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Correlations Between the Charge of Proteins and the Number of Ionizable Groups They Incorporate: Studies Using Protein Charge Ladders, Capillary Electrophoresis, and Debye−Hückel Theory

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Abstract: The values of electrophoretic mobility, $\mu_{electro}$, of bovine carbonic anhydrase II, human carbonic anhydrase II, cytochrome *c*, lysozyme, superoxide dismutase, ovalbumin, and derivatives of these proteins produced by partial neutralization of Lys ϵ-NH_3^+ and/or Asp and Glu carboxyl groups were measured using capillary electrophoresis (CE). For derivatives of these proteins with the lowest overall values of net charge (either positive or negative), the values of $\mu_{electro}$ and the values of charge measured by CE, Z_{CE}, demonstrate a linear correlation with the number of charged groups, *n*, converted to neutral derivatives. For derivatives of these proteins with larger values of net charge, the values of $\mu_{electro}$ and Z_{CE} demonstrate a nonlinear correlation with *n*. Several observations made in this work suggest that shifts in the values of pk_a of the ionizable groups on these proteins likely contribute to the observed nonlinear correlation. Debye−Hückel theory was used to calculate values of electrostatic potential at the surface of the derivatives of all six proteins from the measured values of $\mu_{electro}$. These values were plotted against the values of electrostatic potential calculated by assigning a charge to each protein in direct proportion to *n*. The data for all six proteins fell along a single common curve, regardless of the concentration of monovalent cations in the electrophoresis buffer.

Introduction

The combination of protein charge ladders and capillary electrophoresis (CE) is a useful tool for measuring the electrostatic properties (e.g., the net charge) of proteins and for examining interactions of proteins with charged ligands.[1] A protein charge ladder is a set of derivatives of a protein produced by the partial modification of its Lys ϵ-NH_3^+ and/or carboxyl groups; CE often resolves this set of derivatives into a set of distinct peaks.[2] Each peak or "rung" of the charge ladder is composed of a group of regioisomeric derivatives of the protein that have the same number of modifications and approximately the same value of charge. In this paper, we use the combination of protein charge ladders and CE to explore the way in which the electrophoretic mobility, $\mu_{electro}$, and, by inference, the net charge of proteins depend on their number of charged groups, and the properties of the electrophoresis buffer.

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The correlation of the net charge of proteins with their number of charged groups may appear at first to be a rather simple issue: for example, a reasonable hypothesis may be that the net charge of a protein correlates linearly with the number of charged groups (assuming that the groups under consideration are uniformly charged under the conditions of the analysis and that modification of any number of these groups does not affect the charge of the remaining groups). We have previously shown that for charge ladders of many proteins, the values of $\mu_{electro}$, and, hence, the values of charge estimated by CE, Z_{CE}, of the proteins of lowest overall absolute charge of a charge ladder do correlate approximately linearly with the number of modifications.[2] This linear correlation provides an estimate of the coefficient of friction of the protein and allows for the direct determination of the values of charge of the native protein and its derivatives from values of $\mu_{electro}$.[1]

Linderstrøm-Lang,[3] Tanford,[4] Abe,[5] Kuehner,[6] and others[7] over the last 75 years have used hydrogen ion titration curves to measure the net charge of proteins as a function of the pH of the solution. From the shapes of these curves, they concluded that altering the net charge of a protein influences the values of pK_a of the ionizable groups: the values of pK_a shift to lower (more acidic) values at low values of pH, where the protein has a net positive charge, and shift to higher (more alkaline) values at high values of pH, where the protein has a net negative charge. Values of pK_a may change by more than 1.5 units as the value of pH is varied from 2 to 12.[4] Site-directed mutagenesis has also revealed that the values of pK_a of specific residues may depend significantly on the number of charged groups on proteins: mutagenesis of charged groups on the serine protease, substilisin, resulted in a shift in the value of pK_a of the active site histidine, His_{64}, by +0.08 to −1.0 units; the first value was due to the mutation $Lys_{213} \rightarrow$ Thr (15 Å from His_{64}), the second was due to the mutation $Glu_{156} \rightarrow$ Lys (14.7 Å from His_{64}).[8,9] Altering the number of charged groups on a protein—by changing the value of pH, by site-directed mutagenesis, or by chemical modifications of functional groups—may alter the values of pK_a of other ionizable groups by at least two mechanisms: via electrostatic induced pK_a shifts,[4] which occur when the number of charged groups on a protein is altered, and via hydrophobic induced pK_a shifts,[10] which occur when the number of hydrophobic groups on a protein is altered. The relative effects of these two mechanisms may be determined by altering the ionic strength of the solution under study—only electrostatic effects are altered significantly by changes in ionic strength.

There are parallels between the previous approaches to measuring the net charge of proteins from hydrogen ion titrations[4−6] and the approach used in this work—the combination of charge ladders of proteins and CE. In both experiments the net charge of a protein is varied in a regular way: with hydrogen ion titrations, the charge is varied by altering the activity of hydrogen ions in solution, which, in turn, alters the fraction of protonated acidic and basic groups; with protein charge ladders, the charge is varied by modifying charged groups on the protein chemically while maintaining constant the properties of the solution. The way in which the net charge of the protein is measured differs between the two techniques: for hydrogen ion titrations, the net charge is obtained from the mass balance of hydrogen ions over a range of values of pH of the solution; for charge ladders of proteins, the net charge is estimated from a constant of proportionality between the number of groups modified and the values of $\mu_{electro}$ (Figure 1). In contrast to hydrogen ion titrations, the combination of charge ladders of proteins and CE allows a measure of the net charge of a native protein, as well as the derivatives of the protein that make up the charge ladder, in a single experiment: this approach does not require the careful control of the properties of solution (such as ionic strength) over a range of values of pH, as is required for hydrogen ion titrations.

In this paper, we show that the correlation of the values of Z_{CE} of the proteins that constitute the rungs of a protein charge

