

Electrostatically Driven Protein Adsorption: Charge Patches versus Charge Regulation

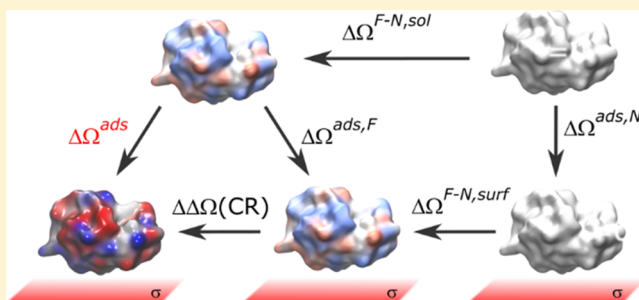
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S Supporting Information

ABSTRACT: The mechanisms of electrostatically driven adsorption of proteins on charged surfaces are studied with a new theoretical framework. The acid–base behavior, charge distribution, and electrostatic contributions to the thermodynamic properties of the proteins are modeled in the presence of a charged surface. The method is validated against experimental titration curves and apparent pK_a s. The theory predicts that electrostatic interactions favor the adsorption of proteins at their isoelectric points on charged surfaces despite the fact that the protein has no net charge in solution. Two known mechanisms explain adsorption under these conditions: (i) charge regulation (the charge of the protein changes due to the presence of the surface) and (ii) charge patches (the protein orients to place charged amino acids near opposite surface charges). This work shows that both mechanisms contribute to adsorption at low ionic strengths, whereas only the charge-patch mechanism operates at high ionic strength. Interestingly, the contribution of charge regulation is insensitive to protein orientation under all conditions, which validates the use of constant-charge simulations to determine the most stable orientation of adsorbed proteins. The present study also shows that the charged surface can induce large shifts in the apparent pK_a s of individual amino acids in adsorbed proteins. Our conclusions are valid for all proteins studied in this work (lysozyme, α -amylase, ribonuclease A, and β -lactoglobulin), as well as for proteins that are not isoelectric but have instead a net charge in solution of the same sign as the surface charge, i.e. the problem of protein adsorption on the “wrong side” of the isoelectric point.



INTRODUCTION

Protein adsorption on charged surfaces is a technologically relevant phenomenon^{1–3} and a very active area of fundamental research.^{4,5} During adsorption, proteins interact with the substrate through a combination of electrostatic, van der Waals, hydrogen-bond, and hydrophobic interactions.^{4,6} On highly charged substrates, electrostatic interactions will be a dominant force and the amount of adsorbed protein will depend on the net charge of the protein. Intuitively, it may be expected that a requisite for electrostatically driven adsorption is that the protein and the surface should have charges of opposite sign. However, various experiments have shown that protein adsorption can occur on bare or polyelectrolyte-modified surfaces even when the charges of the protein and the surface are of the same sign.^{7–12} Furthermore, in many reports, protein adsorption displays a maximum at pHs near the isoelectric point (IEP) of the protein.^{10,13,14} In principle, these observations can be ascribed to the role of nonelectrostatic forces, which will lead to protein adsorption even in the presence of unfavorable electrostatic interactions. However, such conclusion fails to explain many experiments where the amount of adsorbed protein decreases with increasing ionic

strength.^{7,10,11,15–17} Such dependence of adsorption with ionic strength suggests that electrostatic interactions favor adsorption even for proteins with zero net charge or proteins with a charge of the same sign as that of the surface. Two mechanisms have been proposed to explain this result, the charge-regulation (CR) effect^{18–20} and the charge-patch (CP) mechanism.^{15,16,21} In the CR effect, the presence of the charged surface induces a charge of the opposite sign in the protein by displacing the acid–base chemical equilibria of its amino acid, thus favoring adsorption. The CP mechanism is based on the presence of “charge patches” in the protein, which are defined as regions on the protein surface that have an elevated content of amino acids of the same charge. These charge patches allow a favorable protein–surface interaction for protein orientations that place patches with a charge opposite to the surface near them and patch with the same charge as that on the surface far from them. The charge patches are thus related to the charge distribution of the protein, whose first moment, the electric

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dipole of the protein, has been used in the past to explain protein orientation on charged surfaces.²²

The CR effect has been predicted theoretically^{23,24} and confirmed experimentally^{25–27} in different systems, and it is partially responsible for the fact that the pK_a of a free amino acid in solution can be very different from the pK_a of the same amino acid inside a protein.^{28,29} In other words, the protonation state of an acid or basic group depends not only on the pH of the solution but also on the local chemical environment, including the presence of neighboring charged groups and local dielectric properties.^{23,30} These interactions will affect the acid–base equilibrium of an amino acid compared with a bulk solution and will lead to an apparent pK_a (i.e., the pK_a of the amino acid inside the protein) different from the pK_a of the same amino acid free in solution. Placing the protein near a highly charged interface will further modify the pK_a value of that amino acid, which now will be influenced by both the charge of the surface and the charges of neighboring amino acids (which will also be affected by the presence of the surface).^{18,19}

The adsorption of proteins on surfaces of the same charge has been studied by both theory and simulation in the past. For example, atomistic molecular dynamics (MD) simulations have been used to study the adsorption of negatively charged bovine serum albumin on a negative surface.³¹ However, that study, as well as most MD studies of proteins, fixed the charge of the amino acids, thus neglecting the CR effect. Constant-pH MD simulations^{32,33} include the CR effect, but, to the best of our knowledge, they have not been used to study how CR affects protein adsorption. Moreover, the estimation of adsorption free energies is still very difficult for both constant-charge and constant-pH MD simulations due to the difficulty of statistically sampling the free-energy landscape.⁶

The CR mechanism for a protein in solution can be predicted and studied with different theoretical methods, including those based on the Poisson–Boltzmann equation (e.g., DelPhi³⁴ and Fambe-pH,³⁵ among others), electrostatics-conformational methods (e.g., multiconformation continuum electrostatics³⁶), and empirical methods (e.g., PROPKA³⁷). The available implementations of these methodologies do not allow straightforwardly including a charged surface in the calculations, which is possible with the theoretical framework introduced in this work. The relative importance of the CR and the CP mechanisms for the adsorption of proteins on surfaces of the same charge has also been the subject of previous theoretical investigations. For example, de Vos et al. studied the CR and CP mechanisms for brush-modified surfaces using an asymmetrically charged cylinder and concluded that CR is more important than CP at low ionic strengths.³⁸ Longo and Szleifer studied protein adsorption into polyelectrolyte gels using a molecular theory that allowed inhomogeneities in one spatial direction and also identified CR as the dominant mechanism.¹⁹ It is worthwhile to note that these previous theoretical studies either addressed the CR or CP mechanisms individually or considered both effects simultaneously in simplified models that did not include the details of protein structures (i.e., the positions of the amino acids in the three-dimensional space). Moreover, none of these studies provided a clear strategy to isolate the relative thermodynamic contributions of CR and CP to the total adsorption free energy.

This work introduces a new theoretical method to calculate the thermodynamic properties and the state of charge of the

acid–base groups in a protein in the absence or presence of a surface. We first validate our theoretical method by comparing its predictions with experimental titration curves and experimental apparent pK_a s values for individual amino acids. Then, we propose, for the first time, a strategy designed to isolate and analyze the relative importance of the CR and CP mechanisms on the total adsorption free energy. Our results show that both effects are relevant at low ionic strengths, whereas the CP mechanism dominates at high salt concentration. Moreover, we show that the contribution of the CR effect is rather insensitive to protein orientation, which suggests that constant-charge simulations (which neglect CR) can be used to predict protein orientation on a surface without incurring large errors. Finally, our study reveals that the presence of the surface can induce large pK_a shifts in individual amino acids, even for physiological salt concentrations (up to one pK_a unit for a 0.1 M electrolyte). These pK_a shifts may have important implications for protein function in biology and technology, e.g., for the catalytic behavior of enzymes near membranes or on solid substrates, respectively. We present and validate a simple model that provides an explicit expression to estimate the magnitude of the surface-induced pK_a shifts of all acid–base sites in a protein. The input of the model is the distance between each site and the charged surface, which can be determined from the knowledge of the protein structure and its orientation on the surface.

