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Charge regulation in biomolecular solution

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Abstract. Proteins and other biomolecules contain acidic and basic titratable groups that give rise to intricate charge distributions and control electrostatic interactions. ‘Charge regulation’ concerns how the proton equilibria of these sites are perturbed when approached by alien molecular matter such as other proteins, surfaces and membranes, DNA, polyelectrolytes etc. Importantly, this perturbation generates a charge response that leads to attractive intermolecular interactions that can be conveniently described by a single molecular property – the charge capacitance. The capacitance quantifies molecular charge fluctuations, i.e. it is the variance of the mean charge and is an intrinsic property on par with the net charge and the dipole moment. It directly enters the free energy expression for intermolecular interactions and can be obtained experimentally from the derivative of the titration curve or theoretically from simulations. In this review, we focus on the capacitance concept as a predictive parameter for charge regulation and demonstrate how it can be used to estimate the interaction of a protein with other proteins, polyelectrolytes, membranes as well as with ligands.

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1. Introduction

The term ‘charge regulation’ generally describes how the charge state of a polyprotic molecule is changed when approached by alien molecular matter. For example, when a protein comes into close proximity of a lipid membrane, the protonation state (and consequently the charge distribution) changes in order to lower the free energy. The origin of the mechanism is due to protons that migrate to and from titratable sites, thereby enabling the molecule to adapt to changing chemical environments.

Charge regulation was described already in the 1920s by Linderstrøm-Lang at the Carlsberg breweries (Gitlin et al. 2006; Linderstrøm-Lang, 1924) and Kirkwood and Shumaker later used statistical mechanical perturbation theory to show how correlations between the charge distributions of two proteins lead to attractive intermolecular interactions (Kirkwood & Shumaker, 1952a). This theoretical prediction of so-called fluctuation forces was later elegantly verified using light scattering (Timasheff et al. 1955). Since then there has been a range of studies addressing charge regulation for protein–protein interactions (Aguilar et al. 2010; Elcock & McCammon, 2001; Grant, 2001; Kirkwood & Shumaker, 1952b; Lund & Jönsson, 2005; Mason & Jensen, 2008; Phillips, 1974), protein–surface interactions (Biesheuvel & Wittemann, 2005; Biesheuvel et al. 2005; Hartvig et al. 2011; Lund et al. 2005; Shen & Frey, 2005; Ståhlberg & Jönsson, 1996; Sukhishvili & Granick, 2003; Tsao, 2000), polyelectrolytes (Biesheuvel & Wittemann, 2005; da Silva et al. 2006; Gong et al. 2007; de Vos et al. 2010; Shubin & Linse, 1997; Ullner et al. 1994), charge ladders (Gitlin et al. 2003, 2006; Menon & Zydney, 2000; Sharma et al. 2003) and surface interactions (Biesheuvel, 2001; Boon & van Roij, 2011; Borkovec & Behrens, 2008; Carnie, 1993; Chan & Pashley, 1980; Dan, 2002; Keh & Li, 2007; Ninham & Parsegian, 1971; Popa et al. 2010). While charge regulation is most often ascribed to proton equilibria, any binding ion may contribute to charge fluctuations (Kurut & Lund, 2012). Despite the generic nature of the charge regulation mechanism, it is rarely – though with some notable exceptions (Hill, 1956; Tanford, 1961; Wyman & Gill, 1990) – mentioned in text books on physical chemistry. In this text, we give a molecular overview of the mechanism, including tools to predict why and when it becomes important.