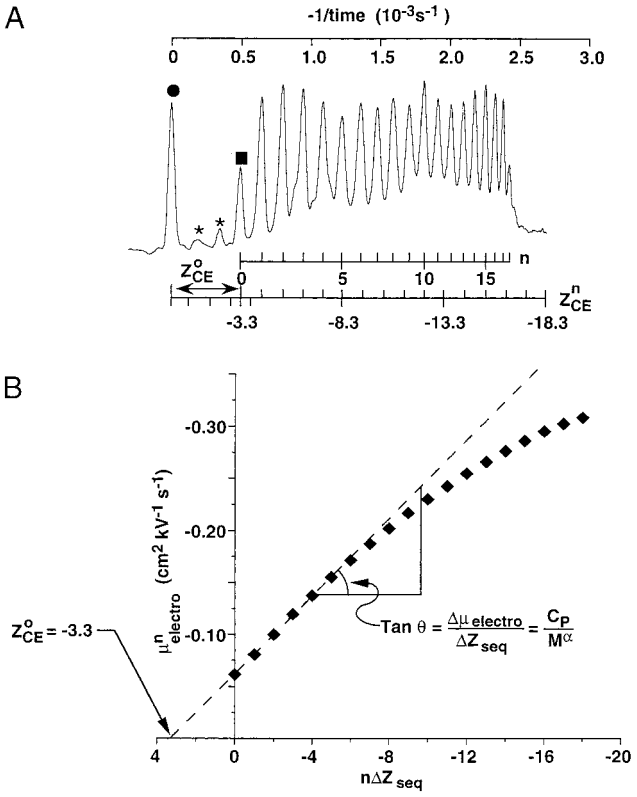

Figure 1. (A) The charge ladder of bovine carbonic anhydrase II (BCA II) produced by the partial acetylation of Lys ϵ-NH_3^+ groups; each acetylation results in an assumed increment of charge, ΔZ_{seq}, due to the conversion of a Lys ϵ-NH_3^+ group to its neutral ϵ-$NHCOCH_3$ derivative ($\Delta Z_{seq} \approx -1$ at pH = 8.4). The number of ϵ-$NHCOCH_3$ groups, *n*, and the net charge of the rungs of the charge ladder estimated by CE, Z^n_{CE}, are indicated below the electropherogram. The −1/time scale is directly proportional to the electrophoretic mobility (Mammen, M.; Colton, I. J.; Carbeck, J. D.; Bradley, R.; Whitesides, G. M. *Anal. Chem.* **1997**, *69*, 2165−2170). The peak marked with (●) is an electrically neutral marker, and the peak marked with (■) is the native protein. The peaks marked with (*) are impurities. **(B)** The electrophoretic mobilities, $\mu^n_{electro}$, of the rungs of the charge ladder of BCA II, plotted as a function of $n\Delta Z_{seq}$. The straight line is a linear least-squares analysis of the first five rungs of the ladder. The slope of this line yields the value of $\Delta\mu_{electro}/\Delta Z_{seq}$, which is an estimate of C_P/M^α; the *x*-intercept gives the charge of the native protein in solution, Z^o_{CE}; the *y*-intercept is the mobility of the native protein, $\mu^o_{electro}$ (see eq 7 in the text). Separations were performed at 25 °C on a 47-cm silica capillary (40 cm from inlet to detector; i.d. 50 μm) using a running buffer of 25 mM Tris−192 mM Gly (pH 8.4). Detection was by direct UV absorbance at 214 nm.

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ladder with the number of modified charged groups, *n*, is dependent on the magnitude of the net charge of the proteins. For the rungs of charge ladders of lowest overall values of charge, the values of Z_{CE} correlate approximately linearly with n; as the net charge increases, the correlation of the values of Z_{CE} with n becomes increasingly nonlinear.

We use Debye−Hückel theory to convert the values of $\mu_{electro}$ and Z_{CE} of the proteins of a charge ladder into values of electrostatic potential by considering each protein as a sphere with a uniformly charged surface. We propose three mechanisms for the observed nonlinearity that are based on changes in the electrostatic potential at the surface of proteins. One mechanism involves a shift in the values of pK_a of ionizable groups using the same model as Tanford used in his interpretation of hydrogen ion titration curves.[4] We also present two other mechanisms that may account for the observed nonlinearity: changes in the conformation of the protein, and changes in the distribution of ions surrounding the protein in solution. We conclude that, regardless of the mechanism, the observed nonlinearity is caused generally by an increase in the electrostatic potential at the surface of these proteins.

Results and Discussion

A protein having a net charge moves through an aqueous buffer under the influence of an electric field (E, V m^{-1}). This protein experiences two forces: an electrostatic force ($f_{electro}$, N) and a hydrodynamic force (f_{hydro}, N) (eqs 1−2), where eZ (C) is the total charge of the protein (e is the charge of a proton), ζ (N s m^{-1}) is its coefficient of friction with the solution, and υ (m s^{-1}) is its velocity. These forces oppose one another: the electrostatic force tends to accelerate the protein; the hydrodynamic force tends to decelerate it. When the velocity of the protein has reached a steady-state value, these two forces are equal, and eq 3 expresses the velocity. The electrophoretic mobility ($\mu_{electro}$, m^2 V^{-1} s^{-1}) of this protein is defined as its velocity per unit electric field strength (eq 4).

$$f_{electro} = eZE \tag{1}$$

$$f_{hydro} = \zeta\upsilon \tag{2}$$

$$\upsilon = \frac{eZE}{\zeta} \tag{3}$$

$$\mu_{electro} = \frac{\upsilon}{E} = \frac{eZ}{\zeta} \tag{4}$$

Equation 5 gives an alternative way of expressing the value of $\mu_{electro}$ of a protein analyzed by CE, where Z_{CE} is its charge, M is its molecular weight and C_P and α are empirical parameters that depend on the properties of the electrophoresis buffer; the ratio of terms M^α/C_P is the effective coefficient of friction of the protein. The value of $\mu_{electro}$ of a protein is measured from its velocity (υ_x) relative to that of an electrically neutral marker (υ_{nm}) per unit of applied electric field ($E_{applied}$) and is calculated using eq 6. In this equation, L_{det} (m) is the length of the capillary from the inlet to the detector; L_{tot} (m) is the total length of the capillary; V (V) is the applied voltage; t_{nm} (s) is the time of migration of an electrically neutral marker from injection to detection, and t_x (s) is the time of migration of an analyte peak. The sign of the numerator in eq 6 is determined by the sign of the value of Z_{CE}; the sign of the value of $\mu_{electro}$ of an analyte is, by convention, the same as the sign of its value of Z_{CE}.

$$\mu_{electro} = \frac{C_P}{M^\alpha} Z_{CE} \tag{5}$$

$$\mu_{electro} = \pm\frac{(\upsilon_{nm} - \upsilon_x)}{E_{applied}} = \pm\frac{\left[\left(\frac{L_{det}}{t_{nm}}\right) - \left(\frac{L_{det}}{t_x}\right)\right]}{\left(\frac{V}{L_{tot}}\right)} \tag{6}$$

The values of $\mu_{electro}$ of the rungs of a protein charge ladder with the lowest overall values of charge correlate approximately linearly with $n\Delta Z_{seq}$, where ΔZ_{seq} is the calculated change in charge per modification.[2] The value of ΔZ_{seq} depends on the pK_a of the Lys ϵ-NH_3^+ or $-CO_2^-$ group that is modified, and the pH of the running buffer. At pH 8.4, the value of pH of the electrophoresis buffer used in our CE experiments, the value of ΔZ_{seq} is -1 for the modification of a Lys ϵ-NH_3^+ group (pK_a $\cong$ 10.7) and is $+1$ for the modification of a Glu- or Asp-CO_2^- group (p$K_a \cong$ 4.4 and 4.0, respectively). Equation 7 describes the relationship between the value of $\mu_{electro}$ of the nth rung of the charge ladder, $\mu^n_{electro}$, and $n\Delta Z_{seq}$, for the rungs with the lowest overall values of charge; Z^o_{CE} and $\mu^o_{electro}$ are the values of Z_{CE} and $\mu_{electro}$ of native protein, respectively. The value of $n\Delta Z_{seq}$ is approximately equal to the difference between the value of Z^n_{CE}, the charge of the nth rung of the charge ladder, and the value of Z^o_{CE} (eq 8). The slope of the best-fit line from a linear least-squares analysis of a plot of $\mu^n_{electro}$ vs $n\Delta Z_{seq}$ is equal to C_P/M^α; the value of Z^o_{CE} is determined from the x-intercept; eq 9 expresses the value of Z^n_{CE}. As an example, the values of $\mu_{electro}$ of the first five rungs of the charge ladder of bovine carbonic anhydrase II (BCA II), produced by the acetylation of its Lys ϵ-NH_3^+ groups, correlate approximately linearly with $n\Delta Z_{seq}$. Extrapolation of the best-fit line to the x-intercept of this plot leads to the value of Z^o_{CE} of -3.3 (Figure 1).

$$\mu^n_{electro} \cong \frac{C_P}{M^\alpha}(Z^o_{CE} + n\Delta Z_{seq}) = \frac{C_P}{M^\alpha} n\Delta Z_{seq} + \mu^o_{electro} \tag{7}$$

$$n\Delta Z_{seq} \cong Z^n_{CE} - Z^o_{CE} \tag{8}$$

$$Z^n_{CE} = \frac{\mu^n_{electro}}{\left(\frac{C_P}{M^\alpha}\right)} \tag{9}$$

The relationship between the values of $\mu^n_{electro}$ and $n\Delta Z_{seq}$ is no longer linear after the first several rungs (i.e., those having lowest overall charge, Z_{CE}) of the charge ladder of BCA II. We previously examined the contribution of mass and volume from different conjugated acyl groups to the coefficient of friction of BCA II by analyzing the values of $\mu_{electro}$ of the rungs of the charge ladders of BCA II formed with acylating agents of different molecular weight.[2] We inferred that for charge ladders of BCA II formed with acylating agents of low molecular weight (e.g., acetic anhydride), the nonlinear change in the values of $\mu^n_{electro}$ as a function of $n\Delta Z_{seq}$ is not directly due to the contribution of the conjugated acyl groups to the coefficient of friction of the protein (for example, by increasing its hydrodynamic radius or its hydrophobic interactions with other species in solution).

