Effects of Cooperativity in Proton Binding on the Net Charge of Proteins in Charge Ladders

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This paper addresses the effects of cooperativity of proton binding on values of net charge and the change in net charge of a protein ΔZ due to the modification of charged groups. Capillary electrophoresis (CE) and charge ladders—collections of protein derivatives that differ in charge—are used to measure the net charge of lysozyme and values of pK_a of the *N*-terminal α-amino group of this protein with different numbers of Lys ϵ-amino groups acetylated. Values of pK_a serve as local thermodynamic reporters of electrostatic potentials and therefore provide a direct measure of cooperativity in proton binding. Measured values are compared with values calculated using a model which combines the Poisson−Boltzmann (PB) equation and atomically detailed models of the distribution of charges on lysozyme with Monte Carlo (MC) sampling of different protonation states of the protein to determine the average extent of protonation of each titratable residue as a function of pH. Calculations with the PB-MC model together with experimental results demonstrate significant cooperativity in proton binding between acetylated Lys ϵ-amino groups and other titratable groups on lysozyme. Two types of interactions are identified: (i) nonspecific interactions between acetylated Lys ϵ-amino group and many other titratable groups on the protein result in the collective reduction in the magnitude of ΔZ of up to 0.1 charge units and (ii) specific interactions between a particular acetylated Lys ϵ-amino group and another titratable group on the protein results in the reduction in magnitude of ΔZ of up to ∼0.3 charge units. These specific interactions occur between groups separated by less than ∼15 Å. Specific interactions are demonstrated for lysozyme at pH 8.4 by the cooperativity in proton binding between the ϵ-amino and *N*-terminal α-amino groups of Lys_1. The PB-MC model effectively identifies both specific and nonspecific cooperative interactions between titratable groups that can affect values of ΔZ. The results of the detailed PB-MC model can be expressed by a simple empirical relationship that describes the cooperativity in proton binding between titratable groups using a screened Coulombic potential. This relationship is useful for the quick and efficient identification of specific cooperative interactions between titratable groups. Finally, an analysis of the effects of uncertainty in ΔZ on the values of net charge and hydrodynamic radius of proteins measured using CE and charge ladders is presented. The percent uncertainty in these parameters are shown to be less than or equal to the percent uncertainty in ΔZ.

Introduction

Charge ladders are collections of protein derivatives produced by the random, partial chemical modification of charged groups on the protein.[1−4] When separated by capillary electrophoresis (CE), these collections separate into discrete peaks: each peak or "rung" of the ladder is composed of a family of protein derivatives that have the same number of chemical modifications and approximately the same net charge. The combination of charge ladders and CE is a useful biophysical tool for measuring the electrostatic properties and interactions of proteins: examples include the net charge[1] and hydrodynamic size[5] of proteins, values of pK_a of titratable groups,[6,7] and the effects of interactions between charged groups on protein−ligand binding[8,9] and protein stability.[10] Applications of charge ladders and CE to the analysis of proteins rely on the assumption that the change in net charge of a protein ΔZ, due to the modification of charged groups, is known. The objective of this paper is to address the effects of cooperativity in proton binding on values of ΔZ and thereby on the interpretation and analysis of protein charge ladders.

Titration curves of molecules with multiple acidic or basic groups demonstrate cooperativity in the binding affinity for protons (expressed by pK_a, the negative log of the proton dissociation constant). In 1923, Bjerrum[11] showed that the titration behavior of diacids could be described accurately only if a term that reflected the electrostatic energy of interaction between the two titrating sites was included. Surfaces of most proteins are characterized by a large number of basic and acidic residues. The pH dependent net charge of a protein therefore depends on (i) the energy of protonation of the individual residues in the absence of formal charge on other titratable groups on the protein and (ii) on the electrostatic interaction energies between charged groups. This second effect, which gives rise to the cooperativity in proton binding, is described by Coulombic interactions. For a given solvent and protein, these interactions will be dependent on the distance between charged groups on the protein and the presence of other charged species, such as dissolved ions, in solution.

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Charge ladders are typically formed by the acetylation of Lys ϵ-amino groups; doing so converts cationic ϵ-NH_3^+ groups to neutral ϵ-$NHCOCH_3$ groups. The change in net charge of the protein ΔZ that results from the acetylation of a Lys ϵ-amino group depends primarily on the charge of the Lys residues Z_{Lys}, which, in turn, depends on the pK_a of this residue (typically $\sim$10.5) and the pH of the solution. In previous applications of charge ladders, it has usually been assumed that $\Delta Z = -Z_{Lys} = -1$. Doing so implies (i) that all of the Lys ϵ-amino groups are fully protonated at the pH of analysis (usually 8.4) and (ii) the values of pK_a of other titratable groups on the protein, and thereby the values of charge carried by these groups, are unaffected by the annihilation of charge on the Lys residues: that is, there are no effects of cooperativity in the binding of protons on ΔZ.

Effects of cooperativity in proton binding on values of ΔZ for protein charge ladders have been addressed previously. For most proteins, the electrophoretic mobility of the rungs of the ladder with the smallest magnitude of net charge correlates linearly with the number of modified charged groups. For the rungs of ladders that have the highest magnitude of net charge, a nonlinear correlation between mobility and number of modified charged groups was observed for several proteins.[12] It was proposed that cooperativity of proton binding between the Lys and His residues on a protein could be one explanation for this effect. Menon and Zydney showed that cooperativity in proton binding could affect values of ΔZ for all the rungs of a charge ladder.[13] They used a model, originally developed by Linderstrøm-Lang,[14] to describe the role of cooperativity in proton titration curves, to predict the contribution of cooperativity in proton binding to ΔZ. The Linderstrøm-Lang model, which Menon and Zydney refer to as charge regulation, after similar calculations done on synthetic colloidal particles,[15] is based on electrostatic potentials calculated by Debye−Hückel theory. This theory treats the protein as a charged sphere with the charge uniformly distributed on its surface. The presence of finite electrostatic potentials at the surface of the sphere is assumed to alter the proton binding by affecting the local concentration of protons. Menon and Zydney used this model to calculate the net charge of the rungs of the charge ladder of bovine carbonic anhydrase. They compared the results of calculations to values of net charge estimated from measured values of electrophoretic mobility. They concluded that cooperativity had a significant effect on ΔZ, changing it from the ideal value of -1 to -0.86 at a value of pH of 8.4. Recently, Gitlin et al. compared values of net charge measured through linear regression analysis of data from CE and charge ladders with values calculated from the Linderstrøm-Lang model and estimated from the mobility of the unmodified protein using the Hückel model of electrophoresis.[16] They concluded that the value of ΔZ for the ladder was -0.93 and hypothesized that the deviations from the ideal value could be due to shifts in the values of the pK_a of the His, Lys, or Zn−OH groups.

The Linderstrøm-Lang model neglects the explicit distance dependence of electrostatic interactions between titratable groups in predicting the cooperativity of proton binding. In contrast, calculations with more detailed electrostatic models[9] and experiments[17] both have shown that interactions between charged groups on a protein have a strong dependence on the distance between groups. A more complete analysis of the effects of cooperativity in proton binding on values of ΔZ for protein charge ladders requires that this issue of distance dependence be addressed. Furthermore, previous studies of the effects of cooperativity of proton binding on ΔZ compared calculated values of net charge with values estimated from measured electrophoretic mobilities. The difficulty with this comparison is that values of electrophoretic mobility depend on both the charge and the hydrodynamic drag of the protein. To determine net charge, values of electrophoretic mobility were fit to an electrokinetic model for uniformly charged, spherical colloids.[18] This model requires the hydrodynamic radius of the protein as an input. Gitlin et al. showed that the values of charge determined in this way are sensitive to the values of hydrodynamic radius used in the model.[16]

In this paper we directly address the effects of distance dependence of electrostatic interactions between titratable groups on the cooperativity of proton binding on values of ΔZ for protein charge ladders. We use CE and charge ladders to measure the pK_a of the *N*-terminal α-amino group of lysozyme with different numbers of Lys ϵ-amino groups acetylated. Values of pK_a serve as local thermodynamic reporters of electrostatic potentials[17,19] and therefore provide a direct measure of cooperativity in proton binding. Analysis of data from CE provides values of pK_a that do not depend on any assumptions about the value of ΔZ or hydrodynamic radius of the protein.

We compare measured values of pK_a of the *N*-terminal α-amino group of lysozyme with values calculated using two different models of cooperativity of proton binding. The first model is that of Linderstrøm-Lang[14] (referred to herein as the LL model) used by Menon and Zydney.[13] The second model is based on electrostatic potentials calculated using numerical solutions of the linearized Poisson−Boltzmann (PB) equation and atomically detailed models of the distribution of charges on lysozyme.[20] We used these potentials together with Monte Carlo (MC) sampling to calculate the average extent of protonation of each titratable residue as a function of pH.[21−23] We refer to this second model as PB-MC.

In this work we use lysozyme as a model protein. The effective values of pK_a of all the titratable groups of lysozyme have been determined experimentally[24] and calculated using continuum electrostatic theory.[21,25] For cooperativity in proton binding to affect significantly values of ΔZ, the pK_a of a titratable group must be within $\sim \pm 2$ pH units of the pH of the solution. Interactions with groups outside of this range of values of pK_a will not affect the charge carried by these groups: they will remain either fully protonated or deprotonated despite these interactions. For lysozyme, the titratable residues that fall within this range for analysis at pH 8.4 are the *N*-terminal α-amino group (on Lys_1) with a pK_a of $\sim$ 7.5 and three Tyr groups with values of pK_a of $\sim$ 9.6; a single His residue (His_{15}) falls just outside this range with a pK_a of $\sim$6.3.[25,26]

We find that significant cooperativity in proton binding only exists between acetylated Lys ϵ-amino groups and other titratable groups on lysozyme separated by less than approximately 15 Å. This effect is demonstrated at pH 8.4 by the cooperativity in proton binding between the ϵ-amino and *N*-terminal α-amino groups of Lys_1. This cooperativity results in reduction in magnitude of ΔZ of nearly 0.2 units of charge, relative to the ideal value of -1, at pH 8.4. CE measures this effect experimentally as a splitting of the first rung of the ladder of lysozyme. This effect is predicted by the PB-MC model, which also shows there is significant cooperativity in proton binding between the imidazole group of His_{15} and the ϵ-amino groups of Lys_1 and Lys_{96} at values of pH between 5 and 7. By examining the distance between Lys ϵ-amino groups and other titrarable groups, we show that there are potentially a large number of specific interactions between groups on lysozyme that can affect values of ΔZ. The LL model does not capture

these specific interactions. We show that the results of the detailed PB-MC model can be expressed by a simple empirical relationship that describes the cooperativity in proton binding between titratable groups using a screened Coulombic potential that depends explicitly on the distance between the two groups on the protein. This relationship is useful for the quick and efficient identification of cooperative interactions between specific groups that can affect values of ΔZ.