2. The charge regulation mechanism

2.1 Phenomenological description

Proteins and a range of other macromolecules contain acidic and basic residues that – depending on solution conditions – can be either protonated or de-protonated. This gives the molecule an often complex electric charge distribution that in turn affects interactions with other molecular matter. To phenomenologically describe the charge regulation mechanism, consider first the equilibrium process of a single ionizable site,

\[ \text{HA} \rightleftharpoons \text{H}^+ + \text{A}^- . \]  

The corresponding equilibrium constant is

\[ K_a = \frac{\gamma_{\text{HA}} \gamma_{\text{A}^-} \rho_{\text{H}^+} \rho_{\text{A}^-}}{\gamma_{\text{H}^+} \gamma_{\text{A}^-} \rho_{\text{HA}}} \]

\[ \Leftrightarrow - \log \frac{\rho_{\text{HA}}}{\rho_{\text{A}^-}} = pK_a = pH - pK_a + \log \frac{\gamma_{\text{HA}}}{\gamma_{\text{A}^-}} . \]
where $\rho$ are average concentrations and $\gamma$ are activity coefficients that take into account excess interactions not accounted for in the reference state (typically infinite dilution). Due to thermal motion, protons migrate to and from the site and the fraction $\rho_{1A}/\rho_A$—directly defines the average charge state of the site. To introduce charge regulation let us now consider the situation where pH equals $pK_a$. At this condition, it is clear from Eq. (2) that the average charge state is determined solely by the activity coefficients. As $\gamma$ accounts for interactions with other solutes, it consequently changes when the site moves into close proximity of other molecules. At equilibrium, the chemical potential or activity, $a_i = \rho_i \gamma_i$, must be constant throughout the medium and if the activity factor changes, so must the concentration. Thus, one could re-write Eq. (2) in terms of $\rho(R)$ and $\gamma(R)$, where $R$ is a spatial coordinate describing the microscopic position of the site, relative to another molecule.

2.2 Statistical mechanical description

It was shown above how the charge regulation mechanism can be explained from basic thermodynamic arguments and we now take a slightly more microscopic route. Consider again a single titratable site that can be either protonated or de-protonated and is exposed to an external electric potential, $\varphi$, due to all other charges in the system. The canonical ensemble average for the charge, $z_i$, then becomes,

$$
\langle z \rangle = \sum_z z_i \exp \left( -\beta e z_i \varphi \right) \left/ \sum \exp \left( -\beta e z_i \varphi \right) \right.,
$$

(3)

where $\beta = 1/kT$ is the inverse thermal energy, $e$ is the electron unit charge and the sums run over all possible charge states. This expectation value for the charge is equivalent to the average charge discussed in the previous section and it is clear that any change in the external potential, i.e. chemical environment, will influence $\langle z \rangle$. Perturbing the system with a small change in $\varphi$ we immediately get that

$$
\frac{\partial \langle z \rangle}{\beta e \partial \varphi} = \langle z^2 \rangle - \langle z \rangle^2 \equiv \iota.
$$

(4)

Here we introduce the capacitance, $\iota$, which is simply the variance of the mean charge and a measure of how much charge can be induced upon exposure to an external electric potential. An identical analysis can be made for the total charge of a macromolecule. Equation (4) is a typical example of linear response where the capacitance is the response function to an external perturbation of the system (Fornés, 2000; Kubo, 1966).

While in most text books a titratable site is characterized solely by its average charge, it is commonly left unsaid that there is a distribution around it as illustrated in Fig. 1. This is unfortunate as it is exactly this distribution – and the possibility to disturb it – that gives rise to the charge regulation mechanism.

2.3 Relation to proton titration curve

Consider the acid dissociation constant given by Eq. (2). Assuming unity activity coefficients, the free energy difference, $\beta e$, between the protonated and de-protonated form is (Sassi et al. 1992;
Ullner et al. (1994)

\[
\beta \epsilon = - \ln \frac{\rho_{HA}}{\rho_{A^-}} = (pH - pK_a) \ln 10, \tag{5}
\]

which we use to set up a microscopic two-state system for a single, titratable site where \( z_H \) is the valency of the titrant (+1 for protons). Defining the de-protonated state as the ground state, \( z_0 = 0 \), we write a molecular partition function and two ensemble averages,

\[
q = \sum_i e^{-\beta \epsilon_i} = 1 + e^{-(pH - pK_a) \ln 10}, \tag{6}
\]

\[
\langle z \rangle = \frac{z_0 + (z_0 + z_H)e^{-(pH - pK_a) \ln 10}}{q}, \tag{7}
\]

\[
\langle z^2 \rangle = \frac{z_0^2 + (z_0 + z_H)^2e^{-(pH - pK_a) \ln 10}}{q}. \tag{8}
\]