One effect of adding increasing concentrations of salt to a solution containing charged molecules is to decrease, or screen, the electrostatic potential that surrounds the molecules. In an

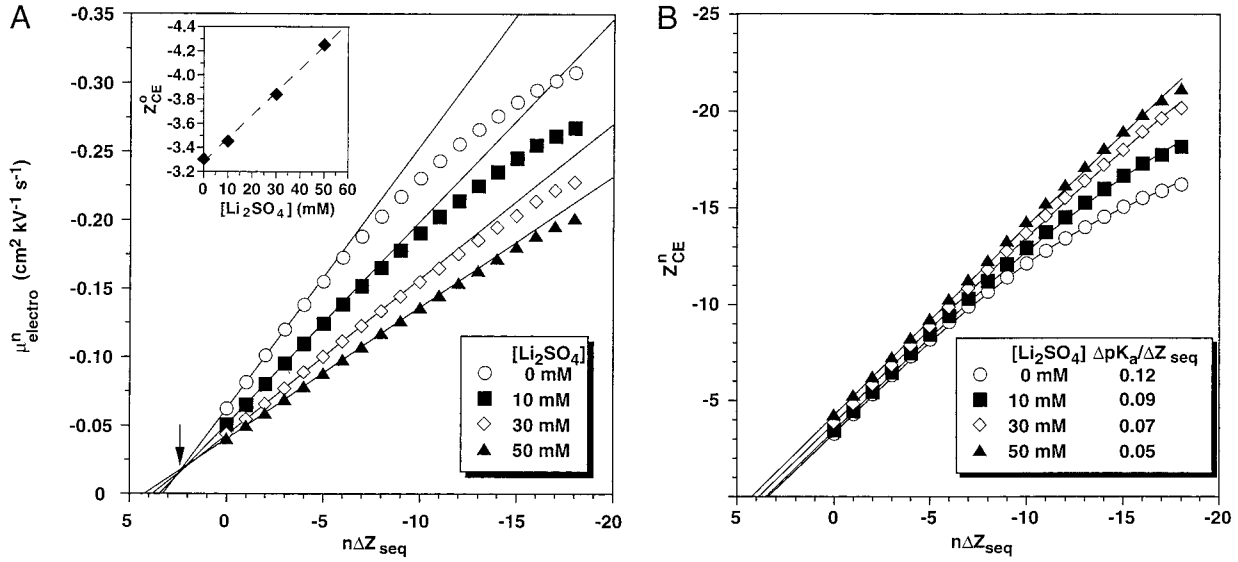

Figure 2. (A) The electrophoretic mobility, $\mu^n_{electro}$, of the rungs of the acetamide charge ladder of BCA II measured at various concentrations of Li_2SO_4 in the electrophoresis buffer (25 mM Tris−192 mM Gly, pH 8.4), plotted as a function of $n\Delta Z_{seq}$. The straight lines are linear least-squares analyses of the first five rungs of the ladders. The arrow indicates where these lines appear to intersect at common point ($\mu^n_{electro} \approx -0.02$, $n\Delta Z_{seq} \approx 2.3$). The inset shows the values of Z^o_{CE} obtained from the *x*-intercept of these lines, as a function of the concentration of Li_2SO_4 in the electrophoresis buffer. The straight line is a linear least-squares analysis of these data. Separation conditions were the same as in Figure 1. The applied voltage was lowered from 15 to 6 kV as the concentration of Li_2S0_4 in the running buffer was increased from 0 to 50 mM to reduce joule heating within the capillary. (Varying the applied voltage from 15 to 6 kV in the absence of Li_2SO_4 had no observable effect on the correlation of $\mu^n_{electro}$ with $n\Delta Z_{seq}$) **(B)** The net charge of the rungs of the charge ladder of BCA II estimated by CE, Z^n_{CE}, from values of $\mu^n_{electro}$ measured at various concentrations of Li_2SO_4 in the electrophoresis buffer, plotted as a function of $n\Delta Z_{seq}$. The solid curves were obtained by performing nonlinear least-squares fitting of the data to eqs 10−13. The data were fit using a value of pK_a of the His residues on the native protein, pK^o_a, of 6.5; the values of the change in pK_a due to acetylation, ΔpK_a, that best fit the data at each concentration of Li_2SO_4 in the electrophoresis buffer are indicated in the legend.

effort to gain some insight into how the values of $\mu_{electro}$ and values of Z_{CE} of BCA II and its acetamide derivatives were influenced by the electrostatic potential at the surface of the protein, we measured values of $\mu^n_{electro}$ of the rungs of the charge ladder of BCA II in the presence of increasing concentrations (0−50 mM) of Li_2SO_4 in the electrophoresis buffer (Figure 2A).[11] We then estimated the values of Z^o_{CE} and of Z^n_{CE} from these data using eq 9 (Figure 2B).

In general, increasing the concentration of Li_2SO_4 in the electrophoresis buffer increased the number of rungs of the charge ladder of BCA II that had values of $\mu^n_{electro}$, and, hence, values of Z^n_{CE}, that correlated linearly with $n\Delta Z_{seq}$. The presence of increasing concentrations of Li_2SO_4 also had a significant effect on the estimated value of the net charge of the native proteins: the magnitude of Z^o_{CE} increased approximately linearly with the concentration of Li_2SO_4 in the electrophoresis buffer—from −3.3 at [Li_2SO_4] = 0 mM to −4.2 at [Li_2SO_4] = 50 mM. From Figure 2A we also observe that the lines from the least-squares analyses of the values of $\mu^n_{electro}$ of the first five rungs of the ladders intersect at a similar point. This common point of intersection is a direct result of the approximately linear dependence of Z^o_{CE} with [Li_2SO_4]. If Z^o_{CE} were independent of the concentration of salt, then these lines would all intersect at the same point on the *x*-axis, $n\Delta Z_{seq} = Z^o_{CE}$. From these results, we infer that values of Z^o_{CE}—and the degree to which $\mu^n_{electro}$ and Z^n_{CE} correlate nonlinearly with $n\Delta Z_{seq}$—are related to the magnitude of the electrostatic potential at the surface of the protein; the effect of increasing concentrations of Li_2SO_4 is to reduce or screen the effects of this potential.

Kuehner, et al.[6] performed hydrogen ion titrations of hen-egg-white lysozyme and free amino acids in solutions containing concentrations of KCl from 0.1 to 2.0 M; from these data they estimated the value of net charge of the protein and the values of pK_a of ionizable groups on the protein as a function of pH and ionic strength. For values of pH below the pI of the protein (i.e., where the protein has a net positive value of charge—for lysozyme, pI = 11.16) the values of pK_a of the ionizable groups on this protein, estimated from fits to hydrogen ion titration curves, were shifted to smaller (more acidic) values, relative to values of pK_a of side chains measured for single amino acids in solution. They also showed that these shifts in values of pK_a decreased, and the net charge of the protein increased, with increasing concentration of KCl in solution: at a value of pH of 2.5, the net charge of lysozyme increased from approximately 15 to 17 as the concentration of KCl was increased from 0.1 to 2.0 M. These results are consistent with our observation that the magnitude of the net charge of the native BCA II increased with increasing concentrations of Li_2SO_4 in the electrophoresis buffer.