Finally, because cooperativity in proton binding can affect values of ΔZ, we provide an analysis of the effects of uncertainty in ΔZ on the values of net charge and hydrodynamic radius of proteins determined from a linear regression analysis of the data from protein charge ladders. We show that the percent uncertainty in net charge and hydrodynamic radius is less than or equal to the percent uncertainty in ΔZ.

Background

Net Charge of Proteins Measured by Linear Regression Analysis of Charge Ladders. During electrophoresis, a molecule undergoes two counteracting forces. The electrostatic force (F_{elec}, N) acting on a molecule is equal to the product of its net charge (Z, C) and the magnitude of the applied electric field (E, V m^{-1}) (eq 1). The hydrodynamic force (F_{hydro}, N) acting on a molecule is equal to the product of its coefficient of friction (f, N s m^{-1}) and its velocity (ν, m s^{-1}) relative to the surrounding solvent (eq 2). The electrophoretic mobility (μ, m^2 V^{-1} s^{-1}) (i.e., the steady-state velocity per unit applied field) is the result of the balance of electrostatic and hydrodynamic forces acting on the charged molecule (eq 3).

$$F_{elec} = ZE \tag{1}$$

$$F_{hydro} = f\nu \tag{2}$$

$$F_{elec} = F_{hydro} \Rightarrow \frac{Z}{f} = \frac{\nu}{E} = \mu \tag{3}$$

Much of the utility of charge ladders results from the ability to separate the measured values of electrophoretic mobility into the individual contributions of charge and hydrodynamic drag.[5] The ability of CE and charge ladders to measure net charge allows the contribution of electrostatics to the energetics of molecular recognition events, such as protein folding[10] and binding of ligands,[4,8] to be measured. It also provides a simple an efficient method to measure changes in the number of bound protons and, thereby, to quantify the thermodynamic linkage between proton binding and protein folding.[10] Knowledge of the hydrodynamic drag f allows an estimate of the conformational properties of proteins, for example, to monitor the transition of a protein from the native to denatured state, and to measure the average hydrodynamic size of chains in the denatured ensemble.[10]

The electrophoretic mobility of the proteins that make up the nth rung of the charge ladder $\mu_{elec}{}^n$, where n is the number of modified charged groups, is determined experimentally using eq 4, where V is the applied voltage, $t_x{}^n$ is the migration time for the proteins in the nth rung of the ladder, t_{nm} is the migration of a neutral marker (typically a neutral organic molecule such as p-methoxy benzyl alcohol added to the injection sample to measure the rate of electroosmotic flow), and l_{tot} and l_{det} are the total capillary length and the length to the detector window, respectively. By convention, the sign of μ_{elec} (+ or −) is assigned to be the same as the net charge of the protein.

$$\mu_{elec}^n = \frac{l_{tot} l_{det}}{V}\left(\frac{1}{t_x{}^n} - \frac{1}{t_{nm}}\right) \tag{4}$$

If we assume that the charge on the nth rung of the ladder is equal to the charge of the unmodified protein Z^0, plus $n\Delta Z$, we obtain eq 5. This equation shows that a linear regression analysis of a plot of μ_{elec}^n vs $n\Delta Z$ provides a measure of both f and the net charge of the unmodified protein Z^0: the x-intercept of the best-fit line determines Z^0 and its slope provides f. It is clear from this approach that the accuracy with which both Z^0 and f are determined depends on the accuracy to which ΔZ is known.

$$\mu_{elec}^n = \frac{Z^0 + n\Delta Z}{f} \tag{5}$$

Net Charge of Proteins from Simulated Proton Titrations. A state of protonation of a protein can be expressed by the protonation vector **x**. Each element of the vector, x_i, gives the number of protons bound to the ith titratable residue. We consider only two possible protonation states ($x_i = 0$ or 1). The proton titration curve of a residue on a protein is the average value of protonation, $\langle x_i \rangle$ as a function of pH. The proton titration curve of a protein,that is, the total number of bound protons vs pH, is just the sum of these values of $\langle x_i \rangle$ over all N titratable residues in the protein. In a similar way, the net charge of a protein is the sum of the charges carried by all the basic and acidic residues on the protein at a particular value of pH (eq 6).

$$Z(\mathrm{pH}) = \sum_{\text{basic}} \langle x_i \rangle - \sum_{\text{acidic}} (1 - \langle x_i \rangle) \tag{6}$$

Values of $\langle x_i \rangle$ can be calculated as a function of pH by simulating proton titration curves. These simulations take as input the binding affinity for protons of individual, isolated amino acids in solution (pK_a^{soln}) and require two separate calculations. First, changes in electrostatic potential in and around the protein that result from the protonation or deprotonation of individual titratable groups on the protein are calculated. We refer to this step as the electrostatics calculation. Second, the average protonation of each titratable residue must be calculated using as input values of pK_a^{soln} and electrostatic potentials calculated in the first step. This second step, which we refer to as the titration calculation, involves averaging over an ensemble of protonation vectors of the protein as a function of pH.

The Linderstrøm-Lang Model. The simplest method to simulate proton titration curves of proteins that incorporates the effects of cooperativity in proton binding is the LL model. In this model the electrostatics calculation uses the solution of the linearized Poisson−Boltzmann equation to relate the charge Z of a uniformly charged sphere to its surface potential, ψ_s (eq 7), where e is the charge on an electron, ϵ is the dielectric constant of the medium surrounding the sphere, ϵ_0 is the permittivity of free space, R is the radius of the sphere representing the protein, and κ is the inverse Debye length.

$$\psi_s = \frac{eZ}{4\pi\epsilon\epsilon_0 R(1 + \kappa R)} \tag{7}$$

The concentration of protons is assumed to follow a Boltzmann distribution; eq 8 describes the concentration of protons at the surface of the sphere $H^+_{surface}$: H^+_{bulk} is the concentration of protons in the bulk (pH = −log H^+_{bulk}), k_B is Boltzmann's constant, and T is absolute temperature. The average extent of

protonation of a titratable group (which varies between 0 and 1) is given by eq 9. This equation is just the Henderson−Hasselbach equation with H^+_{bulk} replaced by $H^+_{surface}$.

$$H^+_{surface} = H^+_{bulk}\ \exp\left(\frac{-e\psi_s}{k_B T}\right) \tag{8}$$

$$\langle x_i \rangle = \frac{1}{1 + 10^{[pH+0.43(e\psi_s/kT) - pK^{soln}_{a,i}]}} \tag{9}$$

The calculation of Z and $\langle x_i \rangle$ at a particular value of pH involves the iterative solution of eqs 6−9. In doing so, every residue of type i is assumed to have the same value of $pK^{soln}_{a,i}$. The net charge of a particular residue is therefore independent of its location on the protein. All groups of a particular type (e.g., carboxyl groups of Asp residues or amino groups of Lys residues) have the same extent of protonation and therefore carry the same net charge at a particular value of pH. Also, the effects of changing the net charge of a particular group (e.g., via the acetylation of a Lys residue) are represented as a change in the uniform charge density of the sphere: that is, there is no explicit representation of the effects of distance between particular groups on the electrostatic interactions between those groups.

The Poisson−Boltzmann Monte Carlo Model. The PB-MC model directly addresses the unique environment of each titratable group and the distance dependence of interactions between groups on a protein when simulating titration curves, albeit through the use of a model that is much more complex than the LL model. In this method, the electrostatics calculation provides the total electrostatic free energy of the protein with a particular protonation vector at a specific value of pH, $G_{elec}(\mathbf{x},\mathrm{pH})$ (eq 10).[22,23]

$$G_{elec}(\mathbf{x},\mathrm{pH}) = \frac{1}{2}\int\int \rho_o(\mathbf{r}) g(\mathbf{r},\mathbf{r}') \rho_o(\mathbf{r}')\, d^3r\, d^3r' - \sum_i \epsilon_i + \sum_i x_i (k_B T \ln 10)(\mathrm{pH} - pK^{int}_{a,i}) + \frac{1}{2}\sum_{\substack{i,j \\ i\neq j}} W_{ij} Q_i Q_j \tag{10}$$

In this expression, the first term is the electrostatic free energy of the protein in the neutral state where $\rho_o(\mathbf{r})$ is the distribution of charge on the protein when all titratable sites are neutral, that is, the distribution of charge that describes the polar nature of covalent bonds in the protein. The function $g(\mathbf{r},\mathbf{r}')$ is the electrostatic Green's function:[23,27] physically this function is the electrostatic potential at $\mathbf{r}$ due to a point charge at $\mathbf{r}'$. The second term, described by the summation of the parameter ϵ_i over all anionic titratable groups, is the intrinsic electrostatic energy of proton binding and includes the self-energy of charged titratable groups and the electrostatic interactions between these groups and the charge distribution, $\rho_o(\mathbf{r})$. The third term in eq 10 is the "intrinsic" energy of proton binding when all other titratable groups are in their neutral states. This energy is described by $pK^{int}_{a,i}$, which is calculated from pK^{soln}_a; each titratable group on the protein has a unique value of $pK^{int}_{a,i}$. The last term describes the interaction free energy between charged titratable groups, where Q_i and Q_j are the net charge on sites i and j (equal to −1, 0, or 1 depending on the value of x_i and whether the site is neutral acidic or basic) and W_{ij} is the electrostatic interaction energy between charged sites i and j and is calculated from values of $g(\mathbf{r},\mathbf{r}')$. In eq 10, only the last two terms depend on pH and values of x_i.

In the PB-MC model, the electrostatics calculations involve the computation of $g(\mathbf{r},\mathbf{r}')$ for all pairs of atoms on the protein that can carry a charge. These computations are done using finite-difference solutions of the linearized Poisson−Boltzmann equation. Values of $g(\mathbf{r},\mathbf{r}')$ are then used to determine values of ϵ_i, pK^{int}_a, and W_{ij}.

In principle, the simulation of proton titration curves can be accomplished by calculating values of $\langle x_i \rangle$ directly using eq 11, where the sum is over the 2^N different protonation states expressed by the vector $\mathbf{x}$.[28,29]

$$\langle x_i \rangle = \frac{\sum_{\mathbf{x}} x_i \exp(-G_{elec}(\mathbf{x},\mathrm{pH})/k_B T)}{\sum_{\mathbf{x}} \exp(-G_{elec}(\mathbf{x},\mathrm{pH})/k_B T)} \tag{11}$$

The time for computing the sums in this equation grows exponentially with N and several approaches have been developed to make these calculations more tractable.[22,23,25,28,30,31] A particularly efficient approach, developed by Beroza et al.,[22,23] is to use Monte Carlo simulations to sample the different protonation states with a probability given by the normalized Boltzmann factor. We make use of this approach and calculate $\langle x_i \rangle$ by averaging x_i over the sampled states.