We can now give expressions for charge fluctuations,

\[
\langle z^2 \rangle - \langle z \rangle^2 = \frac{z_H^2 e^{-(pH - pK_a) \ln 10}}{q^2} \tag{9}
\]

as well as the charge response function to a small pH perturbation,

\[
\frac{\partial \langle z \rangle}{\partial pH} = - \frac{z_H e^{-(pH - pK_a) \ln 10}}{q^2} \ln 10. \tag{10}
\]

Combining (9) and (10) it follows that,

\[
\langle z^2 \rangle - \langle z \rangle^2 = - \frac{z_H}{\ln 10} \frac{\partial \langle z \rangle}{\partial pH} \equiv \epsilon, \tag{11}
\]

where \( \epsilon \) is the same charge capacitance as obtained in Eq. (4). Again, this result can be generalized to the total charge number of a macromolecule, \( Z = \sum z_i \), related to the molecular capacitance,

\[
C = - \frac{1}{\ln 10} \frac{\partial \langle Z \rangle}{\partial pH}. \tag{12}
\]
More on this in the following sections. Note that what we here denote ‘capacitance’ – due to the relation in Eq. (4) – is related to the ‘binding capacity’ used by (Wyman & Gill, 1990).

2.4 Relation to intermolecular interactions

An aqueous solution containing biological molecules can in a general sense be described as an electrolyte solution as it contains simple ions such as Na\(^+\), K\(^+\), Cl\(^-\), etc. as well as macromolecules with a net charge significantly different from unity. DNA, proteins and polysaccharides are important examples of the latter. At low salt concentration, the interaction of these macromolecules is dominated by a direct Coulomb interaction, but when the macromolecular charge is close to zero other non-intuitive interaction terms start to play important roles. It is our intention to discuss the interaction/stability of biological macromolecules in a few generic situations where attractive electrostatic interactions besides the direct ion–ion term are of significance. As shown above, the protonation state of a titratable site depends on the nearby chemical environment and, consequently, electrostatic forces may change when two biomolecules come into close proximity of each other. This ‘induced’ interaction can be formalized in a statistical mechanical perturbation approach (Kirkwood & Shumaker, 1952a; Lund & Jönsson, 2005) and a protein is characterized not only by its average charge distribution, e.g. net charge, dipole moment etc., but also – as shown below – by the charge capacitance introduced above.

Consider two macromolecules A and B, described by two charge distributions \([r_i, z_i]\) and \([r_j, z_j]\), respectively. Their mass centra are separated by \(R\), which means that the distance between two charges \(i\) and \(j\) is \(r_{ij} = |R + r_j - r_i|\). The average net charge of the distributions need not be zero, that is \(n_A = \sum_i z_i \neq 0\). The free energy of interaction can be written as,

\[
\beta A(R) = -\ln \langle \exp \left( -\beta U(R) \right) \rangle_0 \\
\approx \langle \beta U(R) \rangle_0 - \frac{1}{2} \langle \beta U(R)^2 \rangle_0 + \frac{1}{2} \{ \langle \beta U(R) \rangle_0 + \frac{1}{2} \langle \beta U(R)^2 \rangle_0 \}^2,
\]

where \(U(R)\) is the interaction between the two charge distributions and \(\langle \ldots \rangle_0\) denotes an average over the unperturbed system, which in the present case are the two macromolecules at infinite separation in solution. If we for simplicity assume that the interactions are unscreened, that is, the salt concentration is low, then the interaction energy is simply the direct Coulomb interaction between the two charge distributions,