A shift in the value of pK_a of an amino acid residue will have a significant effect on the values of $\mu_{electro}$ and Z_{CE} of a protein only if the value of pK_a is close to the pH of the electrophoresis buffer. For amino acids having normal values of pK_a, it is likely that only shifts in the values of pK_a of His imidazole residues (p$K_a \cong 6.5$) contribute significantly to values of $\mu^n_{electro}$ and Z^n_{CE} at pH 8.4: Lys $\epsilon-NH_3^+$ residues (p$K_a \cong$ 10.2), Arg guanidino residues (p$K_a \cong 12$), and Tyr phenol residues (p$K_a \cong 10.5$) are approximately fully protonated at pH 8.4; Asp and Glu carboxylate residues (p$K_a \cong 4.0$) require extraordinary changes in the electrostatic potential to become significantly protonated at pH 8.4. It is, however, common to find that the pK_a of the *N*-terminal $\alpha-NH_3^+$ group lies between 7 and 10, and the values of pK_a of other groups may often be

(11) Similar results were obtained with various concentrations of K_2SO_4, NaCl and $(NH_4)_2SO_4$ in the electrophoresis buffer.

abnormal, differing from average values by several units of pH.[12] The *N*-terminal α-amino group of native BCA II is acetylated, and we do not include the effects of this group.

We developed a model that describes the nonlinearity observed in plots of $\mu_{electro}$ vs $n\Delta Z_{seq}$ of the rungs of the charge ladder of BCA II as a result of shifts in the values of pK_a of His residues. We expressed the influence of the values of charge of His imidazole residues on the values of Z^n_{CE} of BCA II by eq 10, where Z^{o*}_{CE} is the charge of the native protein that excludes the contributions of charge of His residues (eq 11), N_{His} is the number of ionizable His residues (8)[13] and $\langle Z^n_{His}\rangle$ is the average value of charge of His residues of the proteins in the *n*th rung of the charge ladder. The value of $\langle Z^n_{His}\rangle$ is expressed as a function of the average value of pK_a of His residues of the proteins in the *n*th rung of the charge ladder, pK^n_a, and the value of pH of the electrophoresis buffer (eq 12). We assumed a linear dependence of the values of pK_a of the His residues on the number of acetylated Lys $\epsilon-NH_3^+$ groups, as is expressed in eq 13 where pK^o_a is the value of pK_a of the His residues on the native protein, and ΔpK_a is the change in pK_a per acetylation. We fit eqs 10−13 to values of Z^n_{CE} measured for the acetamide charge ladder of BCA II at various concentrations of Li_2SO_4 in the electrophoresis buffer by assuming $pK^o_a = 6.5$ and solving for the values of ΔpK_a for each of the concentrations of salt (Figure 2B). The values of ΔpK_a obtained from fitting this model vary from 0.12 to 0.05 units as the concentration of Li_2SO_4 is increased from 0 to 50 mM. We infer that modest changes in the value of pK_a (∼0.1 unit of pH per acetylation) can adequately account for the observed nonlinearity in plots of $\mu^n_{electro}$ vs $n\Delta Z_{seq}$. This model is also consistent with the hypothesis that increasing amounts of salt in the electrophoresis buffer decreases the effects of the net charge of the protein on the values of pK_a of ionizable groups.

$$Z^n_{CE} = Z^{o*}_{CE} + n\Delta Z_{seq} + N_{His}\langle Z^n_{His}\rangle \quad (10)$$

$$Z^{o*}_{CE} = Z^o_{CE} - N_{His}\langle Z^o_{His}\rangle \quad (11)$$

$$\langle Z^n_{His}\rangle = \frac{1}{1 + 10^{(pH - pK^n_a)}} \quad (12)$$

$$pK^n_a = pK^o_a + n\Delta pK_a \quad (13)$$

Debye−Hückel Model: We used Debye−Hückel theory to convert values of $\mu_{electro}$ and Z_{CE} to values of electrostatic potential at the surface of the protein. This theory predicts a simple relation between the electrostatic potential, φ_{DH} (V), at the surface of a spherical ion of radius R (m), and charge eZ evenly distributed on its surface (given in eq 14). Here κ (m^{-1}) is the Debye−Hückel parameter, and ϵ (unitless) is the dielectric constant of the solution surrounding the ion (ϵ_o is the dielectric constant of vacuum).[14] The term outside of the parentheses is the potential in the absence of any small ions in solution; the term in parentheses represents the decrease in the potential due to screening by small ions in solution.[15] The Debye−Hückel theory leads to a similar expression for the value of $\mu_{electro}$ of this same ion, given in eq 15, where η (kg m^{-1} s^{-1}) is the viscosity of the solution surrounding the ion. Together, eqs 14−15 predict the direct proportionality between the values of φ_{DH} and $\mu_{electro}$ known as the Hückel equation (eq 16).[16]

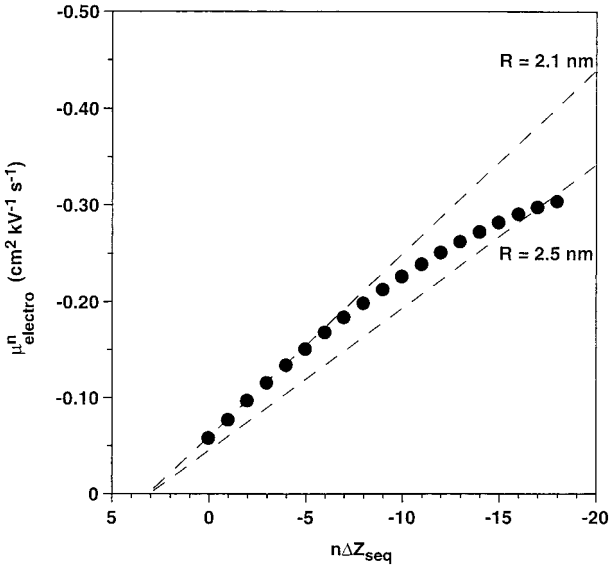

Figure 3. A comparison of measured values of the electrophoretic mobilities, $\mu^n_{electro}$, of the rungs of the charge ladder of BCA II (●) and values predicted by Debye−Hückel theory for spherical ions with a uniformly charged surface. The dashed lines are the values of $\mu^n_{electro}$ calculated using eq 15 for spherical ions of radius $R = 2.1$ and 2.5 nm and values of charge equal to $Z^o_{CE} + n\Delta Z_{seq}$.

$$\varphi_{DH} = \frac{eZ}{4\pi\epsilon_o\epsilon R}\left(\frac{1}{1+\kappa R}\right) \quad (14)$$

$$\mu_{electro} = \frac{eZ}{6\pi\eta R}\left(\frac{1}{1+\kappa R}\right) \quad (15)$$

$$\varphi_{DH} = \frac{3}{2}\frac{\eta}{\epsilon_o\epsilon}\mu_{electro} \quad (16)$$

Here, we consider each protein in a charge ladder to be a spherical ion that has a uniformly charged surface, and a single radius.[17] Using eq 15, we calculated values of $\mu_{electro}$ of the proteins of the acetamide charge ladder of BCA II for two values of the radius, $R = 2.1$ and 2.5 nm, assuming values of charge equal to $Z^o_{CE} + n\Delta Z_{seq}$.[18] We compared these values to measured values of $\mu^n_{electro}$ for this protein (Figure 3). The values of $\mu^n_{electro}$ for the first five rungs of the charge ladder of BCA II are accurately predicted by this model using a value of $R = 2.1$ nm.[18] We conclude that the Debye−Hückel theory provides a simple yet accurate model of the electrophoretic mobility of the first five rungs of the charge ladder of BCA II.

