult to include salting-out effects in the present model. As a starting point one could perhaps use the classical protein solubility relation (eq. [1] from [23]) $\mathrm{Log}(S) = E(I) - K_s \cdot I$, where S is the solubility, $E(I)$ is an electrostatic term (in the classical theory a simple Debye–Hückel activity coefficient), K_s the salting-out constant and I the salt (ionic) strength. These salting-out constants are of course protein and electrolyte specific. In line with the solubility formula, one could perhaps interpret the Henry

coefficient of adsorption similarly, which would lead to 'salting-out' co-adsorption numbers of the type $\Delta r_i(\text{salting-out}) = \text{const.} \cdot \partial I/\partial \ln(a_i) \cong \text{const.} \cdot c_i(1\text{–}1 \text{ electrolyte})$. In the older literature the salting-out is attributed to dehydration [23]. It seems reasonable to suppose that the salting-out co-adsorption numbers are linear in the contact area. This results in formulae of the type $r_i^c(\text{salting-out}) = \text{const}_j \cdot c_j$, which can be added to the right hand side of eq. (7).

Conclusions

Lysozyme ion exchange chromatography on Mono S cation ion exchange resin can be interpreted with a simple model for the compounded ion exchange reactions in the contact layer of the protein–surface complex. The conspicuous features are:

(i) The effective pK's of the protein acid groups in the contact layer are only a few tenths of a pK unit below the solution values.

(ii) Co-adsorption of chloride ion is the sum of and negative co-adsorption due to the displacement of lysozyme counter-ions, and to a minor extent, positive co-adsorption due to the decrease in expulsion of Mono S co-ions.

(iii) Conversely, co-adsorption of sodium ion is the sum of negative co-adsorption due to displacement of Mono S counter-ions, and positive co-adsorption due to decrease of expulsion of lysozyme co-ions [1].

(iv) The fraction f of protein acid and base groups in the contact layer varies with the medium conditions, so that the fraction is higher when the interaction between protein and exchanger surface is stronger.

(v) Indirect evidence points to structural alterations in the upper layers of the Mono S exchanger surface, such that the adsorbed protein is partly buried in the surface.

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Appendix

Calculation procedure

Ion binding numbers in surface phase are defined as (for systems where $c_w \gg c_i$, cf. eqs. (4) and (5) of Part I, with slightly different notation):

$$r_{ab}^{\sigma} = r_{ab}^{L\sigma} - r_{ab}^{R\sigma} \tag{A1a}$$

$$r_{Cl^-}^{\sigma} = r_{Cl^-}^{L\sigma} - r_{Cl^-}^{R\sigma} \tag{A1b}$$

$$r_{Na^+}^{\sigma} = r_{Na^+}^{L\sigma} - r_{Na^+}^{R\sigma} = \Gamma_{ab}^{\sigma} - \Gamma_{Cl^-}^{\sigma} \tag{A1c}$$

where 'ab' is acid/base. Here, ab may be interpreted as H^+ because hydroxyl binding is negligible. $r_i^{X\sigma}$ is defined as $\Gamma_i^{X\sigma}/\Gamma_p$, Γ is adsorbed amount, σ indicates the surface phase, L is the protein solution in contact with surface, R is reference two-phase system. In the present case, where the adsorbed amount of protein is very low, a proper reference for the surface phase is the bare surface in between adsorbed isolated protein molecules.

In view of the definition of f as the fraction of protein acid/base groups in the contact layer, the binding number in the surface phase can also be written as:

$$r_i^{\sigma} = r_i^{c} + (1-f) r_i^{\alpha} \tag{A2}$$

where i = ab, Cl^- or Na^+; 'c' denotes contact layer and 'α' the solution phase.

We define reduced binding numbers $r_i^{c'}$ which are independent of f:

$$r_i^{c'} \equiv r_i^{c}/f \tag{A3}$$

The co-adsorption number Δr_i of i has been defined as (cf. Part I, eq. [3], with $c_w \gg c_i$):

$$\Delta r_i \equiv r_i^{\sigma} - r_i^{\alpha} \tag{A4}$$

Straightforward substitution of eqs. (A1)–(A4) yields the desired expressions for the co-adsorption numbers in terms of f, r_i^{α} and r_i^{c}:

$$\Delta r_i = f \cdot p_i \tag{A5a}$$

where

$$p_i = r_i^{c'} - r_i^{\alpha} \tag{A5b}$$

In this way, we have separated the extensive effects, contained in the fraction f, from the intensive ion exchange effects, contained in the function p_i.

The logic of this fitting procedure is that the parameters of the ion exchange reactions are calculated from a fit of the theoretical ratio p_{Cl^-}/p_{ab} to the experimental ratio $\Delta r_{Cl^-}/\Delta r_{ab}$ [1]. The fraction f, which is not constrained in any way by the fitting procedure, is then calculated as a function of the medium conditions from $f = 0.5(\Delta r_{ab}/p_{ab} + \Delta r_{Cl^-}/p_{Cl^-})$.