\[
\beta U(R) = \sum_i \sum_j \frac{Z_i Z_j}{r_{ij}},
\]

where \(l_B = \beta e^2/4\pi\varepsilon_0\varepsilon_r\) is the Bjerrum length which in an aqueous solution at room temperature is approximately 7.1 Å. Assuming that \(R \gg r_n\) a Taylor series expansion of \(U(R)\) yields an estimate of the free energy decomposed into multipolar terms: ion–ion, ion–dipole and dipole–dipole as well as terms stemming from charge induction. Note that the ion–dipole interaction disappears in first order and that the first non-vanishing dipole term, \(\beta A_{\text{ion-dip}} = -l_B^2 Z^2 \beta \mu^2/6R^4\) is of order \(1/R^4\). We now write an approximation to the free energy including all terms of order up to \(1/R^2\):

\[
\beta A(R) \approx \frac{l_B^2}{2R} \left( \langle Z_A \rangle^2 C_B + \langle Z_B \rangle^2 C_A \right) - l_B^2 \left( C_A C_B \right),
\]

where \(C_A\) and \(C_B\) are the charge capacitance of macromolecules A and B, respectively.
where we have identified the molecular protein charge capacitance as
\[ C = \langle Z^2 \rangle - \langle Z \rangle^2, \] (16)
which is non-zero for molecules with titratable sites. The first term in Eq. (15) is the direct Coulomb term and the following terms are the charge–induced charge and induced charge–induced charge interactions. For identical molecules, \( \langle Z_A \rangle = \langle Z_B \rangle = \langle Z \rangle \), the expression simplifies to
\[ \beta A(R) \approx \frac{l_0 \langle Z \rangle^2}{R} - \frac{l_0^2}{2R^2} (2C \langle Z \rangle^2 + C^2), \] (17)
and if pH equals pI, then \( \langle Z \rangle = 0 \) and the induced charge–induced charge interaction becomes the leading term
\[ \beta A(R) \approx - \frac{(l_0 C)^2}{2R^2}. \] (18)

The above equations show that the fluctuating charge of a protein or macromolecule may under certain circumstances contribute significantly to the net interaction. We have already shown that the capacitance is related to a small change in an imposed external potential, \( \varphi \), which can be used to estimate the amount of induced charge
\[ C = - \frac{\partial \langle Z \rangle}{\beta e \partial \varphi} \Rightarrow \Delta Z = - \beta e \Delta \varphi. \] (19)

The capacitance, \( C \), can also be derived from the experimental titration curve – see Eq. (12). For a single titrating acid the ionization degree, \( \alpha \), can be found in any elementary physical chemistry textbook,
\[ \log K = -\text{pH} + \log \frac{\alpha}{1-\alpha} \] (20)
and taking the derivative of \( \alpha \) with respect to pH gives,
\[ \frac{\partial \alpha}{\partial \text{pH}} = \alpha(1-\alpha) = e \ln 10, \] (21)
where in the second step we have identified the capacitance defined in Eq. (11). We can obtain an approximate value for the capacitance in a protein assuming that there is no interaction between the titrating sites. A protein contains several titrating groups like aspartic and glutamic acid, histidine etc., each with an ideal pK value. Denoting different titrating groups with \( k \) and their number with \( n_k \), the total capacitance can be approximated with
\[ C_{\text{ideal}} = \frac{1}{\ln 10} \sum_k n_k \frac{10^{pH - pK_k}}{(1 + 10^{pH - pK_k})^2} = - \frac{1}{\ln 10} \frac{\partial \langle Z \rangle}{\partial \text{pH}}. \] (22)