(12) Fersht, A. *Enzyme Structure and Mechanism*; 2nd ed.; W. H. Freeman and Co.: New York, 1985.

(13) BCA II has 11 His residues, eight of which are protonatable under the conditions of this study; three of the His residues coordinate with a Zn(II) ion to form the active site of the enzyme. This coordination prevents their protonation.

(14) Tanford, C. *Physical Chemistry of Macromolecules*; J. Wiley: New York, 1961.

(15) When $\kappa^{-1} = R$, the potential is reduced by a factor of $^1/_2$. Typical values of R for proteins are 1 to 5 nm, and $\kappa^{-1} \approx 0.1$, 3, and 10 nm for ionic strengths of 0.10, 0.01 and 0.001 M, respectively.

(16) Evans, D. F.; Wennerström, H. *The Colloidal Domain: Where Physics, Chemistry, Biology and Technology Meet*; VCH Publishers: New York, 1994.

(17) The radius, *R*, of a macromolecule of molecular weight, *M*, is often assumed to be proportional to $M^{1/3}$.

(18) The dimensions of human CA II taken from its crystal structure are 4.1 nm by 4.1 nm by 4.7 nm [Liljas, A.; Kannan, K. K.; Bergesten, P. C.; Waara, I.; Fridburg, K.; Strandberg, B.; Carlbom, U.; Jarup, L.; Lovgren, S.; Petef, M. *Nature New Biol.* **1972**, *235*, 131.].

We then used Debye−Hückel theory to estimate shifts in the values of pK_a of ionizable groups on a protein as a function of its net charge. Using eq 14, we calculated a value of electrostatic potential at the surface of a sphere of radius 2.1 nm of 4.6 mV per unit increase in charge at an ionic strength of 25 mM.[19] This increase in potential corresponds to a shift in the values of pK_a of 0.08 for all ionizable groups (changes in pK_a are directly proportional to changes in electrostatic potential).[20] These results are consistent with the values of ΔpK_a obtained from fitting eqs 10−13 to values of Z^n_{CE} measured for the acetamide charge ladder of BCA II at various concentrations of Li_2SO_4 in the electrophoresis buffer (Figure 2B).

The model of proteins assumed in these calculations—a sphere with uniform charge distributed on its surface—is the same model used by Tanford in his interpretation of hydrogen ion titration curves.[4] Recently, Zhou[21] used this model of proteins to calculate the effects of electrostatics on the values of pK_a of ionizable groups on proteins. He showed that the Debye−Hückel theory, as well as a cavity-function theory, give theoretical results that are in excellent agreement with experimental hydrogen-ion titration data. We infer that this model of the electrostatic properties of proteins accurately captures the average effects of the net charge of a protein on the values of pK_a of ionizable groups.

Since the nonlinearity observed in plots of $\mu_{electro}$ vs $n\Delta Z_{seq}$ appears to be an effect of the electrostatic potential at the surface of the protein, we calculated values of φ_{DH} of the rungs of charge ladders from their values of $\mu_{electro}$ at various concentrations of Li_2SO_4 in the electrophoresis buffer. We compared the value of φ_{DH} of each rung of the acetamide charge ladder of BCA II, calculated from its measured value of $\mu^n_{electro}$, $\varphi^n_{DH,meas}$ (eq 17), with that predicted by assuming a value of charge of each rung equal to $Z^o_{CE} + n\Delta Z_{seq}$, $\varphi^n_{DH,pred}$ (eq 18) (Figure 4). In using eq 18, the value of C_P/M^α is estimated from the best-fit line of the first five rungs of the charge ladder. We found that all of the data fell along a common curve in a plot of $\varphi^n_{DH,meas}$ vs $\varphi^n_{DH,pred}$. There is good agreement between the absolute values of $\varphi^n_{DH,meas}$ and $\varphi^n_{DH,pred}$ at absolute values of $\varphi^n_{DH,pred} <$ 35 mV; the absolute values of $\varphi^n_{DH,meas}$ decrease below those of $\varphi^n_{DH,pred}$ at absolute values of $\varphi^n_{DH,pred} >$ 35 mV. Increasing the concentration of Li_2SO_4 in the electrophoresis buffer lowered the absolute values of $\varphi^n_{DH,meas}$ and $\varphi^n_{DH,pred}$ to the linear region of the common curve.

$$\varphi^n_{DH,meas} = \frac{3}{2}\frac{\eta}{\epsilon_o\epsilon}\mu^n_{electro} \qquad (17)$$

$$\varphi^n_{DH,pred} = \frac{3}{2}\frac{\eta}{\epsilon_o\epsilon}\left[\left(\frac{C_P}{M^\alpha}\right)(Z^o_{CE} + n\Delta Z_{seq})\right] \qquad (18)$$

Debye−Hückel theory is based on the solution of the linearized Poisson−Boltzmann equation, a second-order nonlinear differential equation. This linearization is valid only for electrostatic potentials of magnitude less than 25 mV at 25 °C (i.e., for values of electrostatic potential such that the magnitude of the energy of a unit charge acted on by the potential, $|e\varphi| <$ kT). Violation of this approximation does not lead to the observed nonlinearity of plots of $\varphi^n_{DH,meas}$ vs $\varphi^n_{DH,pred}$; it may limit, however, the range of data for which Debye−Hückel theory provides an accurate estimate of electrostatic potential at the surface of these proteins.

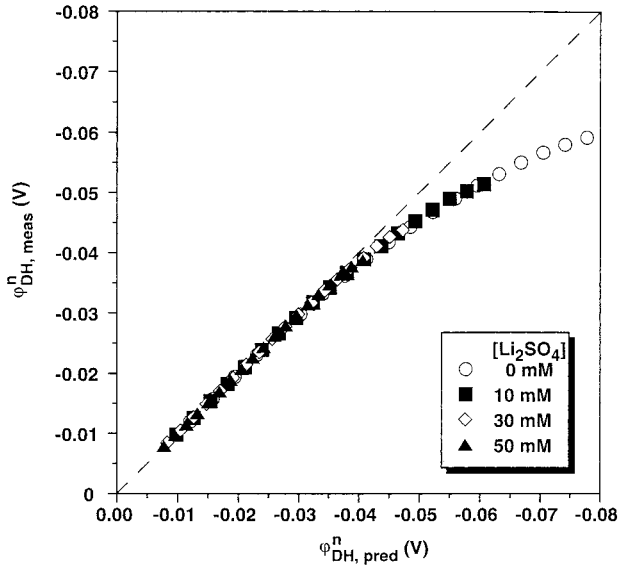

Figure 4. The electrostatic potential, φ_{DH}, of the proteins that make up the rungs of the acetamide charge ladder of BCA II calculated at various concentrations of Li_2SO_4 in the electrophoresis buffer using Debye−Hückel theory. The values of $\varphi^n_{DH,meas}$ are calculated from measured values of $\mu^n_{electro}$ (see eq 17 in the text), and plotted as a function of $\varphi^n_{DH,pred}$, the value of φ_{DH} calculated assuming the value of charge of the proteins that make up each rung of the ladder is equal to $Z^o_{CE} + n\Delta Z_{seq}$ (see eq 18 in the text).