Results and Discussion

The Net Charge of Lysozyme as a Function of Ionic Strength. Cooperativity in proton binding between titratable groups is expected to affect values of net charge. The effects of cooperativity in turn are the result of electrostatic interactions and should thereby be sensitive to the presence of small ions in solution. To study these effects, we measured values of net charge of lysozyme as a function of ionic strength using the linear regression analysis of data from CE and charge ladders.

Figure 1A shows the charge ladder of lysozyme produced by the random partial acetylation of amino groups. The charge ladder was synthesized in a solution at pH 12 such that the Lys ϵ-amino groups are acetylated preferential to the *N*-terminal α-amino group.[6,7] Figure 1B shows the plot μ^n_{elec} vs $n\Delta Z$ for the charge ladder of lysozyme shown in Figure 1A. In plotting the data we assume a value of −1 for ΔZ. A linear regression analysis of these data determined the charge of the unmodified protein Z^0 from the x-intercept of the best-fit line (eq 5). In performing the linear regression analysis for charge ladders of lysozyme, we used data from the last four rungs of the ladder only (i.e., $n = 6$, 5, 4, and 3).

The net charge of lysozyme was measured at pH 8.4 in a buffer composed of 25 mM Tris−192 mM Gly (total ionic strength ∼8 mM) and in this buffer in the presence 25 and 100 mM NaCl (total ionic strengths 33 and 108 mM, respectively). Values of $Z^0 = 7.3 \pm 0.1$ were measured at all values of ionic strength by the linear regression analysis of data from CE and protein charge ladders. There was no measurable dependence of net charge of lysozyme on ionic strength at pH 8.4.

The measured values of net charge were compared with values determined from simulated titration curves calculated using the LL and PB-MC models. Simulated titration curves were calculated for values of pH ranging from 3 to 11. Figure 2 compares the simulated titration curves calculated at an ionic strength of 108 mM to experimental titration data measured at 100 mM KCl. Both models capture the pH dependence of the net charge of the protein measured using pH titrations.[32]

Values of Z^0 at pH 8.4 measured by CE and calculated from simulated titration curves are presented in Table 1. Both models, in agreement with the experiments, show little variation in net

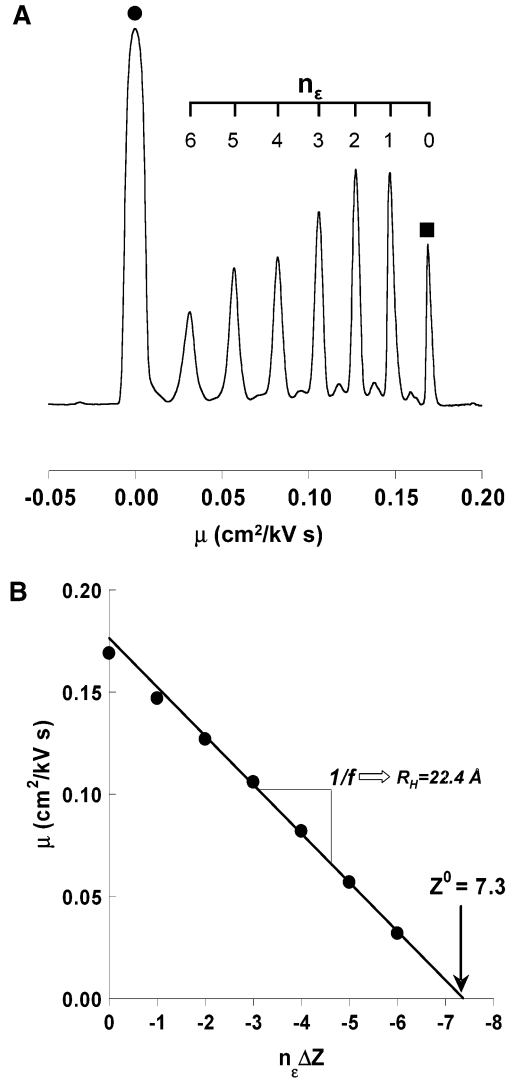

Figure 1. (A) The charge ladder of lysozyme produced by random partial acetylation of the Lys ϵ-amino groups of the protein. The UV absorbance at 214 nm is plotted as a function of electrophoretic mobility μ relative to the neutral marker of electroosmotic flow, denoted by ●. The peak corresponding to the unmodified protein is denoted by ■. The "rungs" of the ladders differ incrementally in the number of modified Lys ϵ-amino groups n_ϵ as indicated above the electropherogram. Separation was done at pH 8.4 in 25 mM Tris-192 mM Gly on a 30 cm capillary (20 cm from inlet to detector, i.d. 50 μm) with an applied voltage of 10 kV. (B) The values of electrophoretic mobility, μ, for the rungs of the ladder shown in (A) plotted as a function of $n_\epsilon \Delta Z$, where an ideal value of $\Delta Z = -1$ has been assumed. The solid line is the result of a linear regression analysis of the data from the last four rungs of the ladder (n_ϵ =3, 4, 5, and 6). The x-intercept of the line gives the negative of the net charge on the unmodified protein; the slope of this line is equal to the inverse of the coefficient of friction ($1/f$) (see eq 5 in the text). From the coefficient of friction, the hydrodynamic radius R_H was estimated using Henry's model of the electrophoresis of colloids.[5]

charge with the ionic strength. At pH 8.4 the Lys residues are fully protonated and the amount of charge carried by each ϵ-amino group is expected to be insensitive to changes in the ionic strength. The values of net charge measured by CE using linear regression analysis show good agreement with values calculated from the PB-MC model; the LL model predicts a smaller value of net charge, in agreement with published values of net charge from proton titration data.[32] Both experiments and calculations show that for lysozyme any effects of cooperativity in proton binding do not show up as a dependence of net charge

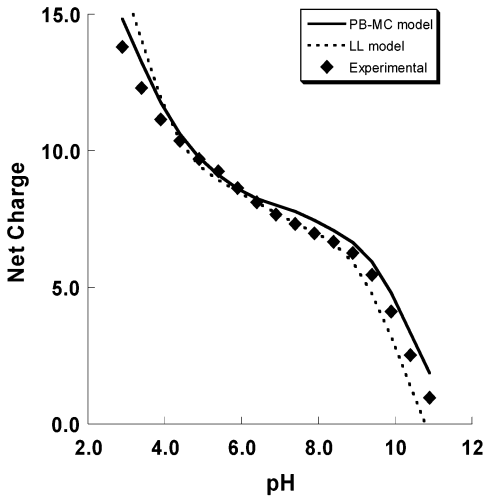

Figure 2. The net charge of lysozyme determined from simulated titrations curves of lysozyme calculated using the PB-MC and LL models. Results from the models are compared with the net charge of lysozyme measured as a function of pH using proton titrations.[32] The calculations were done at an ionic strength of 108 mM; the experiments were performed in 100 mM KCl.

TABLE 1: Values of Net Charge of Unmodified Lysozyme at pH 8.4 with Varying Ionic Strengths Determined By Proton Titrations, Linear Regression Analysis of Data from Charge Ladders, and Capillary Electrophoresis and Calculated from Simulated Titration Curves Using the PB-MC and LL Models

	titration data	linear regression, $\Delta Z = -1$	PB-MC	LL
8 mM[a]		7.3 ± 0.1	6.9	6.3
33 mM[b]		7.3 ± 0.1	7.0	6.5
108 mM[c]	6.7[d]	7.2 ± 0.1	7.1	6.6

[a] The ionic strength of the electrophoresis buffer composed of 25 mM Tris−192 mM Gly is approximately 8 mM at pH 8.4. [b] 25 mM Tris−192 mM Gly, 25 mM NaCl (I = 33 mM). [c] 25 mM Tris−192 mM Gly, 100 mM NaCl (I = 108 mM). [d] Determined from the experimental titration curve measured at 100 mM KCl.[32]

on ionic strength at pH 8.4. This result does not mean cooperativity is absent. It just indicates that values of net charge alone may not always be a sensitive measure of the effects of cooperativity.

Values of the pK_a of *N*-Terminal α-Amino Group of Lysozyme. To further explore the effects of cooperativity in proton binding on the analysis of protein charge ladders of lysozyme, we measured values of pK_a for the *N*-terminal α-amino group of this protein with different numbers of acetylated Lys ϵ-amino groups. In this way, we used values of pK_a as local thermodynamic reporters of electrostatic interactions between titratable groups on the protein.[17] Our analysis focused on two rungs of the ladder: the first rung, where the number of acetylated Lys ϵ-amino groups $n_\epsilon = 0$, and the last rung, where $n_\epsilon = 6$. We choose these rungs because each is composed of a single protein species; rungs of the ladder that have between 2 and 5 acetylations are composed of mixtures of 6−20 different derivatives of proteins.

To determine the degree of protonation of the *N*-terminal α-amino group, we measured the mobility of proteins in these two rungs at different values of pH both with and without the *N*-terminal α-amino group acetylated. The selective acetylation of the *N*-terminal α-amino group of lysozyme at low pH and of Lys ϵ-amino groups of lysozyme at high pH has been described previously.[6] Briefly, to selectively acetylate the

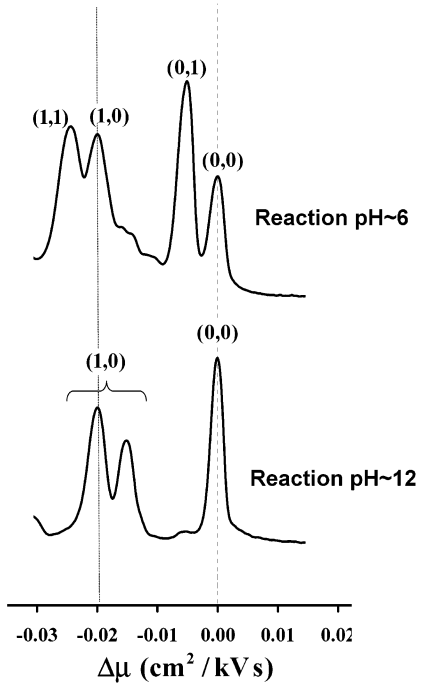

Figure 3. Electropherograms show the selective acetylation of the *N*-terminal α-amino group of lysozyme at pH 6 and of the Lys ϵ-amino group at pH 12. The notation (n_ϵ,n_α) above each peak denotes whether acetylation has occurred at the Lys ϵ-amino and *N*-terminal α-amino groups, respectively. A value of 1 indicates acetylation; a value of 0 indicates no modification. At pH 6 the *N*-terminal α-amino group is selectively acetylated, as shown by the presence of the peaks marked (0,1) and (1,1), which are absent in the ladder formed at pH 12. When the reaction is conducted at pH 12, the Lys ϵ-amino groups are selectively acetylated. The electropherogram from the ladder formed at pH 12 shows a splitting of the (1,0) peak. Separation conditions are the same as those in Figure 1, except the pH has been adjusted to 7.8 to improve the resolution of peaks corresponding to the acetylation of the *N*-terminal α-amino group and 25 mM NaCl has been added (for a total ionic strength of 33 mM) to improve the resolution of the splitting of the (1,0) peak in the ladder formed at pH 12.