We define a test function $t(k)$ for every experimental point k:

$$t(k) \equiv -p_{Cl^-}(k) + 0.5(\rho(k) + 1)p_{ab}(k) \tag{A6}$$

where ρ is the differential coefficient (eq. 14c from [1]):

$$\rho \equiv \left(\frac{\partial\ \mathrm{pH}}{\partial\ \log(a_s)} \right)_{\eta} \tag{A7}$$

The experimental points are taken from the interpolated retention map in Fig. 2b of Part 1, comprising 42 points with pH ranging from 3.75 to 5.75, $\log(a_s)$ from -0.38 to -0.75 and log(R) from -0.21 to 1.00. As explained before [1], in every point the experimental values of R and ρ (and a related differential coefficient) are known from an interpolation scheme. Thus, in (A6) ρ and r_i^{α} are interpolated experimental values ($r_{Cl^-}^{\alpha}$ from the Esin–Markov analysis), and the $r_i^{c'}$ are values from the contact layer model.

The fitting function T is defined as the square root of the sum of $\{t(k)\}^2$ over all points k:

$$T \equiv \left[\sum_k \{t(k)\}^2 \right]^{1/2} \tag{A8}$$

It can readily be shown that in the case of a perfect fit T is zero (combine eq. A5a of this paper with eqs. 15a′ and 15c′ from Part I). In the implementation of the fitting procedure we used a Newton–Raphson iteration method for numerical minimisation of T with respect to the pK's for given constant C, which usually took about 10–15 iterations. The parameter C was found by iterated manual adjustment of C as the input parameter. In the minimum the mean deviation is defined as $\sqrt{(T/\text{number of points})_{\min}}$. The contribution of the experimental noise to the mean deviation can be estimated from the experimental uncertainty in $0.5(\rho + 1)$, this is about 0.5, so that with $f \cong 0.5$ and $\Delta r_{ab} \cong 1$–2 the uncertainty in $t(k)$ is about 0.5–1.0. Mathematics tells us that with only a few parameters ($\ll$ number of points) the fit can never be better than this deviation, even if the theory is exact.

Note that the important feature of the fitting procedure is that f is not constrained in any way: f must be determined by comparison of the model estimated co-adsorption numbers with the experimental co-adsorption numbers.

Rearrangement of eq. (A5) gives two different estimates of f. The first reads:

$$f_{ab}(k) \equiv \Delta r_{ab}(\text{exp.}, k)/p_{ab}(k) \tag{A9}$$

This definition forces the theoretical acid/base co-adsorption to be exactly equal to the experimental (exp.) value. According to eq. (A9) the theoretical co-adsorption numbers can then be calculated from:

$$\Delta r_{ab}(\text{theory}) \equiv \Delta r_{ab}(\text{exp.}) \tag{A10a}$$

$$\Delta r_{Cl^-}(\text{theory}) = f_{ab} \cdot p_{Cl^-} \tag{A10b}$$

The second estimate of f is:

$$f_{Cl^-}(k) \equiv \Delta r_{Cl^-}(\text{exp.}, k)/p_{Cl^-} \tag{A11}$$

This fixes the theoretical chloride ion co-adsorption numbers exactly to the experimental values. The theoretical ion co-adsorption numbers are then calculated from:

$$\Delta r_{ab}(\text{theory}) = f_{Cl^-} \cdot p_{ab} \tag{A12}$$

$$\Delta r_{Cl^-}(\text{theory}) \equiv \Delta r_{Cl^-}(\text{exp.}) \tag{A13}$$

The calculations showed that f_{ab} and f_{Cl^-} never differed by more than about 0.1. For the best estimate of f we therefore simply used the average of f_{ab} and f_{Cl^-}:

$$f = 0.5(f_{ab} + f_{Cl^-}) \tag{A14}$$

The co-adsorption numbers were then calculated with:

$$\Delta r_{ab}(\text{theory}) = f \cdot p_{ab} = 0.5\left\{\Delta r_{ab}(\text{exp.}) + \frac{p_{ab}}{p_{Cl^-}} \cdot \Delta r_{Cl^-}(\text{exp.})\right\} \quad \text{(A15)}$$

$$\Delta r_{Cl^-}(\text{theory}) = f \cdot p_{Cl^-} = 0.5\left\{\frac{p_{Cl^-}}{p_{ab}} \cdot \Delta r_{ab}(\text{exp.}) + \Delta r_{Cl^-}(\text{exp.})\right\} \quad \text{(A16)}$$

The sodium ion co-adsorption number can always be calculated from the electroneutrality condition:

$$\Delta r_{Na^+}(\text{theory}) = \Delta r_{Cl^-}(\text{theory}) - \Delta r_{ab}(\text{theory}) \quad \text{(A17)}$$

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