The same limitation to the validity of the linearized Poisson−Boltzmann equation also points to a simple physical picture for the observation that the absolute values of $\varphi^n_{DH,meas}$ decrease below those of $\varphi^n_{DH,pred}$ at absolute values of $\varphi^n_{DH,pred} >$ 35 mV. The chemical potential of an ion (a proton, or other small ion in solution) far from the surface of the protein is due mainly to its configurational entropy, equal to $kT\ \ln(f)$, where f is the volume fraction of the ions in solution—$\ln(f)$ in aqueous electrolytes is generally of order 10. The chemical potential of an ion adjacent to the charged surface of the protein is due mainly to its energy of interaction with the electrostatic potential, equal to $ze\varphi$, where z is the charge of the ion and φ is the electrostatic potential at the surface of the protein. When $|\varphi| >$ 25.7 mV at 25 °C, the chemical potential of an ion in the presence of this electrostatic potential exceeds thermal energies (kT). If the ion has a charge opposite in sign to that of the protein, the ion can lower its chemical potential by binding, or associating tightly, to the surface of the protein; if the ion has a charge of the same sign as that of the protein, the ion can lower its chemical potential by dissociating from the surface of the protein. If the ion under consideration is a proton, then the changes are expressed as shifts in values of pK_a. This rearrangement of ions will decrease the effective charge of the protein until there is an equilibrium established between the chemical potential of the ions at the surface of the protein and those that are free in solution. The effect of increasing concentrations of salt in solution is to reduce the magnitude of φ for a given distribution of charges on the protein, as shown in Figure 4 for BCA II, and thereby to reduce the tendency of ions to associate strongly with the protein.

This model implies that protons and other ions in solution associate with the surface of charged molecules in a way that maintains a magnitude that does not greatly exceed kT/e. This

(19) The calculations were done at 25 °C assuming a dielectric constant of water equal to 80.

(20) The shift in value of pK_a is related to the potential at the titrating site by the Tanford-Roxby equation, $\Delta pK_a = e\varphi/2.303kT$.

(21) Zhou, Y. *J. Phys. Chem.* **1998**, *102*, 10615−10621.

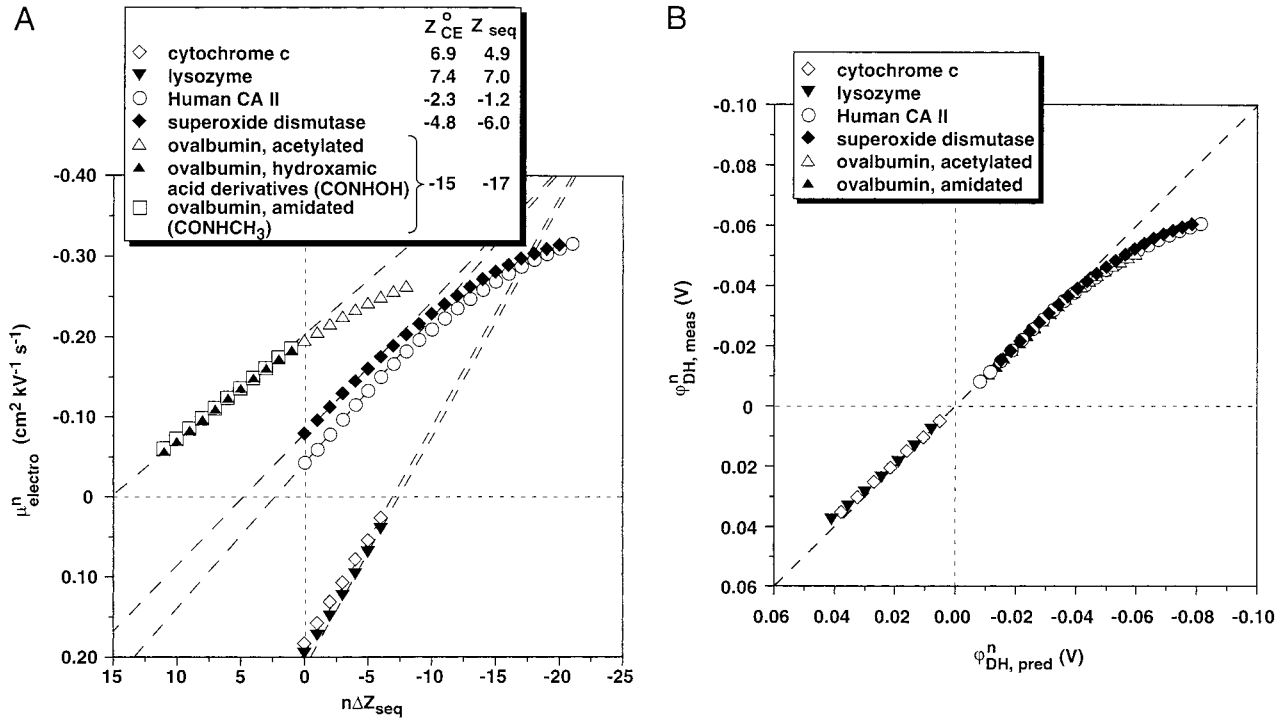

Figure 5. (A) The electrophoretic mobility, $\mu^n_{electro}$, of the charge ladders of human CA II, cytochrome *c*, lysozyme, superoxide dismutase and ovalbumin plotted as a function of $n\Delta Z_{seq}$. The charge ladder of ovalbumin is a combination of the ladder produced by partial acetylation of the Lys ϵ-NH_3^+ groups and the ladder produced by the partial conversion of Glu- and/or Asp-CO_2^- groups to either CONHOH or $CONHCH_3$ groups. The straight lines are linear least-squares analyses of the first five rungs of the ladders of human CA II, superoxide dismutase and ovalbumin, and the first three rungs of the ladders of cytochrome *c* and lysozyme. The values of Z^o_{CE} were determined by extrapolation to the *x*-intercept of the linear least-squares analysis of $\mu^n_{electro}$ vs $n\Delta Z_{seq}$. The values of Z_{seq} were calculated from the sequence of native proteins using the following standard values of pK_a for the residues of the amino acids: Arg = 12; Lys = 10.7; Tyr = 10.2; Cys = 9.3; α-NH_3^+ = 7.4; His = 6.5; α-COOH = 4.9; Glu = 4.4; Asp = 4.0.[2] Separations were performed at 25 °C on a 47-cm silica capillary (40 cm from inlet to detector; i.d. 50 μm) using a running buffer of 25 mM Tris−192 mM Gly (pH 8.4). Detection was by direct UV absorbance at 214 nm. Proteins of pI > 8.4 (cytochrome *c* and lysozyme) and their derivatives were analyzed in reverse polarity on a Polybrene-coated capillary. (B) The electrostatic potential, φ_{DH}, of the proteins that make up the rungs of the charge ladders of human CA II, cytochrome *c*, lysozyme, superoxide dismutase and ovalbumin calculated using Debye−Hückel theory. The values of $\varphi^n_{DH,meas}$ are calculated from measured values of $\mu^n_{electro}$ (see eq 17 in the text), and plotted as a function of $\varphi^n_{DH,pred}$, the value of φ_{DH} calculated assuming the value of charge of the proteins that make up each rung of the ladder is equal to $Z^o_{CE} + n\Delta Z_{seq}$ (see eq 18 in the text).

argument was originally put forward by Alexander, et al.[22] to explain the difference between the effective charge of colloidal particles estimated from measurements of osmotic pressure and bulk modulus, and the charge determined by hydrogen ion titration. This simple model is also consistent with the observation that the linearized Poisson−Boltzmann equation is often a valid assumption for biological systems, regardless of the density of charged groups on a surface of a molecule.[23]

Results for Other Proteins: We found that values of $\mu^n_{electro}$ for charge ladders of five additional proteins (cytochrome *c*, lysozyme, human CA II, superoxide dismutase, ovalbumin) ranging in molecular weight from 14 to 43 kD also demonstrate both linear and nonlinear correlations with values of $n\Delta Z_{seq}$ (Figure 5A). For the rungs of charge ladders with the smallest values of $|Z^o_{CE} + n\Delta Z_{seq}|$, the values of $\mu^n_{electro}$ correlate approximately linearly with $n\Delta Z_{seq}$; as the net charge increases, the values of $\mu^n_{electro}$ deviate from the linear correlation observed for the smaller values of charge. These results are consistent with the results for BCA II (Figure 2A) indicating that the way in which values of $\mu^n_{electro}$, and, therefore, values of Z^n_{CE} depend on the values of $n\Delta Z_{seq}$ is a general property of the proteins included in this study.