α-amino group, the synthesis of charge ladders was carried out at pH 6. At this pH, the *N*-terminal α-amino group, which has a lower value of pK_a and thereby a larger probability of existing in the deprotonated, nucleophilic state than the ϵ-amino groups, acetylates selectively.[7] Charge ladders of lysozyme were also produced at pH 12: at this higher value of pH, the Lys ϵ-amino groups were selectively acetylated.

Comparison of the charge ladders formed at pHs 6 and 12, shown in Figure 3, identified those derivatives that have been acetylated at the *N*-terminal α-amino group. In this figure the electropherograms are plotted as the response of the UV detector as a function of the mobility relative to that of the unmodified protein $\Delta\mu$. The negative values of $\Delta\mu$ reflect a decrease in the net positive charge of lysozyme, and thereby the mobility of this protein, resulting from the acetylation of amino groups. In Figure 3 we label each peak in the figure with the number of acetylated ϵ-amino and α-amino groups, (n_ϵ,n_α). Because we are examining only the first two rungs of the ladder and there is only a single α-amino group on lysozyme, both n_ϵ and n_α are either 0 or 1 for all peaks shown in this figure.

We observed that two products formed in the reaction at pH 6 are only minutely present in the reaction carried out at pH 12. We infer that these products are the result of selective acetylation of the *N*-terminal α-amino group at pH 6 and label them (0,1) and (1,1). At the pH of the separation by CE (pH 7.8), the α-amino group is partially protonated. Acetylation of this group results in a decrease in the net positive charge of the protein and therefore a negative value of $\Delta\mu$. For the pH 12 reaction the Lys ϵ-amino groups are preferentially modified and we see only a small peak corresponding to (0,1); we see no evidence of a peak corresponding to (1,1).

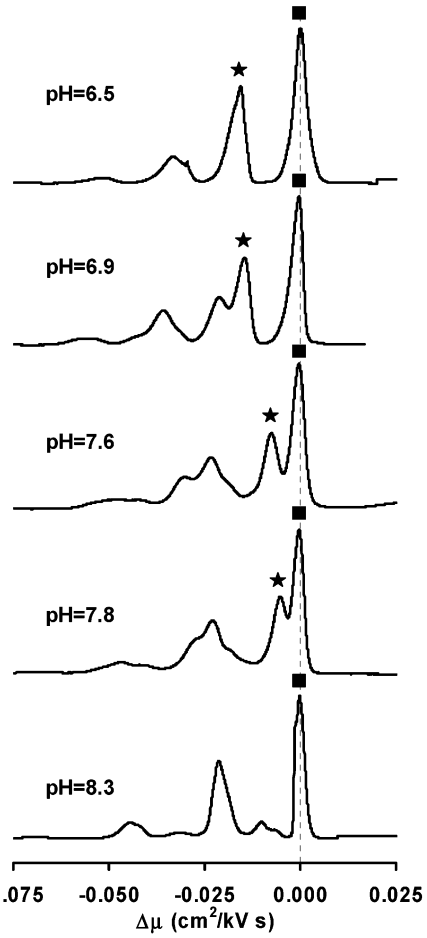

Figure 4. Electropherograms of the first rungs of the charge ladder of lysozyme, produced by the selective acetylation the *N*-terminal α-amino group, at different values of pH of the electrophoresis buffer. The peak that corresponds to protein that has been acetylated at the *N*-terminal α-amino group is denoted by ★. The mobility of this derivative relative to the unmodified protein is a direct measure of degree of protonation of the *N*-terminal α-amino group. Separations were done in 25 mM Tris-192 mM Gly on a 30 cm capillary (20 cm from inlet to detector, i.d. 50 μm) with an applied voltage of 7 kV. The pH was varied though the addition of HCl and NaOH; NaCl was added to keep the total ionic strength constant at 33 mM.

By measuring the change in mobility of lysozyme due to the selective acetylation of the *N*-terminal α-amino group we can determine the degree of protonation of this group. Measurements done over a range of values of pH provide a convenient means to measure the proton titration curve for this group. Figure 4 shows electropherograms, measured at different values of pH, of the charge ladder of lysozyme synthesized at pH 6. At a pH of 8.4 the *N*-terminal α-amino group is almost fully deprotonated and has a value of mobility similar to that of the unmodified protein. As the pH decreases, this group becomes more protonated. Its acetylation results in a change in mobility toward the peak corresponding to $n_\epsilon = 1$. Titration curves were constructed by plotting the change in mobility due to acetylation of the α-amino group relative to the unmodified protein $\Delta\mu_{CE}$ as a function of pH. By fitting these data to eq 12, the pK_a of the *N*-terminal α-amino group was determined for the unmodified protein ($n = 0$) and the peracetylated protein

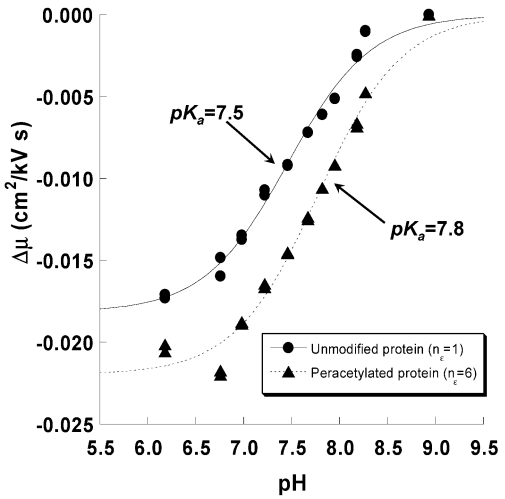

Figure 5. Effects of pH on the electrophoretic mobility of lysozyme selectively acetylated at the *N*-terminal α-amino group for the unmodified ($n_\epsilon = 0$) and peracetylated ($n_\epsilon = 6$) protein. Data are plotted as the change in mobility due to acetylation of the *N*-terminal α-amino group vs pH and are fitted to the Henderson−Hasselbach equation to determine values of pK_a. Separations were done in 25 mM Tris-192 mM Gly on a 30 cm capillary (20 cm from inlet to detector, i.d. 50 μm) with an applied voltage of 7 kV. The pH was varied through the addition of HCl and NaOH; NaCl was added to keep the total ionic strength constant at 33 mM.

($n = 6$) (Figure 5).[6,7,24] In eq 12, C is a constant; it is the difference in mobility of the protein with the α-amino group fully protonated and deprotonated. Because this approach is based on measuring differences in values of mobility with and without the *N*-terminal α-amino group acetylated, other contributions to changes in mobility with pH are eliminated: for example, changes in mobility due to changes in the protonation of titratable groups other than the *N*-terminal α-amino group.

$$\Delta\mu_{CE} = \mu_{CE}|_{(n,0)} - \mu_{CE}|_{(n,1)} = \frac{C}{1 + 10^{(pH-pKa)}} \qquad (12)$$

Values of pK_a are known to be sensitive to the presence of ions in solution, so all pH titrations were done at constant ionic strength. Using this method, the pK_a of the *N*-terminal α-amino group was determined for both the unmodified and the peracetylated protein at ionic strengths of 33 and 108 mM (Table 2). The pK_a of the *N*-terminal α-amino group for peracetylated lysozyme measured at 33 mM ionic strength, 7.8, agrees well with the value of 7.8 which was measured by Cordova et al. using CE and charge ladders;[6] they conducted their experiments at low but nonconstant values of ionic strength.

At both values of ionic strength, the *N*-terminal α-amino group on the peracetylated protein had a value of pK_a greater than that of the unmodified protein. As lysozyme is acetylated, the positive net charge is reduced, and hence, it becomes easier to protonate the residues on the protein, as reflected in the increase in values of pK_a for the *N*-terminal α-amino group. This increase is a direct measure of the cooperativity in proton binding between titratable groups on the protein.

Additionally, our results show that the change in pK_a of the *N*-terminal α-amino group due to peracetylation of the ϵ-amino groups was larger at lower values of ionic strength. The difference between values of pK_a for the *N*-terminal α-amino group on the unmodified and peracetylated protein at high ionic strength is almost within the error of the measurements: this result shows that the addition of salt can effectively screen cooperative interactions between the *N*-terminal α-amino group and the Lys ϵ-amino groups of lysozyme.

TABLE 2: Comparison of Values of pK_a of the *N*-Terminal α-Amino Group of Unmodified and Peracetylated Lysozyme (All Lys Residues Acetylated) at Different Values of Ionic Strength, as Measured Experimentally by CE and Calculated from PB-MC and LL Models

		unmodified protein	peracetylated protein	$\Delta pK_a^{acetylation}$
33 mM	exptl	7.5 ± 0.1	7.8 ± 0.1	0.31 ± 0.03[a]
	PB-MC	7.75	8.30	0.55
	LL	7.00	7.40	0.40
108 mM	exptl	7.9 ± 0.1	8.0 ± 0.1	0.06 ± 0.03[a]
	PB-MC	7.90	8.35	0.45
	LL	7.15	7.43	0.28
ΔpK_a^{salt}	exptl	0.48 ± 0.03	0.23 ± 0.03	
	PB-MC	0.15	0.05	
	LL	0.15	0.03	

[a] The errors reported for measured values of pK_a reflect the uncertainty in the values of pH of the buffers. Because differences in values of pK_a between the unmodified and peracetylated proteins are determined in a single experiment, these errors will largely cancel; consequently, the error in the shift in the value of pK_a upon acetylation is just the error from fitting the curves in Figure 5.

Values of pK_a for the *N*-terminal α-amino group of lysozyme measured by CE were compared to values calculated using the LL and PB-MC models. To calculate the pK_a of the *N*-terminal α-amino group, the net charge of this group Z_{N-term} was determined as a function of pH from the simulated titration curves. The pK_a of this group was identified as the pH at which it was half-protonated (i.e., $Z_{N-term} = 0.5$). The measured and calculated values of pK_a for the *N*-terminal α-amino group on the unmodified and peracetylated protein are compared in Table 2. Both experiments and calculations show that the values of pK_a increase with ionic strength and with the number of acetylated Lys ϵ-amino groups on the protein. Both the screening of the electrostatic potentials surrounding the protein by the addition of salt and the reduction in the net positive charge of the protein by acetylation increases the affinity of the *N*-terminal α-amino group for the binding of protons.