The magnitude of charge regulation is dependent on the system and is usually of the order of \( kT \) or less for two identical proteins at contact. However, the charge regulation can dominate over the direct Coulombic repulsion term if the capacitance is sufficiently large or when one of the macromolecules are at its iso-electric point. Consider two identical proteins,
\[ \beta A(R) \approx \frac{l_0 \langle Z \rangle^2}{R} - \frac{l_0^2 C \langle Z \rangle^2}{R^2} = \frac{l_0 \langle Z \rangle^2}{R} \left[ 1 - \frac{l_0 C}{R} \right]. \] (23)
The free energy of interaction will be less than zero whenever $R < l_B C$ (neglecting the $C^2$ term). For most medium-sized proteins, the capacitance is typically of order unity, while the minimum center-to-center distance is of the order of 30–40 Å, which means that the direct ion–ion repulsion dominates. Still, for a protein sufficiently rich in acidic or basic residues, the capacitance is larger than unity and one can imagine a situation where the charge regulation term dominates at contact. Note also that $C$ increases linearly with the number of amino acid residues, while the protein size, $R_p$, only increases like $N^{1/3}$ and for a sufficiently large protein the charge regulation may dominate at certain pH values. This is of course somewhat speculative, since we have neglected the effect of salt screening.

3. Charge regulation in biomolecules

The capacitance, $C$, is the key parameter for charge regulation and we have calculated it for a number of proteins with different characteristics in terms of number and type of residues – see Fig. 2. To account for internal electrostatic interactions, a MC simulation (or other methods; Baker et al., 2001; Mason & Jensen, 2008) has to be performed at each pH at a given salt and protein concentration. The main difference from the ideal capacitance curve – i.e. when interactions are ignored – is a strong broadening of two peaks corresponding to the response from acidic and basic residues, respectively. If the protein has a significant net charge, the true curve shifts away from the ideal one, as is seen for calbindin at high pH.

The protein hisactophilin is of the same size as calbindin, but it has a very different capacitance curve. Hisactophilin is a histidine-rich protein (HRP), which is reflected in a capacitance maximum for pH 5–6. The downward shift of the maximum compared with the ideal curve is due to the high positive charge of hisactophilin at low pH (+23e at pH 4).

The calcium-binding protein calmodulin is more charged than calbindin and as a consequence it also shows broader capacitance peaks. The capacitance is not particularly sensitive to the details of the atomic structure and if one models calmodulin with a set of spheres each representing only...
the titratable residues, the capacitance curve is virtually identical to the one from an atomistic model (Lund & Jönsson, 2005).

In the following, we will go through a number of examples where charge regulation becomes important.

3.1 Protein–protein interactions

In an elegant light-scattering study of salt-free bovine albumin solutions, Timasheff and co-workers verified early theoretical predictions that attractive interactions between proteins arise due to proton fluctuations (Kirkwood & Shumaker, 1952a; Timasheff et al. 1955). Today, the free energy of interaction – or potential of mean force (PMF) – between protein pairs can be calculated using computer simulations and, by turning proton fluctuations either on or off, the contribution from charge regulation can be quantified. As shown in Fig. 3, left for the interaction between the fictitious calbindin–lysozyme pair, there is a significant difference between the fluctuating and non-fluctuating charge models (Lund & Jönsson, 2005). At large separations both proteins are positively charged (Fig. 3), but as they approach, their average charges change – and much more so for calbindin than for lysozyme. Using the capacitance framework from the previous section this difference can readily be explained by noting that, at pH 4, lysozyme has a large charge and a small capacitance, while the opposite is true for calbindin (see Fig. 2). Knowing the net charges and capacitances for the two proteins – either from experiment or calculations – the regulation free energy and induced charges can be estimated from Eqs. (15) and (19), respectively. As evident from Figs 3, right and 4, excellent agreement is obtained between exact simulation results and the approximative perturbation theory.

An important message is that despite the fact that both calbindin and lysozyme are positively charged at pH 4, there is still an attractive electrostatic interaction between the two. We come back to this issue when discussing protein polyelectrolyte complexation.