THEORETICAL APPROACH

Formulation of the Theoretical Method. We briefly discuss here our theoretical approach to study the different mechanisms involved in the interaction of proteins with a charged surface. The reader is referred to the [Supporting Information](#) (SI) for a detailed description of our method. We model the structure of a single protein in three dimensions at a coarse-grain level of description and fixed atomic positions. Our theory explicitly considers (at a mean-field level) the configurational space of the mobile ions and the state of ionization of all acid–base groups in the system. The theory can be used to model proteins both in solution and on charged surfaces.

Our theoretical approach is based on writing down an approximate grand-canonical free-energy functional (i.e., a grand potential functional) for the system. Note that the grand-canonical free energy, Ω , is the relevant quantity in the present system because the free ions in solution have a fixed chemical potential (including protons, i.e., we work at fixed pH) rather than a fixed number. The grand-canonical free energy is

$$\begin{aligned} \beta\Omega[\rho_i(\mathbf{r}), \psi(\mathbf{r}), f_j] = & \sum_{i=H^+, OH^-, C^+, A^-} \int \rho_i(\mathbf{r}) [\ln(\rho_i(\mathbf{r})/\rho^0) - 1 + \beta\mu_i^0] d\mathbf{r} \\ & + \sum_{j=\text{all sites}} [f_j \ln(f_j) + (1-f_j) \ln(1-f_j)] \\ & + f_j \beta\mu_{j,\text{charged}}^0 + (1-f_j) \beta\mu_{j,\text{uncharged}}^0 - \\ & \sum_{j=\text{acid sites}} [(1-f_j) \beta\mu_{H^+}] - \\ & \sum_{j=\text{basic sites}} [(1-f_j) \beta\mu_{OH^-}] - \\ & \sum_{i=H^+, OH^-, C^+, A^-} \int \beta\mu_i \rho_i(\mathbf{r}) d\mathbf{r} + \int [\langle \rho_Q(\mathbf{r}) \rangle \\ & \beta\psi(\mathbf{r}) - \frac{1}{2} \beta e(\mathbf{r}) (\nabla \psi(\mathbf{r}))^2] d\mathbf{r} + \int_{\text{surf}} \sigma \beta \psi(\mathbf{r}) d\mathbf{r} \end{aligned} \quad (1)$$

where $\beta = (k_B T)^{-1}$, k_B is Boltzmann's constant and T is the absolute temperature (298 K). The grand potential Ω is a functional of $\rho_i(\mathbf{r})$, which is the number density of species i at the position $\mathbf{r}$ in the system ($i = \text{H}^+$, OH^- , C^+ , and A^- for protons, hydroxyl ions, cations, and anions, respectively); f_j , which is the average ionization state of the acid–base group j (i.e., the probability of having the group j in its charged state); and $\psi(\mathbf{r})$, which is the electrostatic potential at position $\mathbf{r}$. Equation 1 contains contributions from the mixing entropy of the ions and their standard chemical potentials (first term); the free energy of the acid–base chemical equilibria for all acid–base groups in the system (second term); the $-N_i \mu_i$ terms of the grand potential, where N_i and μ_i are the number and chemical potential of ions of type i (third, fourth, and fifth terms); and the electrostatic contribution to the free energy³⁹ (sixth and seventh terms).

The equilibrium structure of the system results from finding the extrema of eq 1 with respect to $\rho_i(\mathbf{r})$, f_j , and $\psi(\mathbf{r})$, which leads, after some rearrangements, to expressions for these unknowns. The extremum with respect to $\rho_i(\mathbf{r})$ yields

$$\rho_i(\mathbf{r}) = \rho_i^{\text{bulk}} \exp(-\beta q_i \psi(\mathbf{r})) \quad (2)$$

where ρ_i^{bulk} is the bulk number density of species i (i.e., the density far away from the protein and the charged surface) and q_i is the charge of species i . The functional extremum of $\beta\Omega$ with respect to $\psi(\mathbf{r})$ results in the Poisson equation

$$\nabla(\epsilon(\mathbf{r}) \nabla \psi(\mathbf{r})) = -\langle \rho_Q(\mathbf{r}) \rangle \quad (3)$$

where $\epsilon(\mathbf{r})$ is the static dielectric constant of the medium at $\mathbf{r}$ and $\langle \rho_Q(\mathbf{r}) \rangle$ is the average charge density at $\mathbf{r}$, which has contributions from the charges of the ions and the acid–base groups of the protein. The boundary condition of eq 3 at the bulk solution (solution far from the protein and the surface) is $\psi^{\text{bulk}} = 0$, and the boundary condition at the charged surface (located in the y - z plane at $x = 0$) is

$$\frac{\partial \psi}{\partial X} = -\frac{\sigma}{\epsilon(\mathbf{r})} \quad (4)$$

where σ is the charge density of the surface.

Finally, the functional extremum with respect to f_j results in expressions for the acid–base equilibrium. For example, for acidic groups, we obtain

$$\frac{f_j \rho_{\text{H}^+}^{\text{bulk}}}{1 - f_j} = \rho^\circ \exp(\beta |e| \bar{\psi}(\mathbf{r}_j^\circ) \exp(-\beta \Delta \mu_j^\circ)) \quad (5)$$

where ρ° is a reference density, $\bar{\psi}(\mathbf{r}_j^\circ)$ is the average of the electrostatic potential over the volume of the acid group j (see the SI), $|e|$ is the elemental charge, and $\Delta \mu_j^\circ$ is the change in the standard free energy associated with the charging reaction of the site j (acid deprotonation). The value of $\Delta \mu_j^\circ$ that should be used in eq 5 depends on the choice of the reference state. A distinctive feature of our theory is the method to obtain $\Delta \mu_j^\circ$ to guarantee the correct description of the reference state given by a single acid–base group in the bulk. We first find the values of $\Delta \mu_j^\circ$ for all acid–base groups by solving the theory for each of them isolated in the bulk and enforcing $f = 0.5$ when $\text{pH} = \text{pK}_a$ (where the pK_a values used in these calculations are those experimentally determined and tabulated for the side chains or terminal groups of the amino acids, see the SI). These calculations provide the values of $\Delta \mu_j^\circ$ for all acid–base groups,

which can then be used to solve the theory for the protein at any pH.

To solve the theory, eqs 2–5 and the equation for the acid–base equilibrium of basic groups (see the SI) are discretized in a grid and solved using numerical methods. This calculation provides us with thermodynamic (Ω) and structural ($\psi(\mathbf{r}), \rho_i(\mathbf{r}), f_j$) information and allows us to calculate the total charge of the protein as $Q_{\text{tot}} = \sum_{j=\text{all sites}} f_j q_j$, where q_j is the charge of the acid–base group j in its charged state.

The inputs of the theory are the structure of the protein (which we obtained from the corresponding pdb file from the protein data bank (PDB)); the properties of the solution (salt concentration and pH) and the surface (surface charge density, σ); the pK_a s of the isolated amino acids in the bulk and their volumes (see Table S1 in the SI); the orientation of the protein on the surface, which is unequivocally defined by two angles, φ and ϕ (the azimuthal and polar angles, respectively); and the dielectric constants of the solvent and the protein. Additional details of the protein model and the discretization and numerical solution of the theory are provided in the SI.