At an ionic strength of 108 mM, the value of pK_a of the *N*-terminal α-amino group on the unmodified protein calculated by the PB-MC model agrees well with the measured value of pK_a. Generally, the PB-MC model tends to over-predict values of pK_a, consistent with previous work of others.[17] In contrast, the LL model underestimated values of pK_a. Measured changes in values of pK_a of the *N*-terminal α-amino group due to acetylation $\Delta pK_a^{acetylation}$ and changes in ionic strength of the solution ΔpK_a^{salt} were compared to those from simulated titration curves. Although the LL and PB-MC models captured the qualitative effects of adding salt or acetylating Lys ϵ-amino groups on the pK_a of the *N*-terminal α-amino group, both models overpredicted the effects of acetylation and underpredicted the effect of adding salt, relative to the experimental data. We conclude that these models are useful in estimating the effects of cooperativity in proton binding and thereby in establishing the magnitude of uncertainty in values of ΔZ for charge ladders. In the next section, we show that the PB-MC model is particularly useful in identifying specific residues that contribute significantly to reductions in the magnitude of ΔZ through cooperativity in proton binding.

Cooperativity in Proton Binding Between Individual Lys ϵ-Amino Groups and Other Titratable Groups on Lysozyme. In Figure 3 we also observe a peak from the reaction at pH 12 that is present only as a small shoulder on the right of the (1,0) peak from the reaction at pH 6. Because there is little acetylation

of the α-amino group at pH 12 (i.e., only a small peak corresponding to (0,1) and no observable (1,1) peak), we propose that these peaks reflect a splitting of the peak that corresponds to $n_\epsilon = 1$. This splitting was not observed in the electropherogram shown in Figure 1. The electropherograms in Figure 3 were obtained at a lower pH (7.8 vs 8.4) and higher ionic strength (33 mM vs 8 mM) relative to that in Figure 1. The added salt reduces the mobility of the proteins in the ladder and thereby increases the ability of CE to resolve small differences in charge of proteins within a rung. This increase in resolution, in turn, results in the observed splitting. Because this splitting only occurs in rungs corresponding to $n_\epsilon = 1$ and 2, it does not affect our estimates of Z^0 determined from a linear regression analysis of the four rungs of the ladder with smallest magnitude of net charge.

The observed splitting is consistent with two possible effects: (i) one or more Lys ϵ-amino groups have values of pK_a that are significantly more acidic than the others and thereby are not fully protonated at pH 7.8 and (ii) there are significant cooperative interactions between one or more Lys ϵ-amino groups and other titratable groups on lysozyme such that the acetylation of ϵ-amino groups results in an increase in the protonation of these other groups on the protein. Under conditions where the measured mobility is independent of the strength of the applied field, as is the case in all the experiments described here, the motive force in electrophoresis—the product of field with net charge—is independent of the distribution of charges on the protein: that is, the mobility does not depend on the dipole and higher order moments of the charge distribution. Changes in the dipole moment that result from the acetylation of different Lys residues will not result in the splitting of peaks. We expect the first effect to be insignificant; previous experimental work with lysozyme has shown that the values of pK_a for all the Lys ϵ-amino groups are within a narrow range of 10.3−10.8 [24] and therefore fully protonated at pH 7.8. These results are consistent with simulated titration curves calculated with the PB-MC model, presented below. We propose the peak splitting seen at pH 12 is the result of cooperativity in proton binding between one or more Lys ϵ-amino groups and other titratable groups on lysozyme. We support this hypothesis with the results from the PB-MC model.

To quantify the cooperativity in proton binding between individual Lys ϵ-amino groups and the rest of the protein, we calculated the charge on each of the six Lys ϵ-amino residues of lysozyme $Z_{Lys,i}$, where i is the residue number of a particular Lys in lysozyme. We also calculated the change in net charge of lysozyme due to acetylation of each of these Lys residues ΔZ_i. The results of these calculations at pH 8.4 are given in Table 3. The difference between ΔZ_i and $-Z_{Lys,i}$ for a particular Lys ϵ-amino group is a direct measure of the cooperativity in proton binding between that Lys group and the other titratable groups on the protein. If there is no cooperativity, $\Delta Z_i = -Z_{Lys,i}$. If there is cooperativity, the difference between ΔZ_i and $-Z_{Lys,i}$ is equal to the change in net charge that results from changes in proton binding on the other titratable groups of the protein.

We calculated $Z_{Lys,i}$ for all 6 of the Lys ϵ-amino groups at pH 8.4 on the unmodified protein using both the LL and PB-MC models. For the LL model, all six Lys ϵ-amino groups are treated as identical and therefore have the same charge. In contrast, the PB-MC model predicts a unique value of $Z_{Lys,i}$ for each Lys ϵ-amino group of lysozyme. As shown in Table 3, both the LL and PB-MC models predict that the charge on each the Lys ϵ-amino groups lies between 0.97 and 1 for values of ionic strength that range from 8 to 108 mM.

TABLE 3: Charge on Lys Residues $Z_{Lys,i}$ of Unmodified Lysozyme and Change in Net Charge of the Protein ΔZ_i Due to Acetylation of Lys Residue *i*, Determined at pH 8.4 from PB-MC and LL Models at Different Values of Ionic Strength

PB-MC Model

Lys	$Z_{Lys,i}$[a]	8 mM ΔZ_i	33 mM ΔZ_i	108 mM ΔZ_i
1	0.97	−0.80	−0.81	−0.83
13	0.99	−0.93	−0.95	−0.97
33	0.97	−0.90	−0.92	−0.94
96	1.00	−0.88	−0.91	−0.94
97	1.00	−0.90	−0.93	−0.96
116	0.97	−0.90	−0.93	−0.95
average	0.98	−0.89	−0.91	−0.93

LL Model

	$Z_{Lys,i}$[a]	8 mM ΔZ_i	33 mM ΔZ_i	108 mM ΔZ_i
average of all six Lys ϵ-amino groups[b]	0.98	−0.86	−0.91	−0.94

[a] Values of Z_{Lys}, calculated at pH 8.4 by PB-MC and LL, differed by no more than 0.01 charge units as ionic strength increased from 8 to 108 mM. [b] The Lindstrom-Lang model treats all six Lys ϵ-amino groups as the same.

We calculated values of ΔZ_i for each of the six Lys ϵ-amino groups of lysozyme by subtracting the net charge of the unmodified protein from the net charge of the protein with each amino group fixed in the fully de-protonated state. At pH 8.4, the LL model predicts that the acetylation of a single Lys ϵ-amino group results in values of ΔZ_i that range from −0.86 to −0.94 at values of ionic strength that range from 8 to 108 mM. Values of ΔZ_i calculated from the PB-MC model and averaged over all six Lys ϵ-amino groups are in good agreement with the results from the LL model indicating that the LL model captures the average effects of cooperativity in proton binding, although it provides no information on the effects of the detailed, local interactions. The results in Table 3 from the PB-MC model for the ΔZ_i of individual Lys groups show two types of effects. For Lys_1 we calculated differences between ΔZ_i and $-Z_{Lys,i}$ that range from 0.17 to 0.14 units of charge with increasing ionic strength. In comparison, the other five Lys residues, Lys_{13}, Lys_{33}, Lys_{96}, Lys_{97}, and Lys_{116}, show differences between ΔZ_i and $-Z_{Lys,i}$ that range from 0.02 to 0.1 units of charge. The distinctive value of ΔZ_i for the acetylation of Lys_1 highlights the cooperativity in proton binding between this residue and other titratable residues on lysozyme. From this result, we infer that the local environment of the Lys ϵ-amino groups (i.e., the relative location of other titratable groups on the protein) plays an important role in establishing the effects of cooperativity in proton binding on values of ΔZ.

To further explore the effects of cooperativity in proton binding we calculated values of ΔZ_i for each of the six Lys ϵ-amino groups of lysozyme as a function of pH using both the LL and PB-MC models. The results of these calculations are shown in Figure 6A. The LL model predicts that the value of ΔZ is approximately constant and equal to −0.95 from pH 5 to 8; above this pH, the Lys ϵ-amino groups begin to titrate (pK_a∼10.5), and thereby reduce the magnitude of values of ΔZ_i. The PB-MC model predicts that the value of ΔZ_i averaged over residues Lys_{13}, Lys_{33}, Lys_{97}, and Lys_{116} agrees fairly well with the value of ΔZ_i predicted by the LL model; for Lys_1 and Lys_{96}, however, there are significant differences. At values of pH from 5 to 7, Lys_{96} deviates significantly from the average value of ΔZ_i. Lys_1 shows similar deviations at values of pH from 7 to 9.

On Figure 6A we also indicate the values of pK_a of the single

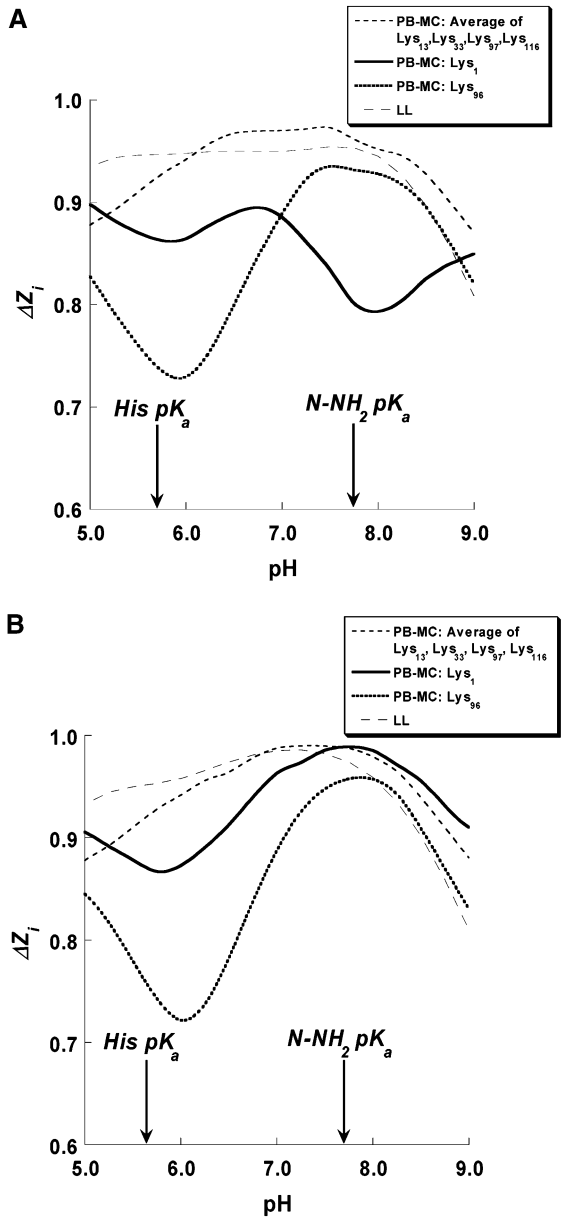

Figure 6. (A) The change in net charge of lysozyme due to the specific acetylation of Lys$_i$ ΔZ_i as a function of pH, predicted by the PB-MC, and the LL models at an ionic strength of 33 mM. Values of ΔZ_i calculated with the PB-MC model and averaged over Lys_{13}, Lys_{33}, Lys_{97}, and Lys_{116} are compared to the individual values of ΔZ_i for Lys_1 and Lys_{96}. The LL model predicts only single value of ΔZ_i for all Lys ϵ-amino groups as this method does not differentiate among different groups of the same type. The values of pK_a for His and *N*-terminal α-amino shown on the plot are results from the PB-MC model (5.7 and 7.7, respectively). (B) Effects of blocking the *N*-terminal α-amino group on values of ΔZ_i are calculated using the PB-MC and LL models.