We define Z_{seq} as the charge of a native protein estimated from its sequence of amino acids using standard values of pK_a and the pH of the electrophoresis buffer. We compared the measured values of Z_{CE} of the native protein, Z^o_{CE}, with the values of Z_{seq}, and, as we showed previously,[2] found that there is good agreement between the two measures of charge. The differences observed between Z^o_{CE} and Z_{seq} likely arise from the fact that the values of pK_a of the ionizable groups are not constant and may depend on the net charge of the protein and the properties of the solution (e.g., temperature and ionic strength). Also, individual ionizable groups on many proteins have values of pK_a that differ substantially from standard values: for example the pK_a of Glu_{35} on hen-egg-white lysozyme is estimated to be 6.1 from hydrogen ion titration data;[24] the value of pK_a for the side chain of the free glutamic acid is 4.2. We believe that the values of Z_{CE} include these effects and therefore give a more accurate estimate of the true charge of proteins than values of Z_{seq}.

We produced charge ladders of ovalbumin by the partial acetylation of Lys ϵ-NH_3^+ groups and by the partial conversion of Glu- and/or Asp-CO_2^- groups to either CONHOH or $CONHCH_3$ groups. The values of $\mu^n_{electro}$ of these derivatives of ovalbumin all fell along a common curve (Figure 5A). The agreement between Z^o_{CE} and Z_{seq} for ovalbumin is improved,

(22) Alexander, S.; Chaikin, P. M.; Grant, P.; Morales, G. J.; Pincus, P.; Hone, D. *J. Chem. Phys.* **1984**, *80*, 5776−5781.
(23) Honig, B.; Nicholls, A. *Science* **1995**, *268*, 1144−1149.
(24) Kuramitsu, S.; Hamaguchi, K. *Biochem.* **1980**, *87*, 1215−1219.

relative to our previous measurements of Z_{seq} based on only the values of $\mu_{electro}$ of the rungs of a charge ladder produced by acetylation of Lys ϵ-NH_3^+ groups. This improvement is likely due to the use of data from charge ladders of this protein prepared by the partial conversion of Glu- and/or Asp-CO_2^- groups to either CONHOH or $CONHCH_3$ groups; this set of derivatives allowed an estimate of C_P/M^α that was more accurate than that obtained from derivatives of this protein produced by the partial acetylation of Lys ϵ-NH_3^+ groups.

The modification of carboxylic acid residues of ovalbumin with hydroxylamine results in the formation of hydroxamic acid groups (CONHOH): values of pK_a of hydroxamic acids range from 8 to 9;[25] acetohydroxamic acid ($CH_3CONHOH$) has a value of pK_a of 9.0 (whether hydroxamic acids are O acids or N acids is a topic of continued debate[26]). We observed a similar change in values of $\mu_{electro}$ of the protein with the conversion of Glu- and/or Asp-CO_2^- groups to either CONHOH or $CONHCH_3$ groups. This observation implies that the hydroxamic acid groups on this protein are approximately fully protonated at pH 8.4; that is, the spacing of the rungs of the charge ladder are consistent with a value of $\Delta Z_{seq} \approx +1$ at pH 8.4, due to the annihilation of the charge associated with the carboxylate ion and the formation of either a neutral CONHOH or $CONHCH_3$ group. We infer that the pK_a of the hydroxamic acid groups of the derivatives of ovalbumin is significantly affected by local electrostatic potentials, and is, thus, shifted to a value >10.0.

We calculated values of $\varphi^n_{DH,meas}$ from the values of $\mu^n_{electro}$ of these charge ladders and plotted them against their corresponding values of $\varphi^n_{DH,pred}$ (Figure 5B). The data from this plot fell along a common curve with an onset in nonlinearity at a magnitude of $\varphi^n_{DH,pred}$ of approximately 35 mV, in agreement with our results for BCA II obtained at different concentrations of Li_2SO_4 in the electrophoresis buffer. This result suggests that the nonlinearity observed in plots of $\mu^n_{electro}$ vs $n\Delta Z_{seq}$ is a general phenomenon for the proteins included in this study, and is related to the magnitude of the electrostatic potential at the surface of the protein. As the magnitude of this potential exceeds kT/e, the association of protons and other ions with the protein changes in such a way as to reduce the magnitude of the potential to a value that is closer to kT/e.

The Debye−Hückel theory—the theory used in this work—was the first approach used to calculate the electrostatic properties of proteins.[14] Most recent computations of the electrostatic properties of proteins have been done using atomic descriptions of the protein and a continuum model of the aqueous electrolyte solution surrounding the protein, as reviewed by Honig, et al.[23] The Debye−Hückel theory embodies the same model of the solution surrounding the protein as the atomic-level models; both are based on the linearized Poisson−Boltzmann equation,[27] but the Debye−Hückel theory uses a much simpler model of the protein (i.e., a sphere of uniform charge distributed on its surface), rather than an array of charged atoms whose positions are obtained from crystallographic data of the protein. The ability of such a simple model to describe the observed nonlinear correlation between $\mu^n_{electro}$ and $n\Delta Z_{seq}$ is likely related to the observation that the electrophoretic mobility of proteins appears to depend only on their net charge and not on the locations of the charged groups on the protein.

Neither the Debye−Hückel theory nor the more detailed models based on numerical solutions of the Poisson−Boltzmann equation *predict* a nonlinear correlation of mobility and net charge of proteins with the number of modifications of Lys ϵ-NH_3^+ or carboxyl groups. What these theories are able to do is describe the electrostatic potential for a given structure and distribution of charges (in the Debye−Hückel theory, the structure and distribution of charges is described by the radius and density of charge of the surface of a sphere). In this study, we used Debye−Hückel theory not to predict the nonlinear correlation of $\mu^n_{electro}$ vs $n\Delta Z_{seq}$, but to *describe* this nonlinear correlation in terms of the measured and predicted values of electrostatic potential. Previously, we demonstrated that Debye−Hückel theory failed to *predict* differences correctly in the electroosmotic flow inside a silica capillary in the presence of monovalent and divalent cations in solution.[28] We hypothesized that this failure was due to specific association of these ions with the siloxide wall of the capillary. The observed nonlinearity of plots of $\mu^n_{electro}$ vs $n\Delta Z_{seq}$ for charge ladders of proteins may lead to similar conclusions about the association of ions, including protons, to the surface of proteins.