3.2 Ligand binding

Binding of a ligand to a protein is often accompanied by charge regulation (Aguilar et al. 2010; Di Cera, 1991; Linse et al. 1991; Svensson et al. 1993). Let us take two examples: binding of four...
calcium ions to calmodulin and the binding of a short cationic (+7\(e\)) peptide to calmodulin. Calmodulin has 43 acidic and 16 basic residues, where the latter are all charged for pH < 10. Figure 5 shows the number of protons dissociated from calmodulin upon calcium binding. At low pH it closely reflects the calmodulin capacitance, while at basic pH values the co-variation of the release of protons and the capacitance is less pronounced. This is because the acidic groups titrating at low pH are closer in space to the four calcium-binding sites than the basic residues. One can note that at pH 4, the binding of four Ca\(^{2+}\) with a total charge of +8\(e\) gives rise to the release of almost eight protons. The binding of the heptavalent peptide shows the same pattern, although the proton release is slightly weaker. This is understandable from the fact that calmodulin already binds four divalent calcium ions. Again the proton release closely follows the variation of the capacitance.

At this stage, we should point out that charge regulation phenomena associated with ligand binding to proteins have been extensively covered within the thermodynamic linkage framework developed by Wyman & Gill (1990). While we have here taken a statistical mechanical viewpoint and focus on regulation effects on intermolecular interactions, future work rigorously connecting with the linkage theory would be desirable.

3.3 Protein–membrane interactions

Biological membranes are often negatively charged and nearby macromolecules are consequently exposed to an electric potential different to that in the bulk. As demonstrated in the previous sections – and quantified by Eq. (26) – such an electrostatic perturbation may trigger charge regulation but the question is if such a mechanism is relevant in a biological setting? Most proteins have capacitance peaks at acidic and alkaline pH (see Fig. 2) yet biomolecules rich in histidines are expected to peak at neutral pH. Examples are hisactophilin, HRP2 as well as histatin saliva proteins. In this section, we focus on hisactophilin and as seen in Fig. 2 this protein has a high capacitance at pH 6 while at pH 8, \(C\) is reduced almost by a factor of 3.

By simulating hisactophilin close to a negatively charged surface we investigate the free energy of interaction for a situation where the protein can titrate and one where the residue charges are fixed (Lund \textit{et al}. 2005). The results, shown in Fig. 6 for different salt concentrations and pH values, are in excellent agreement with the capacitance picture described above: Going from pH...
7.5 to pH 6.5 we see a strong increase in the contribution from charge regulation. At the same time the effect of salt is strong, yet even at physiological salt conditions the charge regulation mechanism contributes with approximately $-1.5 \ kT$. Using Eq. (26) and Gouy–Chapman theory, salt screening is expected to dampen the interaction by $\exp (-2kr)$, i.e. much stronger than for direct Coulomb interactions.

Interestingly, the binding of hisactophilin to phospholipid membranes is biologically governed by minor intra-cellular pH changes and the picture is exactly as observed in the simulations: binding at low pH and release at high pH (Hanakam et al. 1996). That the capacitance for hisactophilin peaks exactly at conditions where the protein needs to bind is hardly a coincidence, but rather a sophisticated evolutionary design to control a biologically relevant interaction.

3.4 Protein–polyelectrolyte complexation

The complexation of polyelectrolytes and proteins is extensively used in pharmaceutics, food and cosmetics (de Kruif et al. 2004; Doublier et al. 2000; Girard et al. 2003; Hubbell, 2003;
Jiang et al. 2002; Schmitt et al. 1998; Simon et al. 2004; Zancong & Mitragotri, 2002). The subject has been addressed by a number of authors exploring it from experimental measurements (de Kruif et al. 2004; Girard et al. 2003; Hallberg & Dubin, 1998; Seyrek et al. 2003) to theoretical modeling (Carlsson et al. 2001; de Vries, 2004; Grymonpré et al. 2001). The strength of interaction is to a large extent regulated by electrostatic interactions, governed by key parameters such as pH and salt concentration.