histidine of lysozyme, His_{15} (p$K_a = 5.7$), and of the *N*-terminal α-amino group (p$K_a = 7.7$) calculated from the PB-MC model. We observe that minima in the magnitude of ΔZ_i for Lys_{96} and Lys_1 correlate with the values of pK_a of these groups, implying that cooperativity in proton binding between these groups and Lys_{96} and Lys_1 may be the source of the observed variation in ΔZ_i with pH.

To test this idea we repeated the calculations of ΔZ_i as a function of pH with the *N*-terminal α-amino group "blocked": that is, fixed in the deprotonated (neutral) state. The results of these calculations are shown in Figure 6B. Because it is blocked, the *N*-terminal α-amino group can no longer accept a proton and becomes charged. There is a clear effect of blocking the *N*-terminal α-amino group on the pH dependence of ΔZ_i due to acetylation of Lys_1. Above pH 7, the value of ΔZ_i due to acetylation of Lys_1 is now similar to the value of ΔZ_i, averaged over residues Lys_{13}, Lys_{33}, Lys_{97}, and Lys_{116}, and to the value of ΔZ_i predicted by the LL model. We conclude that the pH dependence of ΔZ_i due to acetylation of Lys_1 between pH 7 and 9 shown in Figure 6A is the result of a specific interaction between the *N*-terminal α-amino group and the ϵ-amino group of Lys_1. This conclusion seems reasonable considering that both amino groups are on the same residue (i.e., Lys_1 is the *N*-terminal amino acid of lysozyme). From the crystal structure of lysozyme we estimate that these groups are only approximately 7 Å apart. The pH dependence of ΔZ_i shown in Figure 6 for Lys_{96} between pH 5 and 7 is consistent with cooperativity in the binding of protons between this residue and His_{15}. From the crystal structure of lysozyme we observe that the ϵ-amino group of Lys_{96} and the imidazole group of His_{15} are separated by approximately 8 Å. From the pH dependence of ΔZ_i for Lys_1 between pH 5 and 7 we infer that this residue also has a cooperative effect on the binding of protons to His_{15}, although this interaction appears weaker than the interactions with the *N*-terminal α-amino group. This weaker interaction is consistent with a separation of 12 Å between the ϵ-amino group of Lys_1 and the imidazole group of His_{15}. The difference between results from the LL model and the average of Lys_{13}, Lys_{33}, Lys_{96}, Lys_{97}, and Lys_{116} below pH 6 is likely due to the fact that Lys_{97} has signficant cooperative interactions with Asp_{101} (p$K_a \sim 4.5$): the distance between these groups is only 5.5 Å.

From these calculations we conclude that the large difference between ΔZ_i and $-Z_{Lys,i}$ for Lys_1 at pH 8.4 is primarily the result of a specific interaction between this ϵ-amino group and the *N*-terminal α-amino group. Specific interactions between pairs of groups on the protein can result in large deviations of ΔZ from the ideal value of -1. For example, at pH 6.0, the interaction between Lys_{96} and His_{15} results in a magnitude of ΔZ_i that is almost 0.3 units of charge less than that of $Z_{Lys,i}$.

The values of ΔZ_i calculated from the PB-MC model are consistent with the electropherograms shown in Figure 3. Differences in electrophoretic mobility between rungs of a protein charge ladder are directly proportional to values of ΔZ. The PB-MC model predicts that at pH 7.8 and an ionic strength of 33 mM the species acetylated at Lys_{13}, Lys_{33}, Lys_{96}, Lys_{97}, and Lys_{116} will have an average ΔZ_i of approximately -0.96 while the protein acetylated at the Lys_1 residue will have a $\Delta Z_i \sim -0.8$. As predicted, the electropherogram of the 1st rung of the charge ladder formed at pH 12 and separated at pH 7.8 an ionic strength 33 mM shows 2 distinct peaks corresponding to (1,0): that is, there are different derivatives of lysozyme with a single ϵ-amino group acetylated that are separated by CE and therefore differ significantly in net charge. The splitting of the (1,0) peak of the charge ladder of lysozyme is a direct measure of the effects of cooperativity in proton binding on ΔZ.

Cooperative Interactions That Affect Values of ΔZ Can Be Classified as One of Two Types. From the results in Table 3 and Figures 6, we identify two types of cooperative interactions that can affect values of ΔZ: we characterized these interactions as specific and nonspecific. Specific interactions are the results of cooperativity in proton binding between a particular Lys ϵ-amino group and another specific titratable group on the protein; a single specific interaction can results in large deviations of ΔZ (up to 0.3 units of charge) from the ideal value of -1. Nonspecific interactions are the result of cooperativity between a particular Lys ϵ-amino group and a number

TABLE 4: Distance between Each Lys ϵ-Amino Group on Lysozyme and Other Titratable Groups on the Protein[a]

residue	exptl[32] pK_a	Lys_1	Lys_{13}	Lys_{33}	Lys_{96}	Lys_{97}	Lys_{116}
Asp_{18}	2.0	20.4	**7.6**	20.3	**11.6**	20.8	19.2
Asp_{87}	2.1	**9.5**	16.9	21.3	**11.7**	18.4	30.0
Glu7	2.6	**8.6**	**15.0**	**12.2**	20.3	29.6	28.2
C-term	3.1	16.5	**4.9**	20.1	16.1	27.3	27.0
Asp_{101}	4.5	28.5	25.6	29.0	**14.3**	**5.0**	20.4
His_{15}	5.8	**11.8**	**13.1**	22.0	**7.8**	16.6	28.2
Glu_{35}	6.1	19.5	24.5	**12.2**	19.6	20.5	16.0
N-term	7.9	**7.2**	23.4	**14.9**	21.3	26.5	30.7
Tyr_{23}	9.8	26.8	20.1	21.8	15.4	16.2	**9.0**
Lys_{97}	10.3	26.6	23.8	29.8	**11.6**	-	24.1
Tyr_{20}	10.3	23.3	16.7	25.7	**6.2**	**7.9**	20.2
Lys_{96}	10.8	18.5	**12.4**	24.1	-	**11.6**	23.8

[a] Groups that are within 15 Å of a particular Lys ϵ-amino group (distances shown in bold) are expected to make a specific contribution to cooperativity at over a range of values of pH corresponding approximately to values of p$K_a \pm 1$ unit of pH. Arginine residues were not included because of their high values pK_a ($\sim$12.5).

of other titratable groups; each interaction is relatively weak, the combined effect of these may impact ΔZ by up to 0.1 units of charge.

For the cooperative interaction between a Lys ϵ-amino group and another titratable group to be classified as specific the distance between the groups must be $<\sim$15 Å. From Figure 6 we infer that for a specific cooperative interaction to make a signficant impact on ΔZ beyond the effects of nonspecific interactions (e.g., >0.1 units of charge) the titratable group must have a value of pK_a within $\sim$1 pH unit of the solution. Charge ladders are typically analyzed at pH 8.4. This condition implicates groups with values of pK_a in the range of 7−9: typically His and Tyr residues and the *N*-terminal α amino group. Effects of specific cooperative interactions on ΔZ are maximal when the pH of the solution is equal to the pK_a of the interacting titratable group.

These two conditions are satisfied for lysozyme at pH 8.4 by the interactions between the α-amino (p$K_a \sim 7.5$) and ϵ-amino groups of Lys_1; these amino groups are $\sim$7 Å apart. Cooperative interactions between these two amino groups can be large enough to result in a splitting of peaks within a single rung of the charge ladder of lysozyme that can be resolved by CE, as shown in Figure 3.

The interactions between α-amino and ϵ-amino groups of Lys_1 are typical of a large number of interactions between titratable groups in lysozyme. Table 4 summarizes the number of possible specific cooperative interactions between the Lys ϵ-amino groups of lysozyme and other titratable groups on the protein. This table summarizes the distance between each Lys ϵ-amino group on lysozyme and other titratable groups on the protein. The titratable groups are listed in order of increasing values of pK_a to indicate at what range of values of solution pH these specific interactions could affect values of ΔZ: that is, values of pH corresponding approximately to values of p$K_a \pm 1$ unit of pH. Even for a relatively small protein such as lysozyme there are a large number of different interactions that can result in specific cooperative interactions that occur over values of pH spanning from 2 to 12, the useful range of solution pH for the study of proteins. This result highlights the importance of the distance dependence of interactions between titratable groups in determining the effects of cooperativity in proton binding on values of ΔZ. While the LL model does not predict these specific interactions, they are captured by the PB-MC model and can be identified and largely quantified using a simple empirical relationship described in the next section. Because of the magnitude of these interactions, they can be measured by shifts in values of pK_a, for example, by NMR,[17,19] and in values of electrophoretic mobility measured by CE (and thereby values of net charge), as shown in Figure 3.

In comparison, nonspecific interactions are difficult to measure directly because they result in changes in values of pK_a and net charge that are outside of the resolution of the techniques used to measure these quantities. By the nature of the model, however, these nonspecific interactions are captured by the LL model; they are also captured by the PB-MC model.

Cooperativity in Proton Binding between Titratable Groups Can Be Described Using a Simple Electrostatic Model. The results from the PB-MC model shown in Figure 6 illustrate that cooperativity in proton binding on values of ΔZ is largely the result of interactions between Lys ϵ-amino groups and specific titratable residues on the protein. The effect of acetylation of a Lys ϵ-amino group on the net charge carried by other titratable residues on the protein depends on the pH, the values of pK_a of these residues, and the distance from the Lys ϵ-amino group that is acetylated. The PB-MC model predicts these effects. Because these calculations are time-consuming, a more rapid and efficient approach to estimating effects of cooperativity on values of ΔZ is preferred.