RESULTS AND DISCUSSION

Validation of the Theoretical Methodology. Figure 1 shows the secondary structure of the proteins selected for this

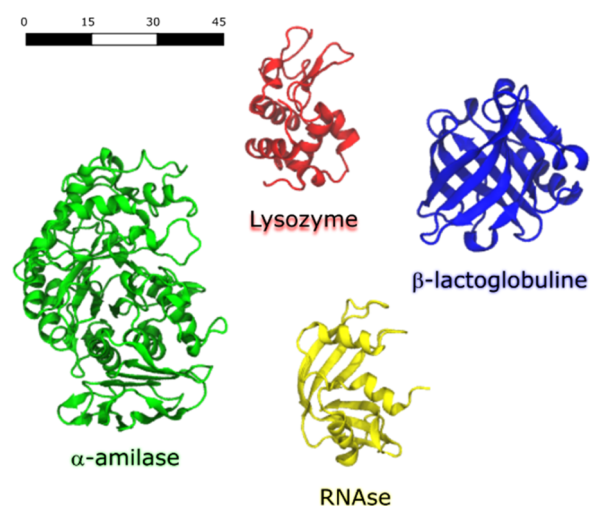


Figure 1. Representation of the proteins (as secondary structure) used in this work. The structures were obtained from crystallographic atomic coordinates taken from the PDB files: hen egg-white lysozyme (in red, IEP: 11.2, PDB ID: 3RZ4), ribonuclease A (RNase, in yellow, IEP: 9.9, PDB ID: 1KF5⁴⁰), human salivary α -amylase (in green, IEP: 6.4, PDB ID: 1SMD⁴¹), and ovine β -lactoglobulin (in blue, IEP: 5.1, PDB ID: 4CK4⁴²). The bar shows the scale in angstroms.

study: lysozyme, α -amylase, ribonuclease A (RNase), and β -lactoglobulin. These four proteins span a wide range of molecular sizes, types of folding, and isoelectric points (IEPs). We chose lysozyme, RNase, and β -lactoglobulin due to the availability of experimental titration curves and/or single-amino acid pK_a values. Since the most relevant conclusions of this work are of general validity, we will mostly present results for lysozyme in the main text and refer the reader to the SI for selected results of the other proteins.

To validate our theoretical approach, we calculated the titration curves (total protein charge, Q_{tot} vs pH) for lysozyme, β -lactoglobulin, and RNase and compared them with the experimental titration curves from the literature; see Figure 2.

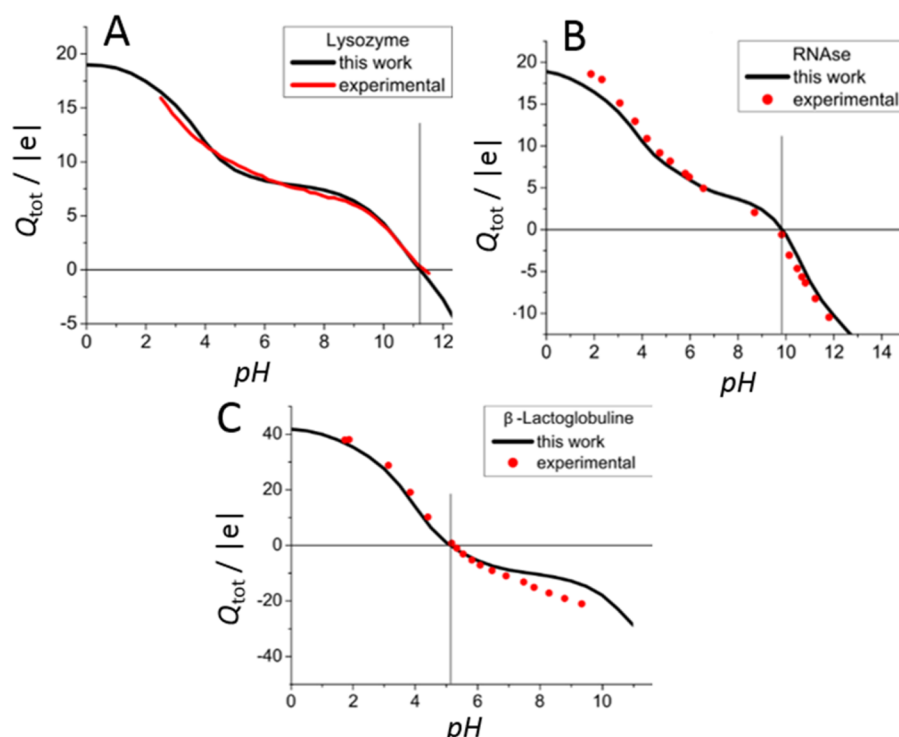


Figure 2. Comparison of experimental and calculated titration curves (net protein charge, Q_{tot} , vs pH) for lysozyme ((A) experimental data was originally plotted as a solid line),⁴⁵ RNase (B),⁴⁶ and β -lactoglobulin (C).⁴³ The isoelectric point (IEP) of each protein ($Q_{\text{tot}} = 0$) is indicated with straight lines.

We observe a good agreement between theory and experiment for lysozyme and RNase in the whole pH range under study. In the case of β -lactoglobulin, a good agreement is observed for acidic pHs, although our prediction deviates from the experimental results for $\text{pH} > 5$. We speculate that this discrepancy may arise from the fact that the crystallographic structure of β -lactoglobulin used in the calculations corresponds to a sample crystallized at $\text{pH} 5.6$,⁴² but β -lactoglobulin is known to undergo conformational changes above $\text{pH} 4.5$, followed by unfolding above $\text{pH} 9$.^{43,44} Therefore, the structure used in our calculations may appreciably differ from that present in solution for $\text{pH} > 5$.

As an additional validation, we applied our methodology to calculate the apparent pK_{a} s, $\text{pK}_{\text{a}}^{\text{app}}$, of each titratable amino acid in lysozyme (the $\text{pK}_{\text{a}}^{\text{app}}$ of an acid–base site is defined as the solution pH for which the site has an average ionization fraction of $f = 0.5$). Lysozyme is usually used to benchmark the performance of pK_{a} calculations^{28,35,36} because the $\text{pK}_{\text{a}}^{\text{app}}$ of most of its amino acids have been experimentally determined⁴⁷ and because this enzyme is very stable to changes of temperature, pH, and salt concentration. Our results (see Figure S2 in the SI) show an average difference of 0.87 pK_{a} units between the $\text{pK}_{\text{a}}^{\text{app}}$ predicted by our method and the experimental values. This difference is similar to those obtained with other pK_{a} calculation programs: PROPKA³⁷ (0.95), Fambe-pH³⁵ (1.16), and Delphi³⁴ (0.75).

So far, we showed that our method can correctly predict titration curves in bulk and the apparent pK_{a} s of individual amino acids with the same level of accuracy as that of other methods in the literature. A key advantage of our method, compared with available pK_{a} -predicting tools, is that it allows straightforwardly incorporating the presence of a charged surface in the system.

Interaction between Isoelectric Proteins and Charged Surfaces. Let us consider the interaction between proteins that are at their isoelectric point (IEP) and thus have zero net charge in bulk (isoelectric proteins) with charged surfaces. We first determine the grand-canonical free energy of adsorption $\Delta\Omega^{\text{ads}}$, defined as

$$\Delta\Omega^{\text{ads}} = \Omega^{\text{prot/surf}} - \Omega^{\text{prot/sol}} - \Omega^{\text{surf}} \quad (6)$$

where $\Omega^{\text{prot/surf}}$, $\Omega^{\text{prot/sol}}$, and Ω^{surf} are the free energies (determined with eq 1) of the protein–surface system, the protein in the bulk solution, and the surface in the absence of the protein, respectively. It is worthwhile to recall that in this work, we are interested in studying the electrostatic contribution to the adsorption free energy (where “electrostatic contribution” is broadly defined and includes effects such as counter-ion release and charge regulation). Therefore, the functional Ω (eq 1) and the values of $\Delta\Omega^{\text{ads}}$ derived from it contain only contributions from the electrostatic energy, mixing entropies of the free ions, and the free-energy contributions from chemical equilibria. They do not include other contributions, such as protein–surface hydrogen bonds and hydrophobic interactions, which also contribute to the thermodynamics of the adsorption⁶ (unlike the electrostatic interactions, these contributions are very dependent on the chemical nature of the surface⁴⁸). Therefore, $\Delta\Omega^{\text{ads}}$ does not allow to unequivocally predict whether adsorption will occur or not but it rather indicates whether electrostatic interactions favor adsorption or not.