Other Interpretations of Nonlinearity. There are interpretations of the nonlinear correlation of $\mu^n_{electro}$ with $n\Delta Z_{seq}$ that are based on mechanisms other than changes in values of pK_a of ionizable groups. A second interpretation of the nonlinearity is that increasing the electrostatic potential at the surface of a protein changes its conformation due to unfavorable electrostatic interactions between the remaining charged groups; these changes in conformation result in changes in the coefficient of friction. Previously, Aviram, et al., demonstrated that increasing the number of Lys ϵ-NH_3^+ groups modified with maleic anhydride produced changes in the CD spectra of cytochrome *c*, indicating disruption of the native structure of this protein.[29]

The binding affinities of the proteins that constitute the acetamide charge ladder of BCA II for neutral benzenesulfonamide inhibitors are, however, essentially independent of the number of acetylated Lys ϵ-NH_3^+ groups.[30] This observation implies that the structure of at least the active site of this protein is retained across the rungs of its charge ladder. The active site of this protein is buried approximately 15 Å from the surface; even if it remains intact, the periphery of the protein may still change and, thereby, affect the coefficient of friction of the protein. If changes in the conformation of the protein were the only mechanism that caused the observed nonlinearity in plots of $\mu^n_{electro}$ vs $n\Delta Z_{seq}$, then from Figure 3, we would infer that an increase in the radius of BCA II of approximately 0.4 nm across the rungs of the ladder would be required to account for the observed nonlinearity. Such a change in radius could arise, for example, from an increase in extension of the side chains of Glu and Asp as the protein becomes more negatively charged.

A third interpretation of the nonlinearity is that increasing the electrostatic potential at the surface of a protein changes the distribution of small ions surrounding it during the electrophoresis experiment (an effect referred to as counterion relaxation); this effect reduces the effective field acting on the protein and, thereby, increases the measured value of v_x and decreases the magnitude of the calculated value of $\mu_{electro}$ of the protein

(25) Bagno, A.; Comuzzi, C.; Scorrano, G. *J. Am. Chem. Soc.* **1994**, *116*, 916−924.

(26) Ventura, O. N.; Rama, J. B.; Turi, L.; Dannenberg, J. J. *J. Am. Chem. Soc.* **1993**, *115*, 5754−5761.

(27) Atomic-level models have been developed based on numerical solutions of both the linear and the nonlinear Poisson−Boltzmann equation; primarily the linear version of this equation is used in modeling the electrostatic properties of proteins.

(28) Mammen, M.; Carbeck, J.; Simanek, E. E.; Whitesides, G. M. *J. Am. Chem. Soc.* **1997**, *119*, 3469−3476.

(29) Aviram, I.; Myer, Y. P.; Schejter, A. *J. Biol. Chem.* **1981**, *256*, 5540−5544.

(30) Gao, J.; Mammen, M.; Whitesides, G. M. *Science* **1996**, *272*, 535−537.

(eq 6). Allison et al.[31] estimated the effects of counterion relaxation on the electrophoretic mobility of lysozyme; they found that the magnitude of this effect increases as the charge on the protein is increased, consistent with the general trends observed in our experiments.

Conclusions

We have observed that the values of μ^n_{electro} of the charge ladders of a group of six proteins and, hence, the values of their net charge estimated by capillary electrophoresis, Z^n_{CE}, demonstrate both linear and nonlinear correlations with the number of charged groups they incorporate. For the rungs of charge ladders with the smallest values of charge (either positive or negative), the values of Z_{CE} correlate approximately linearly with the number of modified groups; as the net charge increases, the values of Z_{CE} of the proteins that constitute the rungs of a protein charge ladder deviate from the linear correlation observed for the smaller values of charge. We expressed these correlations in terms of the value of electrostatic potential at the surface of a sphere of uniform surface charge calculated by the Debye−Hückel theory. In expressing the data in this way, we have shown that all the data fall along a single common curve, regardless of the ionic strength of the electrophoresis buffer: the onset of nonlinearity occurred for absolute values of the calculated electrostatic potential >35 mV for charge ladders of all six proteins.

A simple model for this effect is based on the fact that when the magnitude of the electrostatic potential at the surface of the protein is greater than kT/e, ions of charge opposite that of the protein may lower their chemical potential by binding, or associating tightly, to the surface of the protein and thereby reduce the net charge of the protein. For protons, this process is reflected as shifts in values of pK_a of ionizable groups. The association of ions will continue to reduce the charge of the protein until there is an equilibrium established between the chemical potential of ions at the surface of the protein and those that are free in solution. We conclude that ion binding will always occur to maintain a magnitude of surface potential that does not greatly exceed kT/e.

Although changes in the values of pK_a are sufficient to account for the observed dependence of the charge of proteins included in this study on the number of charged groups they incorporate, we cannot rule out at least two other mechanisms by which an increasing electrostatic potential at the surface of the protein may give rise to the observed nonlinearity: it may change the conformation of the protein, and the distribution of small ions in solution surrounding the protein. We conclude that the way in which a protein responds to a change in the number of ionizable groups it incorporates may represent a combination of several effects, all of which indicate significant interactions between charged groups on the protein, and between these charged groups and small ions in solution: the combination of charge ladders of proteins and capillary electrophoresis is a useful tool with which to measure these effects.

(31) Allison, S. A.; Potter, M.; McCammon, J. A. *Biophys. J.* **1997**, *73*, 133−140.

Experimental Section

Materials. Carbonic anhydrase II (bovine; pI 5.9 and 5.4; E.C. 4.2.1.1), carbonic anhydrase II (human; pI 7.6; E.C. 4.2.1.1), cytochrome *c* (horse heart; pI 10.0), superoxide dismutase (bovine erythrocytes; pI 6.6; E.C. 1.15.1.1), ovalbumin (chicken egg; pI 5.1), hydroxylamine·HCl, methylamine·HCl and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were purchased from Sigma (St. Louis, MO). Lysozyme (egg white; pI 10.9; E.C. 3.2.1.17) was purchased from Worthington (Freehold, NJ). Hexadimethrine bromide (Polybrene) was purchased from Aldrich (Milwaukee, WI). Fused silica capillaries with an internal diameter of 50 μm were purchased from Polymicro Technologies (Phoenix, AZ). NICK spin columns containing G-50 Sephadex gel were purchased from Pharmacia Biotech (Piscataway, NJ).

Acetylation of Amino Groups of Proteins. Proteins were dissolved in water at a concentration of ~0.1 mM, and 10 vol % of 0.1 N NaOH was added to bring the pH to ~12. Five to 20 equiv of acetic anhydride (100 mM in dioxane) was immediately added to the solution of protein, and the reactants were quickly mixed by vortexing. Reactions were usually complete within 1 min. The sample was diluted in electrophoresis buffer (25 mM Tris−192 mM Gly, pH 8.4) prior to analysis.

Amidation of Carboxylate Groups of Ovalbumin. To 200 μL of a solution of ovalbumin (0.1 mM in deionized H_2O) was added 20 μL of EDAC (50 mM in deionized H_2O) and 50 μL hydroxylamine·HCl or methylamine·HCl (500 mM in deionized H_2O). The reaction mixture was vortexed and allowed to react for at least 20 min. The samples were purified using NICK spin columns. The sample was diluted in electrophoresis buffer (25 mM Tris−192 mM Gly, pH 8.4) prior to analysis.

Capillary Electrophoresis (CE). CE experiments were conducted on a Beckman P/ACE 5500. Proteins of pI < 8.4 and their derivatives were analyzed at 25 °C on an uncoated capillary of fused silica with the length of the capillary from the inlet to the detector, $L_{\text{det}} = 40$ cm, and the total length of the capillary, $L_{\text{tot}} = 47$ cm; separations were done using 25 mM Tris−192 mM Gly buffer, pH 8.4 and an applied voltage of 15 kV. The applied voltage was lowered from 15 to 6 kV as the concentration of Li_2S0_4 in the running buffer was increased from 0 to 50 mM to prevent joule heating within the capillary. Proteins of pI > 8.4 and their derivatives were analyzed at 25 °C with reverse polarity on a Polybrene-coated capillary[32] ($L_{\text{tot}} = 47$ cm, $L_{\text{det}} = 40$ cm), using 25 mM Tris−192 mM Gly buffer, pH 8.4 and an applied voltage of 30 kV.

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