Recently Lee et al. measured the distance dependence and salt sensitivity of energies of interaction between pairs of titratable residues on staphylococcal nuclease (SNase).[19] They measured values of pK_a by ^{1}H NMR spectroscopy of His residues in mutants of SNase that involved both charge-reversal and charge-neutralization. Changes in values of pK_a relative to values for the wild-type protein are a direct measure of the change in electrostatic potential around the protein due to the mutation of charged residues. They expressed the change in electrostatic potential $\Delta\psi_{ij}$ at site i that results from a change in charge at site j, Δz_j, using Coulomb's law with a Debye−Hückel term to account for the effects of small ions in solution (eq 13). In this equation, r_{ij} is the distance between the two sites and ϵ_{eff} is the effective dielectric constant of the medium between the sites. This equation can be derived from the linearized version of the Poisson−Boltzmann equation and forms the centerpiece of the Debye−Hückel theory of electrolyte solutions. Equation 14 relates the change in electrostatic potential to the change in value of pK_a of site i.

$$\Delta\psi_{ij} = \frac{e}{4\pi\epsilon_o\epsilon_{\text{eff}}}\frac{\Delta z_j}{r_{ij}}\exp(-\kappa r_{ij}) \tag{13}$$

$$\Delta \text{p}K_{a,ij} = \frac{e\Delta\psi_{ij}}{2.303k_BT} \tag{14}$$

Lee et al. showed that these equations reproduced the measured sensitivity to salt and distance dependence of changes in the values of pK_a of His due to different charge mutations. They used this model as an empirical relationship to describe pairwise electrostatic interactions between titratable residues on a protein; the value of the dielectric constant was varied to match the measured dependence of shifts in values of pK_a with distance between titratable groups and concentration of salt. A dielectric constant near 80 reproduced the salt sensitivity and distance dependence of these interaction energies. We compared the predictions of this empirical relationship to the results obtained from the PB-MC model. Figure 7 shows values of ΔpK_a predicted by the PB-MC model for the *N*-terminal α-amino group of lysozyme due to acetylation of each of the 6 Lys ϵ-amino residues plotted as a function of the distance between

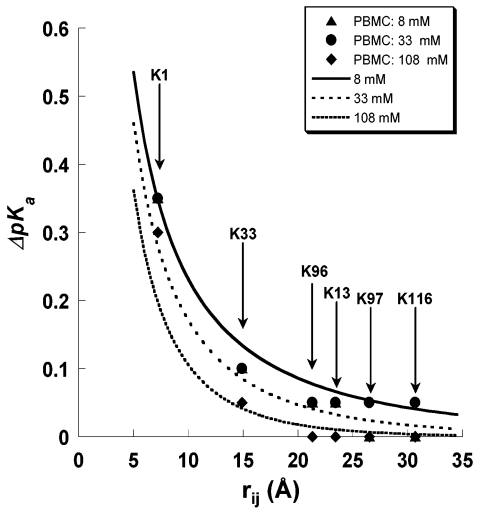

Figure 7. Changes in the value of pK_a of the *N*-terminal α-amino group of lysozyme at different values of ionic strength due to the acetylation of Lys ϵ-amino groups on this protein, plotted as a function of the distance between the α-amino and ϵ-amino groups r_{ij}. Individual points are results from the PB-MC model; curves are the results from the screened Coulombic potential expressed in eqs 13 and 14 and calculated using a value of $\epsilon_{eff} = 80$.

the α- and ϵ-amino groups at pH 8.4. The curves in Figure 7 are the results of eqs 13 and 14 for solutions with values of ionic strength of 8, 33, and 108 mM and a dielectric constant of 80. In applying this relationship we assume $\Delta z_j = -1$ due to the acetylation of a fully protonated Lys ϵ-amino group. This plot shows the expected dependence of interaction energy on distance between groups and, more specifically, the significant cooperativity that exists between the α- and ϵ-amino groups of Lys_1. The large increase in ΔpK_a as r_{ij} approaches very short distances is also consistent with the experimental results found by Sundd et al. in which the shift in the values of pK_a of acidic residues on ubiquitin were measured as Lys residues on the protein were mutated.[33] They found that the mutation of Lys_{11} caused the pK_a of Glu_{34} to shift by 0.8 units; Glu_{34} is located 3.3 Å from Lys_{11}.

Figure 7 also shows that Lys residues that are separated from the *N*-terminal α-amino group by more than approximately 15 Å have negligible interaction energies with this group; the acetylation of these groups will not affect the value of pK_a and thereby the net charge of this residue. The fact that eqs 13 and 14 accurately capture the results from the more detailed calculations using the PB-MC model suggests that titratable residues on the surface of proteins interact as if they were a dilute solution of ions in an electrolyte solution.

We conclude that this empirical model captures the dependence of values of ΔpK_a on distance and concentration of salt, predicted by the much more complex PB-MC model. These results highlight the importance of including structural information, such as the distance between titratable residues, when predicting the effects of cooperativity in proton binding on values of ΔZ for protein charge ladders. They also show that these effects can be estimated with similar accuracy using a simple screened Coulombic potential; this empirical potential reproduces the results of the more complex and time-consuming PB-MC model.

Analysis of Effects of Uncertainty in ΔZ on the Net Charge and Hydrodynamic Radius of Proteins Determined by CE and Charge Ladders. Values of ΔZ for protein charge ladders are in general affected by cooperativity in proton binding, leading to uncertainty in the value of this parameter. In this section we establish the relationship between uncertainty in ΔZ with uncertainty in values of Z^0 and f (and, thereby, hydrodynamic radius R_H) determined from the linear regression analysis of data from charge ladders and CE. If we assume both f and ΔZ are independent of n (a reasonable assumption if the values of μ^n_{elec} correlate linearly with n, as is the case when the linear regression analysis is applied), then the following set of relationships, shown as eqs 15 and 16, can be derived directly from eq 5.

$$Z^0 = \frac{\mu^o_{CE}}{\mu^1_{CE} - \mu^o_{CE}}\Delta Z = \frac{2\mu^o_{CE}}{\mu^2_{CE} - \mu^o_{CE}}\Delta Z = \frac{3\mu^o_{CE}}{\mu^3_{CE} - \mu^o_{CE}}\Delta Z = \frac{n\mu^o_{CE}}{\mu^n_{CE} - \mu^o_{CE}}\Delta Z \quad (15)$$

$$f = \frac{1}{\mu^1_{CE} - \mu^0_{CE}}\Delta Z = \frac{2}{\mu^2_{CE} - \mu^o_{CE}}\Delta Z = \frac{3}{\mu^3_{CE} - \mu^o_{CE}}\Delta Z = \frac{n}{\mu^n_{CE} - \mu^o_{CE}}\Delta Z \quad (16)$$

These series indicate that both f and Z^0 are directly proportional to ΔZ. We define the uncertainty in ΔZ as $\delta\Delta Z$, the percentage deviation of ΔZ from the ideal value, ΔZ_{ideal} (eq 17). The uncertainty in Z^0 and f, expressed as a percentage of the ideal value determined by assuming ΔZ_{ideal}, is equal to $\delta\Delta Z$.

$$\delta\Delta Z \equiv \frac{\Delta Z_{ideal} - \Delta Z}{\Delta Z_{ideal}} \quad (17)$$

Values of R_H are determined from f using eq 18, which describes a model developed by Henry[18] for the electrophoretic mobility of a colloidal particle; this model is appropriate to the linear regime of plots of μ^n_{elec} vs $n\Delta Z$.[5] For most situations involving proteins, the value of κR_H is <1 and $g(\kappa R_H) \approx 1$.

$$f = \frac{6\pi\eta R_H(1 + \kappa R_H)}{g(\kappa R_H)} \quad (18)$$

Rewriting eqs 16 and 18 as eqs 19 and 20 and solving for R_H yields eq 21, which shows that the relative uncertainty in R_H will always be less than the relative uncertainty in ΔZ.

$$f = A_2\Delta Z \quad (19)$$

$$f = A_1 R_H(1 + \kappa R_H) \quad (20)$$

$$R_H = \frac{\sqrt{1 + 4\kappa A_2\Delta Z/A_1} - 1}{2\kappa} \quad (21)$$

where

$$A_1 = \frac{6\pi\eta}{g(\kappa R_H)}$$

and

$$A_2 = \frac{1}{\mu^1_{CE} - \mu^0_{CE}} = \frac{2}{\mu^2_{CE} - \mu^o_{CE}} = \frac{3}{\mu^3_{CE} - \mu^o_{CE}} = \frac{n}{\mu^n_{CE} - \mu^o_{CE}}.$$

Figure 8 demonstrates the relationship between uncertainty in ΔZ and values of Z^0 and R_H determined for lysozyme by linear regression analysis of data from charge ladders and CE.

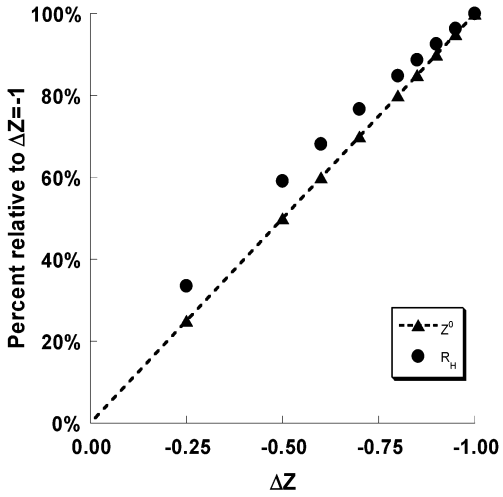

Figure 8. Effect of differences in ΔZ on values of the hydrodynamic radius R_H and the net charge Z^0 of lysozyme determined from the linear regression analysis of data obtained from charge ladders and CE. Values of R_H and Z^0 are expressed as a percentage of the values obtained assuming the ideal value of $\Delta Z = -1$: $Z^0 = 7.32 \pm 0.03$ and $R_H = 22.35 \pm 0.35$ Å. Error bars, which are the standard deviation of at least three runs, are smaller than the symbols shown.

In this example, we assume $\Delta Z_{ideal} \approx -1 \Rightarrow \delta\Delta Z = 1 + \Delta Z$. We plot the percent change in values of Z^0 and R_H from the values calculated assuming the ideal value of $\Delta Z = -1$ as a function of ΔZ. We see that the relative difference in the Z^0 and R_H from those determined using the ideal value ΔZ will always be less than or equal to the relative uncertainty in ΔZ itself.