Since $\Delta\Omega^{\text{ads}}$ depends on the orientation of the protein and its distance from the surface, we performed calculations fixing the distance between the surface and the center of the bead closest to it to 0.6 nm and we sampled the orientational space of the protein on the surface by considering 404 different

orientations, each one characterized by two rotation angles, φ and ϕ ; see the SI. Figure 3 shows color maps of $\Delta\Omega^{\text{ads}}$ as a

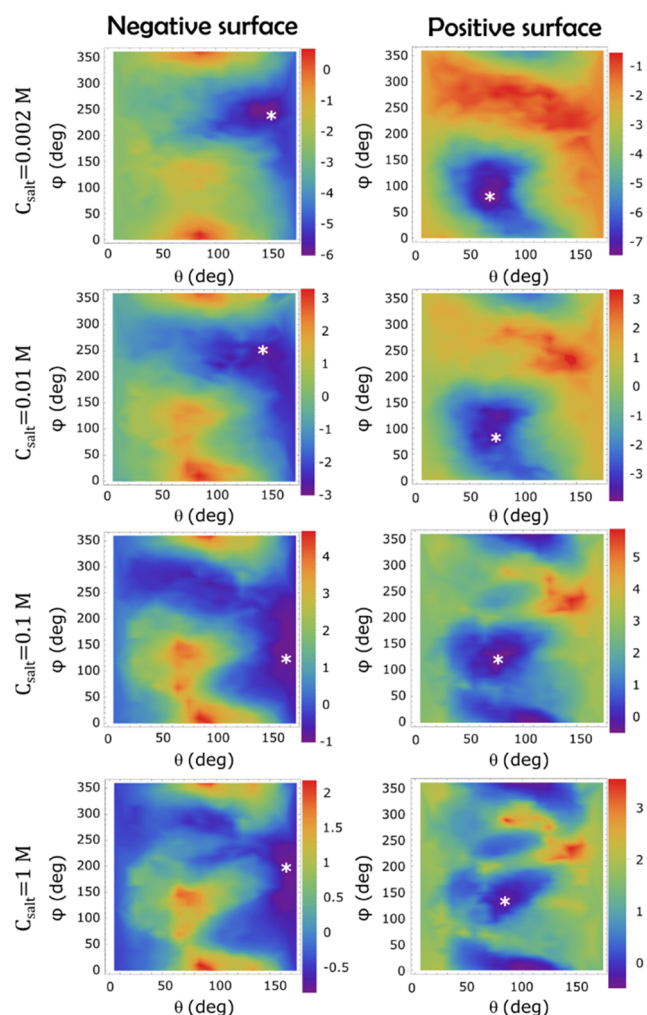


Figure 3. Color maps showing the adsorption (grand canonical) free energy, $\Delta\Omega^{\text{ads}}$, for lysozyme (in units of $k_B T$) as a function of the angles φ (azimuthal angle, y axis) and θ (polar angle, x axis), which determine the orientation of the protein on the surface. The plots correspond to negatively (left panels, $\sigma = -1 \text{ l e l nm}^{-2}$) and positively (right panels, $\sigma = 1 \text{ l e l nm}^{-2}$) charged surfaces and different concentrations of added 1:1 electrolyte, C_{salt} . White asterisks indicate the orientations of the protein with the lowest $\Delta\Omega^{\text{ads}}$ for each plot.

function of φ and ϕ for the adsorption of lysozyme at different ionic strengths and on negative and positive surfaces (similar plots for the other proteins are presented in Figures S3–S5 in the SI). In all calculations, we used a surface charge density, $\sigma = -1 \text{ l e l nm}^{-2}$ (negative surfaces) or 1 l e l nm^{-2} (positive surfaces), which is in the same order of magnitude as that of model silica surfaces, which have $\sigma = -2.6 \text{ l e l nm}^{-2}$.³¹ We observe that $\Delta\Omega^{\text{ads}}$ strongly depends on protein orientation. For some orientations, $\Delta\Omega^{\text{ads}}$ is negative, indicating a favorable electrostatic contribution to the adsorption of the isoelectric protein. As expected for an attraction of electrostatic nature, the minima of $\Delta\Omega^{\text{ads}}$ become less negative as the added salt concentration increases (see scales of the color maps in Figure 3). The effect of the sign of the surface charge and concentration of added salt on protein adsorption shows some interesting features: (i) the maxima in the plots for the

positive surface are close to but not exactly in the same position as the minima in the plots of the negative surface (and vice versa); (ii) the most stable orientation on a given surface changes with the concentration of added salt. These two effects are a consequence from the complex interplay between electrostatic attractions and repulsions in the system, which is modulated by charge regulation and the screening of charges by the electrolyte.

Analysis of Lysozyme–Surface Interaction. We will now provide a detailed analysis of the determinants of protein adsorption for a selected case. Figure 4A,B shows color maps

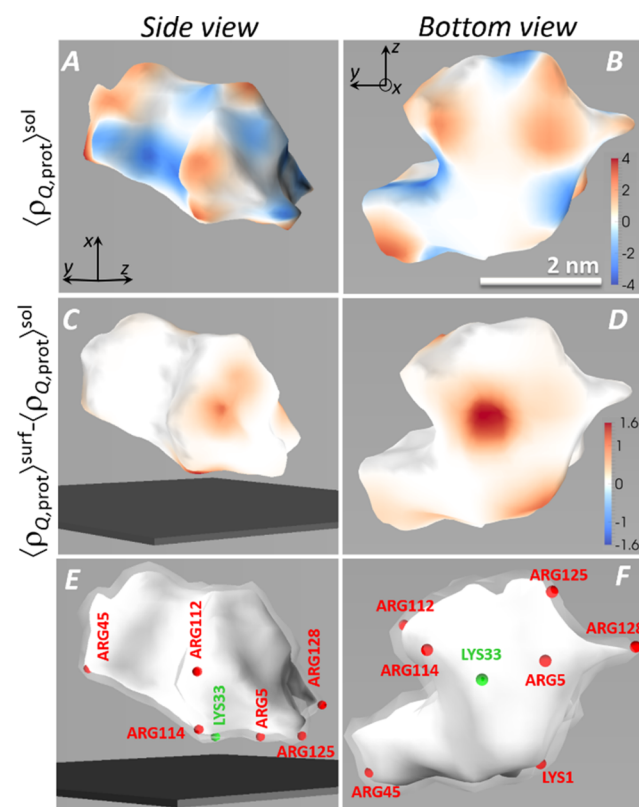


Figure 4. (A, B) Color map of the charge density of the amino acids of lysozyme in the bulk, $\langle\rho_{Q,\text{prot}}\rangle^{\text{sol}}$ (units of l e l nm^{-3}). Red and blue regions indicate positive and negative charge densities, respectively. The bottom view (B) shows the side of lysozyme that will interact with the charged surface. (C, D) Color maps showing the change of the charge density of the amino acids of lysozyme upon introducing the negatively charged surface, $\langle\rho_{Q,\text{prot}}\rangle^{\text{surf}} - \langle\rho_{Q,\text{prot}}\rangle^{\text{sol}}$. Red regions indicate an increase of the charge density upon adsorption. (E, F) Plots showing the position of some relevant amino acids on the surface of lysozyme.

of the average charge density of lysozyme in solution (in units of elemental charges per nm^3) for $\text{pH} = \text{IEP}$ and $C_{\text{salt}} = 2 \text{ mM}$. Let us now consider the interaction of lysozyme with a negatively charged surface. The bottom view (Figure 4B) shows the side of lysozyme that will face the surface for the orientation with minimal $\Delta\Omega^{\text{ads}}$ (orientation indicated with an asterisk in the topmost right panel of Figure 3). Figure 4E,F indicates with red dots the positions of the amino acids that are relevant for the lysozyme–surface interaction. Note that each of these amino acids corresponds to a positive charge density (red spot) in Figure 4A,B. The amino acids Arg114, Arg125, Arg128, Arg5, and Lys1 are very close to the surface and almost fully dissociated; therefore, these amino acids

377 contribute the most to the protein–surface attraction. Other
378 amino acids that are almost fully dissociated are Arg45 and
379 Arg112, but they are farther from the surface than the other
380 five amino acids mentioned above.