A particularly interesting observation is the apparently paradoxical formation of soluble complexes at conditions where the net charges of protein and polyelectrolyte have the same sign. Experimental studies of Dubin, Kruif and co-workers (de Kruif et al. 2004; de Vries et al. 2003; Grymonpré et al. 2001) have demonstrated this counterintuitive property referred to as ‘complexation on the wrong side’ of the isoelectric point of the protein. The molecular interpretation initially focused on the assumption of ‘charged patches’ on the protein surface (de Kruif et al. 2004; de Vries, 2004; Hattori et al. 2000; Seyrek et al. 2003), but it has later been suggested that charge regulation has a significant if not dominating role (Biesheuvel & Wittemann, 2005; da Silva et al. 2006; de Vos et al. 2010).

A formal way to describe the interaction between oppositely charged patches on two macromolecules is in terms of a multipole expansion and using simulated capacitances and dipole moments we can analytically calculate the ion-induced charge and ion–dipole contributions to the interaction free energy according to an expansion as in Eq. (15).

In Fig. 7 we have calculated the interaction between a polyanion ($\zeta = -21e$) and different proteins (da Silva et al. 2006). The regulation term is by far the most important term for lysozyme, while for $\alpha$-lactalbumin and $\beta$-lactoglobulin the ion–dipole terms are of comparable magnitude. While the curves in Fig. 7 should be regarded as qualitative they still give – as will be seen below – a correct picture of the behavior of the three proteins. The regulation term decays slower than the ion–dipole term and thus gains in relative importance at larger separation. This means that even if the two terms are comparable at contact, the regulation term can still dominate the contribution to, for example, the second viral coefficient.

Fig. 7. Ion-induced charge (solid lines) and ion–dipole (dashed lines) contributions to the free energy of interaction between a polyanion and three different proteins. Calculated from Eq. (15) augmented with an ion–dipole term using simulated capacitances and dipole moments (da Silva et al. 2006).
In order to test the perturbation results in Fig. 7 we have performed two different simulations for each protein–polyelectrolyte complex: (A) all residue charges in the protein are kept fixed to their average bulk values and (B) the residue charges are allowed to titrate.

The calculated free energy of interaction, \( A(R) \), for the three proteins at their respective pI all show a clear minimum, see Fig. 8. The relative strength of the minima are in qualitative agreement with perturbation calculations, cf. Figure 7, while the actual numbers are approximately half the values predicted by second-order perturbation theory. The minima appear at roughly the same separation despite the fact that \( \beta \)-lactoglobulin is more than twice as big as the two others. This can be explained by the elongated form of the former, which also results in a more long-ranged attraction. The separation \( R \) can approach zero, which corresponds to a situation where the polyelectrolyte wraps around the protein. Note, however, that \( A(0) \) is repulsive indicating that the ‘wrapping’ of the chain around the proteins is an entropically unfavorable structure.

The attractive minimum in the protein–polyelectrolyte complex is reduced upon addition of salt (de Vries, 2004) and we can use the free energy minima, \( A(R_{\text{min}}) \), from Fig. 8 to estimate the critical ionic strength. Assuming that the salt screening follows simple Debye–Hückel theory and that the complex is dissociated when the interaction is less than \( kT \), we get the relation,

\[
\exp \left( -2\kappa R_{\text{min}} \right) |A(R_{\text{min}})| \leq kT
\]  

where the factor of 2 in the exponent is because the second-order terms dominate the interaction. Following this recipe we find that approximately 10 and 20 mM salt is sufficient to dissociate the \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin polymer complexes, respectively.