Conclusions

Much of the utility of charge ladders results from the ability to separate the measured values of electrophoretic mobility of a protein into the individual contributions of net charge and hydrodynamic drag. The accuracy with which these quantities are determined depends directly on the accuracy of our estimate of ΔZ, which is the change in net charge of the protein that results from the chemical modification of charged groups. Our work, in agreement with the previous work of Menon and Zydney,[13] shows that changes in the net charge of proteins due to the acetylation of Lys ϵ-amino groups ΔZ does not necessarily equal the ideal value of -1, even if the amino groups that are acetylated are fully protonated. This difference is due to cooperativity in proton binding between Lys ϵ-amino group that are acetylated and other titratable groups on the proteins.

Measured changes in values of pK_a of the N-terminal α-amino group of lysozyme due to acetylation of Lys ϵ-amino groups show that cooperativity of proton binding can be significant under the conditions of low ionic strength ($\sim$8 mM) and alkaline pH (8.4) typically used in the analysis of protein charge ladders. These experiments also show that the effects of cooperativity in proton binding between titratable groups can be largely eliminated if analysis by CE is done at an ionic strength of $\sim$100 mM.

We identify two types of cooperative interactions that can affect values of ΔZ. We characterized these interactions as specific and nonspecific. Specific interactions are the results of cooperativity in proton binding between a particular Lys ϵ-amino group and another specific titratable group on the protein. Individual interactions of this type can affect values of ΔZ by up to 0.3 units of charge. Specific interactions can be measured by shifts in values of pK_a and as a splitting of the rungs of the charge ladder separated by CE. These interactions are expected to be important in solutions of low ionic strength when two conditions are satisfied: (1) the titratable group is within $\sim$15 Å of a Lys ϵ-amino group that has been acetylated and (2) the titratable group has a value of pK_a within $\sim$1 pH unit of the solution.

Nonspecific interactions are the result of cooperativity between a particular Lys ϵ-amino group and a number of other titratable groups; each interaction is relatively weak and, therefore, is difficult to measure. The combined effects of these nonspecific interactions can have significant impact, affecting values of ΔZ by as much as 0.1 units of charge.

This result highlights the importance of the distance dependence of interactions between titratable groups in determining the effects of cooperativity in proton binding on values of ΔZ. It also shows that a simpler mode used previously, that of Linderstrøm-Lang, which treats the protein as a uniformly charged sphere and thereby accounts for cooperativity only in an average sense, does not capture these specific interactions. Both specific and nonspecific effects of cooperativity in proton binding on values of ΔZ can be predicted by simulating the proton titration curves of proteins using a combination of Poisson−Boltzmann theory to calculate electrostatic potentials and Monte Carlo simulations to determine the average degree of protonation of individual residues on a protein as a function of pH. Results from these calculations could also be used as input for models of the electrophoresis of proteins that use a similar level of atomic detail.[34,35]

A simple empirical relationship based on a screened Coulombic potential can accurately capture the results of the detailed titration calculations. The fact that this model accurately captures the results from the more detailed calculations using Poisson−Boltzmann theory suggests that titratable residues on the surface of proteins interact largely as if they were point charges separated by a specific distance defined by their location on the protein, but otherwise interacting through a solution of composed only of small ions and water. We expect this empirical relationship will be useful in estimating effects of cooperativity in proton binding on the analysis of charge ladders.

Uncertainty in values of ΔZ can be incorporated into an error analysis of the values of net charge and hydrodynamic radius of proteins determined by CE and charge ladders. This analysis shows that the uncertainty in values of net charge and hydrodynamic radius are always less than or equal to the uncertainty in values of ΔZ. Uncertainty in values of ΔZ can be reduced through the use of electrostatics calculations that use, as a minimum, the distance between titratable groups on the protein (as obtained from structural data from NMR and/or X-ray diffraction). These calculations can also be used to identify specific groups that could be mutated to reduce the effects of cooperativity on ΔZ.

Experimental Details

Materials. Fused silica capillaries (i.d. = 50 μm; o.d. = 360 μm) were purchased from Polymicro Technologies (Phoenix, AZ). Trizma base, glycine, lysozyme (chicken egg white), sodium phosphate monobasic, and sodium phosphate dibasic were obtained from Sigma Chemical (St. Louis, MO). Poly(diallyldimethylammonium chloride) (PDADMAC) (high molecular weight, 20 wt % solution), acetic anhydride, 1,4-dioxane, and *p*-methoxybenzyl alcohol (PMBA) were obtained from Aldrich (Milwaukee, WI). Sodium chloride, 1 N NaOH, 1 N HCl, and RBS 35 were obtained from Fisher Scientific (Fair Lawn, NJ) and Pierce (Rockford, IL), respectively. All chemicals

were used as received, and all solutions were made using 18 MΩ deionized water.

Synthesis of Protein Charge Ladders. Protein charge ladders of lysozyme were made by the selective acetylation of the Lys ϵ-amino groups at pH 12. Lysozyme was dissolved in deionized water at a concentration of 1 mM; 500 μL of this protein solution was mixed with 500 μL of the reaction buffer (50 mM phosphate buffer, pH 12). Two to 10 μL of a 1 vol % acetic anhydride solution (in 1,4-dioxane) were added to the protein solution, and the reaction products were analyzed by capillary electrophoresis without further purification. Further aliquots of the acetic anhydride solution were added until a desired distribution of products was achieved. The *N*-terminal α-amino group of lysozyme was also selectively acetylated using a similar procedure. The only difference was the substitution of 100 mM phosphate buffer, pH 6, for the reaction buffer.[3]

Capillary Electrophoresis. Capillary electrophoresis (CE) experiments were ran either on a Beckman P/ACE MDQ or on a Beckman P/ACE 5500. Capillaries ranging in total length from 30 to 50 cm in total length were used. All experiments were performed at a fixed temperature (25 °C) using the internal cooling system of the instruments to dissipate Joule heating.

Because lysozyme is positively charged at values of pH below 10.9 (the pI of the protein), interactions between the protein and the negatively charged silica wall had to be minimized.[6,36] To reduce these interactions, PDADMAC was physically absorbed on to the walls of the capillary.[36] The coating was done by first rinsing the capillary with a 2 vol % solution of RBS 35 for 15 min. The coating solution, 2 wt % polymer in 0.1 N NaOH, was flushed through the capillary for an hour. Prior to the first separation, the capillary was rinsed with the running buffer for 15 min. Capillaries coated in this manner underwent separations in the "reverse polarity" mode with the cathode at the inlet.

An injection sample, with a total volume of 100 μL, was typically prepared by diluting the charge ladder, as synthesized, 10-fold in electrophoresis buffer. PMBA was added to the injection sample at a concentration of 0.0025 vol % as a neutral marker of electroosmotic flow. Separations were performed at an applied potential of 20 kV for electrophoresis buffer with values of ionic strength <50 mM; an applied potential of 10 kV was used for buffers with values of ionic strength > 50 mM. Peak detection was by UV absorbance at 214 nm.

Consecutive separations of charge ladders with CE in buffers of different values of pH or ionic strength were done without changing the capillary. Before changing the composition of the electrophoresis buffer, the capillary was flushed with the polymer solution for 10 min. The capillary was then rinsed with the new electrophoresis buffer for 15 min before performing a separation. Using this approach the variation in measured electrophoretic mobility run-to-run was less than 5%.

pH Titrations of the *N*-Terminal α-Amino Group. pH titrations were conducted at constant ionic strength using a buffer composed of 25 mM Tris-192 mM Gly (ionic strength ~8 mM). The pH was adjusted by the addition of 1 N NaOH and 1 N HCl; NaCl was added to bring the total ionic strength to the desired value (33 or 108 mM).

Simulations of Proton Titration Curves. *Linderstrøm-Lang Model.* Calculations of simulated titration curves using the Linderstrøm-Lang model (as described in the Background section) require as input the number of each type of titratable group, the values of pK_a^{sol} of these groups, and the radius of the sphere representing the protein. We used the following values for the number and pK_a^{sol} of the titratable groups on lysozyme: C-terminal carboxyl group (1, $pK_a^{sol} = 3.8$), Asp (7, $pK_a^{sol} = 4.0$), Glu (2, $pK_a^{sol} = 4.4$), Tyr (3, $pK_a^{sol} = 9.6$), His (1, $pK_a^{sol} = 6.3$), Arg (11, $pK_a^{sol} = 12.5$), Lys (6, $pK_a^{sol} = 10.5$), and *N*-terminal α-amino (1, $pK_a^{sol} = 7.5$).[25,26] These calculations also require as input the radius of the sphere that represents the protein. We assumed a value of 21 Å (an average of the radii measured using charge ladders in 8, 33, and 108 mM solutions). The net charge of lysozyme was relatively insensitive to the value of the radius: a change in radius of 20% caused the net charge to change by less than 0.3 units.

The pK_a of the *N*-terminal α-amino group was calculated as the value of pH at which $\langle x_i \rangle$ given by eq 9 was equal to 0.5. In performing calculations for acetylated proteins, it was assumed that acetylated Lys ϵ-amino groups were neutral could not be protonated at any value of pH.

The Poisson−Boltzmann Monte Carlo Model. The program developed by Beroza et al.[21,23] (available at ftp.scripps.edu:/pub/beroza) was used to calculate electrostatic free energies based on solutions of the linearized Poisson−Boltzmann equation and to then sample the different protonation states of the protein using a Monte Carlo algorithm. The details of this program are given in the literature.[23]

The protein used in these calculations is the triclinic form (1LZT in the Brookhaven Protein Databank). Hydrogen atoms were placed on the protein using the HBUILD[37] facility of CHARMM.[38] The interior of the protein was treated as a low dielectric medium ($\epsilon_p = 20$), while the exterior solvent was treated as a high dielectric ($\epsilon_s = 80$). All calculations were done using the CHARMM PARAM22 all-atom parameter set. The finite difference Poisson−Boltzmann equation was solved assuming a temperature of 298K. A Debye−Hückel boundary condition was used together with, a Stern layer thickness of 2 Å, a focus factor of 0.25, an initial grid spacing of 4 Å, and a maximum number of iterations of 14.

To simulate protonation curves, Monte Carlo simulations were done over a range of values of pH, values which can be computed. Values of pK_a^{int} were calculated as described,[23] using the same values of pK_a^{sol} used in the calculations with the Linderstrøm-Lang model. These simulations were run with 75000 normal steps, during which the protonation state of individual titratable groups were varied, and with 75000 "pairwise" steps, in which two strongly interacting groups (interacting by an energy greater than one unit of pK_a) were varied simultaneously.[22]

Lys ϵ-amino groups were "acetylated" computationally by setting the value of pK_a to zero and excluding interaction energies between the acetylated group and any other titratable groups on the protein, including the other nonacetylated Lys groups.

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