381 Upon protein adsorption on the negatively charged surface,
382 the amino acids regulate their charge in response to the charge
383 of the surface. The negatively charged surface inhibits the
384 dissociation of negatively charged amino acid and enhances the
385 ionization of positive ones. This charge regulation (CR)
386 mechanism results in an increase of the net charge of the
387 protein, which favors protein adsorption. It is important to
388 stress that the CR mechanism cannot be understood in terms
389 of the charge density of the amino acid within the protein in
390 bulk solution rather it is necessary to examine the charge of
391 lysozyme in the presence of the surface. Figure 4C,D, depicts
392 color maps of the change in the charge density of the protein
393 induced by the presence of the negative surface (red spots
394 indicate an increase of the charge density upon adsorption).
395 Notably, Lys33 (indicated with a green spot in Figure 4E,F)
396 resides very close to the surface and its degree of ionization for
397 $\text{pH} = \text{IEP}$ changes from weakly dissociated ($f = 0.09$) in
398 solution to strongly dissociated ($f = 0.72$) in the presence of
399 the surface. Therefore, it is very interesting that Lys33 strongly
400 contributes to the adsorption process, even though it is only
401 weakly charged in the bulk protein.

402 It is worthwhile to compare the results in Figure 4 with the
403 predictions of constant-charge MD simulations in the
404 literature, which modeled the adsorption of lysozyme on
405 negatively charged silica surfaces at pH 7^{49,50} and 8.⁵¹ The
406 orientation predicted by our model for lysozyme at its
407 isoelectric point (pH 11.2) on a negative surface is very
408 close to the most stable orientation predicted by these MD
409 simulations.^{49–52} Moreover, MD studies have identified the
410 same amino acids as in our study (Lys1, Arg5, Arg125, and
411 Arg128) as the amino acids that reside closest to the surface
412 and contribute the most to the interaction with the silica
413 substrate.

414 In Figure 5, we analyze the CR effect in further detail. Figure
415 5A,B shows the titration curves (total protein charge, Q_{tot} vs
416 pH) for lysozyme in solution and in the presence of the
417 negatively charged surface for total ionic strengths of $I = 2$ mM
418 (Figure 5A) and 100 mM (Figure 5B). For $I = 2$ mM,
419 lysozyme on the surface has between one to four positive
420 charges (depending on the pH) more than lysozyme in
421 solution, which shows a strong CR effect. On the other hand,
422 for $I = 100$ mM, the differences between the bulk and the
423 surface titration curves are very slight but the CR effect is still
424 operative and it still can produce important shifts in the
425 apparent pK_{a} s of individual amino acids. This effect is
426 demonstrated in Figure 5C, which shows the degree of
427 ionization, f , as a function of pH for Lys33 and Asp119 in
428 lysozyme in solution and on a negative surface for $I = 100$ mM.
429 Interestingly, even at this relatively large ionic strength, there
430 are large shifts in the $\text{pK}_{\text{a}}^{\text{app}}$ of the amino acid upon
431 adsorption: $\Delta\text{pK}_{\text{a}}^{\text{app}} = +0.89$ for Lys33 and $+0.60$ for Asp119,
432 where we defined $\Delta\text{pK}_{\text{a}}^{\text{app}} = \text{pK}_{\text{a}}^{\text{app,surf}} - \text{pK}_{\text{a}}^{\text{app,sol}}$, see Figure
433 5C. Surface-induced pK_{a} shifts can be very important for
434 enzymes whose catalytic activity rely on the acid–base
435 properties of the amino acid in the active site.^{53–55} In this
436 type of enzymes, one can expect that shifts in the apparent
437 pK_{a} s of the amino acid in the active site upon adsorption will
438 affect the catalytic efficiency and/or shift the pH of optimal
439 catalytic activity of the enzyme.^{3,56,57}

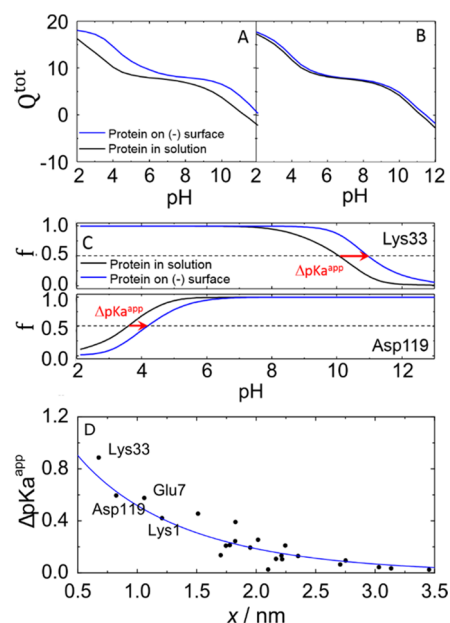


Figure 5. (A, B) Titration curves (total protein charge vs pH) for lysozyme in bulk (black line) and on a negatively charged surface (blue line) for ionic strengths of 2 mM (A) and 100 mM (B). (C) Titration curves (average fraction of ionization, f , vs pH) for Lys33 and Asp119 for an ionic strength of 100 mM. The plots show titration curves for the amino acid inside lysozyme in bulk solution (black line) and on a negatively charged surface (blue line). The changes of the apparent pK_{a} s upon adsorption, $\Delta\text{pK}_{\text{a}}^{\text{app}}$, are shown with horizontal red arrows. (D) Change in $\text{pK}_{\text{a}}^{\text{app}}$ upon adsorption, $\Delta\text{pK}_{\text{a}}^{\text{app}}$, as a function of the distance from the charged surface, x , for the amino acid in lysozyme. The blue solid line shows the prediction of eq 7. In all cases, the orientation of lysozyme on the surface corresponds to that with the minimal adsorption free energy (minimal $\Delta\Omega^{\text{ads}}$ in the corresponding plots of Figure 3).

50 far, we have shown that the charge of the surface induces 440
shifts in the $\text{pK}_{\text{a}}^{\text{app}}$ of the amino acid upon adsorption (up to 1 441
 pK_{a} unit). The magnitude of these shifts (i.e., the magnitude of 442
 $\Delta\text{pK}_{\text{a}}^{\text{app}}$) is expected to decrease for increasing distance 443
between the amino acid and the surface. This prediction is 444
confirmed in Figure 5D, which shows $\Delta\text{pK}_{\text{a}}^{\text{app}}$ for different 445
amino acids as a function of the distance of the amino acid 446
from the surface for $I = 100$ mM (some arginines that have 447
 $\text{pK}_{\text{a}}^{\text{app}} > 13$ are not included in the plot). 448

The trend observed in Figure 5D can be captured by a 449
simple model that considers the effect of the local electrostatic 450
potential on the $\text{pK}_{\text{a}}^{\text{app}}$. The model, which is derived in the SI, 451
relates the surface-induced $\text{pK}_{\text{a}}^{\text{app}}$ shift of an amino acid with 452
its distance to the charged surface, x 453

$$\Delta\text{pK}_{\text{a}}^{\text{app}} = 1.74 \cdot a \cdot \tanh \left[\tanh \left(\frac{1}{2} a \sinh \left(\frac{\sigma}{(8/\epsilon k_{\text{B}} T)^{1/2}} \right) \right) \right] \exp(-\kappa x) \quad (7)$$

where κ is the Debye length of the solution and ϵ is the 455
dielectric constant of the medium. 456

The prediction of eq 7 is shown with a blue solid line in 457
Figure 5D. The simple model captures very well the general 458
trend displayed by the more detailed calculations of our 459
theoretical method. The differences between the predictions of 460

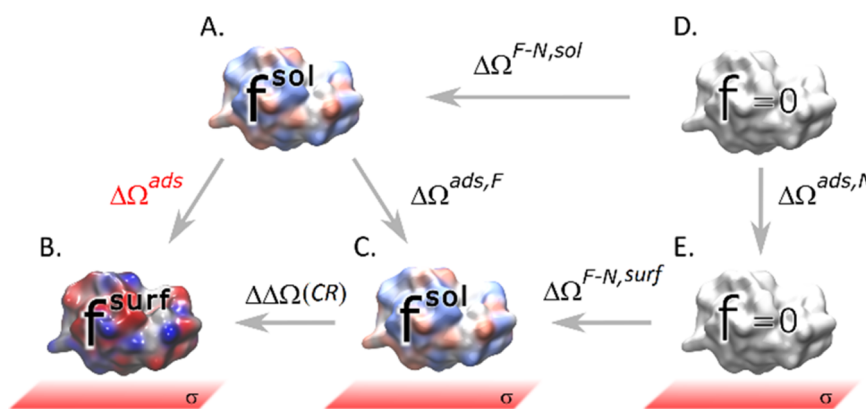


Figure 6. Thermodynamic cycle used to study the contributions from CR and CP to the adsorption of isoelectric proteins onto charged surfaces. The states represented in the cycle correspond to: (A) protein far from the charged surface (i.e., in the bulk solution); (B) protein on a charged surface, where the average state of ionization of each amino acid changes with respect to state (A) due to the charge-regulation mechanism; (C) hypothetical state, where the protein is placed on the charged surface but retains the same state of charge as that of the protein in the solution (i.e., same charge state as that of the protein in state (A)); (D) protein in solution in a hypothetical state, where all acid–base groups are nonionized; and (E) same as (D) but for the protein on a charged surface.