Thus, we have shown that a polyanion can form a complex with a neutral protein molecule. To determine whether this attractive intermolecular interaction is due to charge ‘patches’ or if it comes from charge regulation, we perform additional simulations – this time with a fixed protein charge distribution, set to that in bulk. These results, shown in Fig. 9, show that the free energy minimum for lysozyme is solely due to charge regulation; if the charge distribution on lysozyme is considered fixed, the polyanion–lysozyme interaction is essentially everywhere repulsive. This also means that the ion–dipole interaction gives only a very small attractive contribution, while the effect from higher order moments is negligible.
The polyanion interacts more strongly with α-lactalbumin than with lysozyme and charge regulation increases the depth of the minimum from approximately $-4kT$ to $-6kT$. In this case, the attraction is due to a combined effect from both charge patchiness and charge fluctuations.

4. Conclusion

Charge regulation is a generic mechanism present for all macromolecules with titratable sites. Using statistical mechanics and computer simulations we can quantify the importance of charge regulation for the interaction between a protein with other proteins, polyelectrolytes, membranes and ligands. We have seen that charge regulation drives the complexation between a neutral protein and a polyelectrolyte, and that regulation interactions may be responsible for a biological pH-sensitive switch for binding proteins to phospholipid membranes.

From basic statistical mechanical arguments it is shown that charge regulation can be captured by a single, intrinsic molecular parameter, namely the charge capacitance, which:

- is an intrinsic molecular property on par with the net charge and dipole moment;
- measures how easy it is to distort proton equilibria of titratable groups;
- is strongly dependent on pH and the $p\mathcal{K}_a$-values of the titratable groups;
- can be obtained from the derivative of the proton titration curve or from calculations; and
- enters the expression for the free energy between macromolecules.

5. A Probability distribution ansatz

Let us briefly discuss an alternative approach to derive the interaction between charge regulating matter (Lund, 2010). Figure 1 shows how the charge of a single titratable site fluctuates around the mean charge and extending this to molecular charge fluctuations we write the intrinsic probability of observing the charge $Z$ by an arbitrary probability function $P(Z)$. Exposing the molecule to an external electric potential, $\varphi$, the interaction free energy is

$$
\beta A = - \ln \int P(Z) \exp(-\beta \varphi Z) dZ.
$$

(25)
While we here use a mean field approach, the external potential can equally well be a function of $Z$ so as to include possible correlations.

Approximating $P(Z)$ by a normal distribution, Eq. (25) can be solved exactly

$$\beta A \approx -\ln \frac{1}{\sqrt{2\pi C}} \int_{-\infty}^{\infty} \exp \left[ -\frac{(Z - \langle Z \rangle)^2}{2C} - \beta e\phi Z \right] dZ$$

$$= \beta e\phi \langle Z \rangle - \frac{1}{2} C (\beta e\phi)^2. \quad (26)$$

This result is identical to the ion–ion and ion–induced ion terms in Eq. (15) and hence the first-order perturbation theory applied in the previous section simply corresponds to ‘attaching’ charge by a harmonic potential with a force constant inversely proportional to the capacitance. For a fixed charge distribution, the capacitance is zero and the force constant infinite.

For complex biomolecules, proton exchange also leads to fluctuations in higher order moments (Fornés, 2000; Kirkwood & Shumaker, 1952b) and in a Gaussian ansatz one could – for example – consider the free energy of a fluctuating dipole, $\mu$, in an external electric field, $E(R)$, by integrating over all angles as well as dipole moments,

$$\beta A(R) = -\ln \frac{1}{4\pi} \int_{0}^{2\pi} d\phi \int_{0}^{\pi} \sin \theta d\theta \int_{-\infty}^{\infty} P(\mu) d\mu. \quad (27)$$

Here, the unperturbed $P(\mu)$ can be obtained by assuming that fluctuations around $\langle \mu \rangle$ follow a normal distribution with variance $\langle \mu^2 \rangle - \langle \mu \rangle^2$.

The remaining question is whether or not the distribution of charge and dipole moment actually follow normal distributions? This can be verified in a computer simulation where site titration and intermolecular interactions are explicitly included. As shown in Fig. 10 both the net charge and molecular dipole moment are excellently described by a Gaussian distribution.

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