eq 7 and those of our theoretical method are due to the local environment of each amino acid inside the protein. We believe that eq 7 can be used by experimental groups working with adsorbed proteins to estimate the magnitude of surface-induced pK_a^{app} shifts of single amino acid. The main difficulty in using the equation is to determine the distance between the amino acid and the surface. We suggest that this parameter can be estimated by combining the crystallographic structure of the protein (i.e., obtained from PDB files) with experimental or theoretical information about its orientation on the substrate.

Analysis of the Contributions from Charge Regulation (CR) and Charge Patches (CP) to Protein Adsorption. We will address in this section the different contributions to $\Delta\Omega^{ads}$, with special focus on the relative strength of two electrostatic mechanisms that promote the adsorption of proteins: charge regulation (CR) and charge patches (CP). It is worthwhile to mention that these contributions are not explicit terms in our free energy functional, eq 1, and they can only be extracted from our theory by comparing the free energies of different states of the protein/surface system. For this purpose, we devised the thermodynamic cycle in Figure 6. This cycle relates different states of the system. Some of them are hypothetical states that do not correspond to real states of the protein/surface system. The charge distribution of the protein in each state in Figure 6 is unequivocally defined by the values of the average fractions of ionization of all acid–base groups, i.e., the values of f_j for all j . We will refer to this set of values as f . The free-energy differences between the different states in Figure 6 are calculated using our theoretical method, as described in the Theoretical Approach section, although in the hypothetical states, f is fixed to a given value instead of being determined from the theory (i.e., by solving eq 5 for acidic groups or the equivalent equation for basic groups). In other words, in these hypothetical states, the free energy is no longer required to be an extrema with respect to the ionization fractions f_j .

The state A in Figure 6 corresponds to the protein very far from the charged surface (charge state f^{sol}), and the state B corresponds to the protein on the charged surface (charge state f^{surf}). The change of the grand-canonical free energy Ω between states A and B is $\Delta\Omega^{ads}$, which has been already defined in eq 6 and discussed in Figure 3. State C in Figure 6 is

a hypothetical state in which the protein is located on the surface but it keeps the same state of charge as that in bulk solution (i.e., it has a charge state f^{sol}). The free-energy difference between states C and A is, therefore, the free energy of adsorption of a protein that cannot regulate its charge, which we denote $\Delta\Omega^{ads,F}$ (where the superscript F indicates fixed charge). On the basis of these definitions, we identify the contribution of charge regulation to the total adsorption free energy as

$$\Delta\Delta\Omega(CR) = \Delta\Omega^{ads} - \Delta\Omega^{ads,F} \quad (8)$$

States D and E in the cycle of Figure 6 are hypothetical states of a protein far from the surface (state D) or on the surface (state E) in which the charges of all amino acids have been set to zero (i.e., $f = 0$). Note that this situation differs from that in states A and B, where the net protein charge is zero (because $pH = IEP$), but each acid–base group has a nonzero average charge. The free-energy difference between states D and E is $\Delta\Omega^{ads,N}$, which is an adsorption free energy for a hypothetical protein that has nonionized acid–base groups. The value of $\Delta\Omega^{ads,N}$ is always positive (i.e., favors protein–surface repulsion) because of the combination of two effects: (i) placing a protein with $f = 0$ near the charged surface displaces the counterions that are compensating the charge of the surface, which results in an increase in system's free energy; (ii) the protein has a dielectric constant smaller than that of the solvent; thus, placing it in the electric field generated by the surface results in an energetic penalty. Additional calculations (presented in Figure S6 in the Supporting Information) show that the first mechanism (disruption of electrical double layer by the protein) accounts for 72–85% of the total value of $\Delta\Omega^{ads,N}$.

At this point, we can isolate the contribution of charge patches to the total adsorption free energy, $\Delta\Delta\Omega(CP)$. This contribution is equal to the adsorption free energy of the hypothetical protein with nonregulating acid–base groups (i.e., a protein that has charge patches but cannot regulate charge), $\Delta\Omega^{ads,F}$ minus the adsorption free energy of the protein with fully nonionized acid–base groups, $\Delta\Omega^{ads,N}$

$$\Delta\Delta\Omega(CP) = \Delta\Omega^{ads,F} - \Delta\Omega^{ads,N} \quad (9)$$

542 Analysis of the thermodynamic cycle in Figure 6 provides an
543 alternative definition of $\Delta\Delta\Omega(\text{CP})$

$$544 \quad \Delta\Delta\Omega(\text{CP}) = \Delta\Omega^{\text{F-N,surf}} - \Delta\Omega^{\text{F-N,sol}} \quad (10)$$

545 where $\Delta\Omega^{\text{F-N,surf}}$ and $\Delta\Omega^{\text{F-N,sol}}$ are the free-energy changes
546 due to introducing the charge patches (changing the state of
547 charge from $f = 0$ to f^{ol}) in a protein on the surface and in a
548 protein in solution, respectively. Therefore, according to this
549 definition, $\Delta\Delta\Omega(\text{CP})$ is the difference between the free energy
550 of introducing the charge patches in a protein on the surface
551 and the free energy of introducing them in a protein in the
552 solution.

553 Adding eqs 8 and 9 results in

$$554 \quad \Delta\Omega^{\text{ads}} = \Delta\Delta\Omega(\text{CR}) + \Delta\Delta\Omega(\text{CP}) + \Delta\Omega^{\text{ads,N}} \quad (11)$$

555 which shows that the total adsorption free energy can be split
556 into three contributions: one from charge regulation, $\Delta\Delta\Omega(\text{CR})$;
557 one from charge patches, $\Delta\Delta\Omega(\text{CP})$, and one from the
558 adsorption of a neutral protein, $\Delta\Omega^{\text{ads,N}}$. Equation 11 is the
559 most important result from the thermodynamic cycle in Figure
560 6 because it shows that the total adsorption free energy in our
561 theory can be split into three contributions that are attributable
562 to well-defined processes.

563 In Figure 7, we plot $\Delta\Delta\Omega(\text{CR})$, $\Delta\Delta\Omega(\text{CP})$, and $\Delta\Omega^{\text{ads,N}}$ as
564 a function of $\Delta\Omega^{\text{ads}}$ for lysozyme under the same conditions of
565 Figure 3 (positive and negative surfaces and four different
566 concentrations of added salt). Note that each point in the plots
567 of Figure 7 corresponds to a different orientation of the protein
568 on the surface. The most stable orientations (points with
569 minimal $\Delta\Omega^{\text{ads}}$) are, therefore, located on the left side of each
570 plot.

571 As explained above, the contribution from the adsorption of
572 the neutral protein, $\Delta\Omega^{\text{ads,N}}$, is always positive. It is also rather
573 insensitive to protein orientation because it depends only on
574 the volume distribution of the protein along the direction
575 normal to the surface. This volume distribution does not
576 change too much with protein orientation unless the protein is
577 highly elongated.

578 The contribution from charge regulation, $\Delta\Delta\Omega(\text{CR})$, is
579 always negative and similar for all orientations. We attribute
580 the insensitiveness of $\Delta\Delta\Omega(\text{CR})$ to orientation to the fact that
581 all amino acids in the protein can regulate charge (although
582 differently, depending on their pK_a^{app} and solution pH).
583 Therefore, in all orientations there are amino acids close to the
584 surface that can contribute to $\Delta\Delta\Omega(\text{CR})$. At this point, it is
585 useful to highlight that most MD simulations aiming to
586 determine the orientation of proteins on surfaces use fixed
587 charges and therefore neglect CR effects. The fact that
588 $\Delta\Delta\Omega(\text{CR})$ is relatively insensitive to protein orientation
589 suggests that fixed-charge MD does not introduce an important
590 error by neglecting CR when comparing the relative stability of
591 different protein orientations, thus validating the use of
592 constant-charge simulations for this purpose. Furthermore,
593 the insensitiveness of $\Delta\Delta\Omega(\text{CR})$ with protein orientation
594 observed in Figure 7 for $\text{pH} = \text{IEP}$ is maintained even when
595 the protein has a nonzero charge in solution (pH not equal to
596 IEP); see below.

597 The contribution from CP, $\Delta\Delta\Omega(\text{CP})$, strongly depends on
598 protein orientation, and it can be either positive or negative.
599 There is a strong linear correlation between $\Delta\Delta\Omega(\text{CP})$ and
600 $\Delta\Omega^{\text{ads}}$, which indicates that $\Delta\Delta\Omega(\text{CP})$ is mainly responsible
601 for determining the most favorable orientation on the surface.

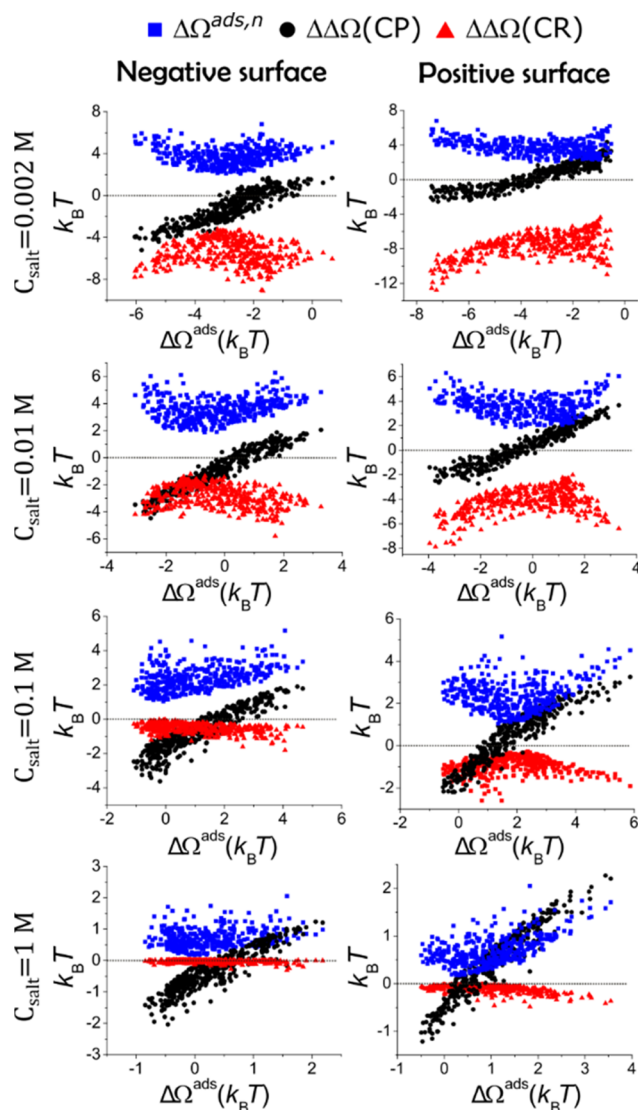


Figure 7. Contribution from charge patches ($\Delta\Delta\Omega(\text{CP})$, black circles), charge regulation ($\Delta\Delta\Omega(\text{CR})$, red triangles), and free energy of adsorption of neutral protein ($\Delta\Omega^{\text{ads,N}}$, blue squares) to the adsorption free energy as a function of the total adsorption free energy, $\Delta\Omega^{\text{ads}}$. Each point corresponds to a different orientation of the protein on the surface. The plots correspond to lysozyme at $\text{pH} = \text{IEP}$ for the same conditions as those in Figure 3.

At this point, we ask the question of which mechanism, CP or CR, contributes the most to the adsorption free energy of isoelectric proteins. The contributions to $\Delta\Omega^{\text{ads}}$ for the most stable orientation (orientations with most negative $\Delta\Omega^{\text{ads}}$) in each panel of Figure 7 show that the answer to this question depends on the salt concentration of the solution. For low salt concentration (2 mM), both CP and CR contribute to protein adsorption, with charge regulation accounting for $\sim 80\%$ of the total stabilization free energy ($\Delta\Delta\Omega(\text{CP}) + \Delta\Delta\Omega(\text{CR})$) for the negative surface and $\sim 65\%$ for the positive surface. On the other hand, for a high concentration of added salt (1.0 M), the CR contribution is almost zero and therefore the CP contribution dominates. The CR effect vanishes at high ionic strength because its magnitude depends on the intensity of the electrostatic potential generated by the surface at the position of the acid–base group; see eq 7. Therefore, as the ionic strength of the solution increases, salt ions screen the charge of

the surface and decrease the magnitude of the local electrostatic potential at the position of the acid–base groups. As a consequence, the charge regulation effect weakens and $\Delta\Delta\Omega(\text{CR})$ approaches zero. Note that the contribution due to charge patches, $\Delta\Delta\Omega(\text{CP})$, also decreases in magnitude upon increasing the ionic strength of the solution, but this decrease is less dramatic than that of $\Delta\Delta\Omega(\text{CR})$. In other words, although both mechanisms, CR and CP, depend on the magnitude of the electrostatic potential at the position of the protein, the CR effect is more affected by this variable than the CP mechanism.

The conclusions reached so far for lysozyme are valid for the other proteins as well. Figure 8 compares the different

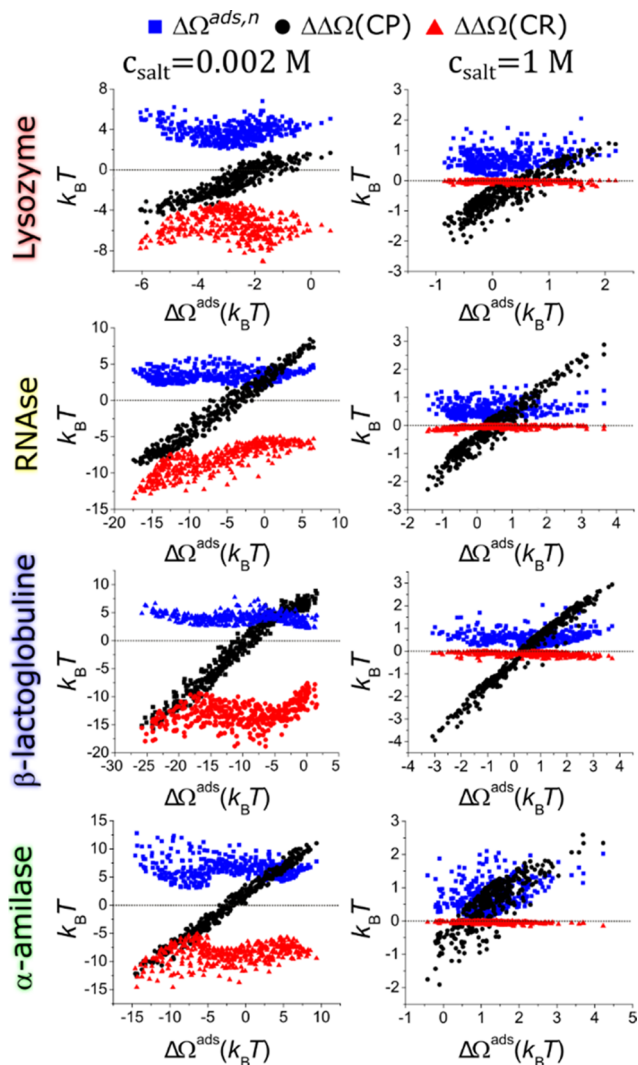


Figure 8. Same as Figure 7 for different proteins adsorbed on negatively charged surfaces ($\sigma = -1 \text{ lel nm}^{-2}$) and salt concentrations of 2 mM (left panels) and 1 M (right panels).

contributions to $\Delta\Omega^{\text{ads}}$ for the four proteins considered in this study on negatively charged surfaces for salt concentrations of 2 mM and 1.0 M. We observe that the results for all proteins qualitatively follow the same trends discussed in Figure 7 for lysozyme. Figures S7–S9 in the Supporting Information show additional plots for β -lactoglobulin, α -amylase, and RNase for other salt concentrations and positively charged surfaces.

We finally briefly address the case discussed in the introduction where the protein has a net charge of the same sign as that on the surface, i.e., the problem of protein adsorption on the “wrong side” of the isoelectric point. In Figure 9, we analyzed the adsorption of β -lactoglobulin on

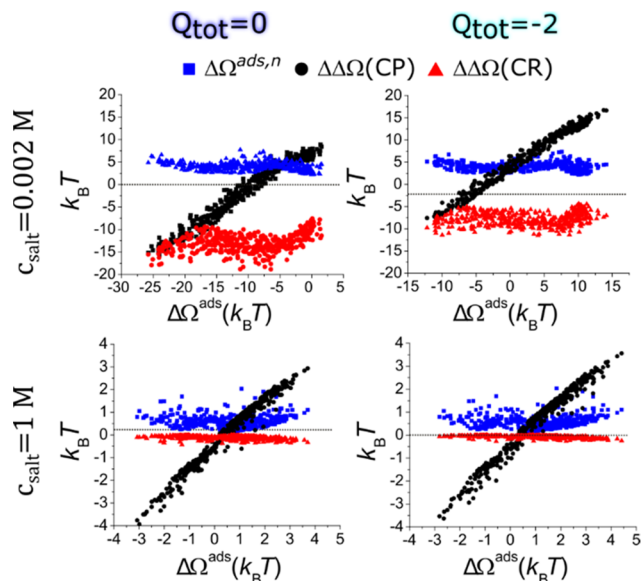


Figure 9. Comparison of the CP and CR contributions to the adsorption of β -lactoglobulin on negatively charged surfaces ($\sigma = -1 \text{ lel nm}^{-2}$) for $Q_{\text{tot}} = -2 \text{ lel}$ (pH 5.8) and $Q_{\text{tot}} = 0$ (pH 5.1) for salt concentrations of 2 mM and 1 M.

negatively charged surfaces at pH 5.8, where the net charge in solution is -2 lel (the IEP of β -lactoglobulin is 5.1). Most of the conclusions obtained in Figure 7 for the isoelectric protein hold for the case of a protein with the same charge as that of the surface: (i) there are orientations on the surface that have $\Delta\Omega^{\text{ads}} < 0$; thus, electrostatics favors adsorption even on the wrong side of the IEP; (ii) the contribution of the CR effect is rather insensitive to protein orientation and strongly decreases with increasing salt concentration; and (iii) the CP effect is still responsible for dictating the most stable orientation for the protein on the surface. Note that at low ionic strength, the magnitude of the CR effect is similar to that observed for the isoelectric protein for β -lactoglobulin in Figure 9.

It is worthwhile to mention that in the analysis of Figure 9, we kept the same definitions for the components of $\Delta\Omega^{\text{ads}}$ used for isoelectric proteins; therefore, $\Delta\Delta\Omega(\text{CP})$ now accounts for the free energy of both bringing the net charge of the protein from 0 to -2 lel and introducing the charge patches (the separation of these two processes is possible although not free of ambiguities and it will not be discussed in this work). However, it is worthwhile to note that the difference of $\Delta\Delta\Omega(\text{CP})$ between the most stable and the least stable orientations for β -lactoglobulin is similar for a net charge of -2 lel (Figure 9, lower and upper right panels) and a net charge of zero (Figure 9, lower and upper left panels). This result suggests that the stabilization due to charge patches is similar in both cases; therefore, our conclusion that the CP contribution is similar to that of CR at low ionic strength and dominates at high ionic strengths also holds for proteins on the wrong side of the isoelectric point.

675 ■ CONCLUSIONS

676 In this work, we introduced a new theoretical framework to
677 study the acid–base and electrostatic properties of proteins
678 and validated it against experimental titration curves and
679 pK_a^{app} values. Although there exist several methods in the
680 literature for predicting the pK_a^{app} of amino acid within
681 proteins, our method has the key advantage to allow easily
682 incorporating the presence of a surface in the calculations. Our
683 method is also computationally efficient; for instance, in this
684 work, we performed systematic calculations for four different
685 proteins, 404 orientations, two types of surfaces (positive and
686 negative), four different concentrations of added salt, and
687 seven different states (the states in the thermodynamic cycle of
688 Figure 6), which account for a total of $\sim 9 \times 10^4$ different cases.
689 Systematic studies of this size would be prohibitive with more
690 accurate (and more expensive) calculation methods, such as
691 constant-pH MD simulations.

692 We combined our theoretical framework with a strategy
693 designed to isolate the contributions from the CR and CP
694 mechanisms to the total adsorption free energy. This
695 theoretical development allowed us to address for the first
696 time the competition between CR and CP in a realistic protein
697 model. Interestingly, the predictions of our study show some
698 differences from those in previous works, which analyzed
699 protein adsorption on soft surfaces using other levels of
700 theoretical approximation.^{19,38} For example, these works
701 suggested that the CR mechanism was the largest contribution
702 to the free energy of adsorption at low ionic strengths,^{19,38}
703 whereas we show that for the adsorption on a planar charged
704 surface, both the CR and CP mechanisms have similar
705 relevance (for $C_{\text{salt}} = 2$ mM). In agreement with all previous
706 literature reports, we found that the importance of the CR
707 effect decreases for increasing ionic strength. Notably, we
708 found that the magnitude of the CP effect also decreases with
709 increasing salt concentration, although to a lesser degree than
710 that in the CR mechanism.

711 An interesting prediction of our study is that the
712 contribution from the CR mechanism is rather insensitive to
713 the orientation of the protein on the surface; therefore, the
714 orientation is mainly determined by the CP mechanism. This
715 conclusion suggests that neglecting the CR effect when
716 predicting protein orientations on a surface (which is the
717 rule in constant-charge MD simulations) is a good
718 approximation. Note, however, that neglecting CR can still
719 result in an incorrect picture of the adsorption mechanism;
720 e.g., in Figure 4, we show that Lys33 contributes to the
721 adsorption of lysozyme onto a negatively charged surface, even
722 when this amino acid is uncharged in bulk solution. This
723 information is inaccessible to constant-charge calculations.

724 We showed that the effect of the surface on the pK_a^{app} of
725 individual amino acids decays with increasing distance to the
726 surface, and we provided an explicit expression (eq 7) that
727 captures the effect. Surface-induced pK_a^{app} shifts for functional
728 amino acids, such as those involved in enzymatic catalytic sites,
729 can be very important for the behavior of immobilized
730 proteins. Our expression will allow to estimate the magnitude
731 of the effect, provided that the position of each amino acid can
732 be estimated from the structure of the protein (e.g., from the
733 protein data bank) and knowledge of the orientation of the
734 protein on the surface.

735 In future work, we plan to extend the capabilities of our
736 theoretical framework to study some poorly understood

phenomena, such as protein adsorption on porous and
nanomaterials, the effect of interprotein interactions on
adsorption (which is a relevant problem under high-surface-
coverage conditions), and the effect of adsorption on the
functional properties of the proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the
ACS Publications website at DOI: 10.1021/acs.lang-
muir.8b03411.

Detailed description of theoretical methods, comparison
between all-atom and coarse-grained representations of
lysozyme, comparison between theoretical predictions
and experiments for the pK_a^{app} of amino acid in
lysozyme, adsorption free energy and its components
for β -lactoglobulin, RNase, and α -amylase, derivation of
eq 7, contributions to the free energy of adsorption of
neutral proteins and method to sample protein
orientations on the surface (PDF)

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Author Contributions

The manuscript was written through contributions of all
authors. All authors have given approval to the final version of
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Notes

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