Measuring the Size and Charge of Proteins Using Protein Charge Ladders, Capillary Electrophoresis, and Electrokinetic Models of Colloids

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Separation of macromolecules by electrophoresis is an essential tool in molecular biology. Advances in the electrophoretic separations of nucleic acids helped increase the rate of sequencing of the human genome over the last several years.[1,2] Challenges arising in functional genomics and proteomics require methods for the separation and identification of patterns of protein expression, protein−ligand binding, and protein modification.[2] Separations of proteins in free solution by capillary electrophoresis (CE) may address some of these challenges. In this paper we (i) present a methodology for measuring both the hydrodynamic size and net charge of a protein in a single CE experiment and (ii) demonstrate the importance of ion relaxation and polarization in protein electrophoresis.

We have used charge ladders of proteins[3] (collections of derivatives of proteins produced by the partial modification of charged groups) and CE to examine correlations between values of electrophoretic mobility (μ_{elec}, $m^2\ V^{-1}\ s^{-1}$) of proteins and the number of ionizable groups they incorporate.[4] Figure 1 shows values of μ_{elec} of the charge ladder of human carbonic anhydrase II (HCAII) reported in ref 4: derivatives with the lowest overall net charge demonstrate a linear correlation of μ_{elec} with the number of charged groups, *n*, converted to neutral derivatives. For derivatives of this protein with larger values of net charge, μ_{elec} demonstrate a nonlinear correlation with *n*. In this work, we re-interpret these data and show that the "standard model" of the electrokinetic properties of colloids[5] accurately describes μ_{elec} for protein charge ladders at different concentrations of added salt (Figure 2). Charge ladders and CE have also been used to measure the net charge of proteins.[6] In this work, we extend the combination of charge ladders and CE to the measurement of values hydrodynamic radius of proteins (Figure 1, inset).

The electrophoretic mobility of a molecule in free solution is, in general, a function of the net charge and hydrodynamic drag of the molecule and the properties of the solution (the viscosity, concentration, and mobility of dissolved ions). The ability to predict values of μ_{elec} requires knowledge of the size and charge of the protein. One empirical expression that relates μ_{elec} to the charge and size of proteins is given in eq 1

$$\mu_{elec} = C_p \frac{eZ}{M^{\alpha}} \tag{1}$$

where *eZ* is net charge, *M* is molecular weight, and C_P and α are empirical parameters that depend on the ionic composition and viscosity of the solution. An attempt to develop empirical values for C_P and α met with limited success.[9] A key point is that eq 1 expresses μ_{elec} as a linear function of *Z*.

In 1931, Henry[10] established a theoretical model of μ_{elec} for colloids. Equation 2 combines Henry's model with Debye−

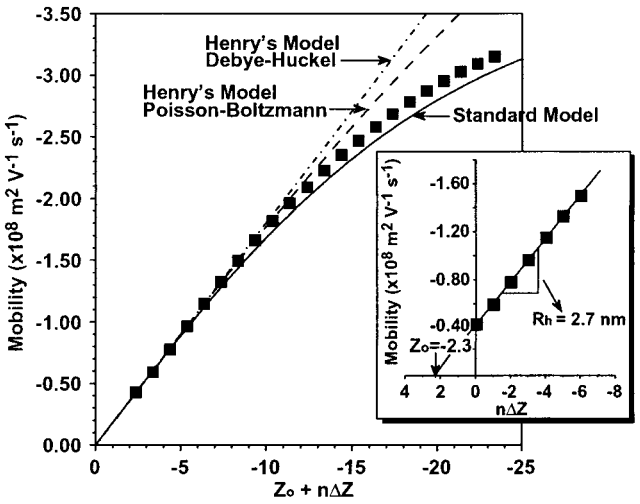

Figure 1. Values of the electrophoretic mobility (μ_{elec}) from ref 4 of the rungs of the charge ladders of human carbonic anhydrase II (HCAII), produced by the partial acetylation of Lys ϵ-NH_3^+ groups, plotted as a function of net charge ($Z_o + n\Delta Z$):[7] Z_o is the charge of the unmodified protein; each acetylation results in an assumed increment of charge, ΔZ, due to the conversion of a Lys ϵ-NH_3^+ group to its neutral ϵ-$NHCOCH_3$ derivative ($\Delta Z \approx -1$ at pH = 8.4). Dashed lines show values of μ_{elec} predicted by Henry's equation using the electrostatic potential surrounding the protein calculated from either the Debye−Huckel equation (eq 2, − • − • − •), or the nonlinear Poisson−Boltzmann equation (eq 3, − − −); the solid line shows values of μ_{elec} predicted by the standard model.[8] *Inset:* Values of μ_{elec} of the first seven rungs of the charge ladder of HCAII, plotted as a function of $n\Delta Z$ and fit to eq 2 using linear least-squares analysis: the *x*-intercept gives the net charge of the unmodified protein, Z_o; the hydrodynamic radius, R_h, is determined from the slope of the line.

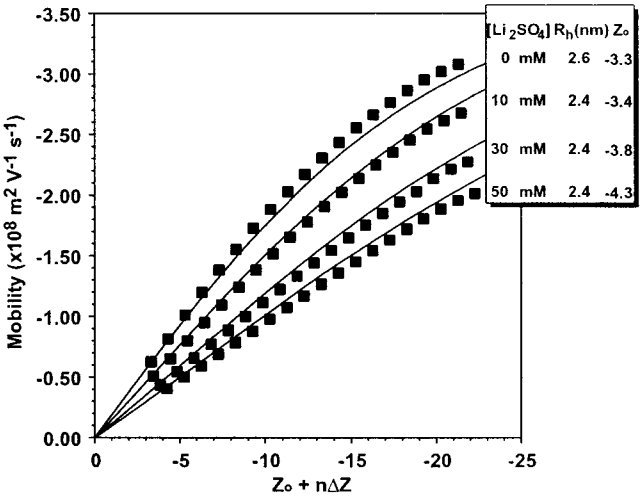

Figure 2. Values of the electrophoretic mobility (μ_{elec}) from ref 4 of the rungs of the charge ladders of bovine carbonic anhydrase II (BCAII) with different concentration of Li_2SO_4 added to the electrophoresis buffer;[7] values of R_h and Z_o are determined as described in Figure 1. The solid curves show μ_{elec} predicted by the standard model.[8] Values of R_h for BCAII decrease by ~2 Å upon the addition of 10 mM Li_2SO_4 to the electrophoresis buffer; R_h is approximately constant as more Li_2SO_4 is added.

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(1) From ~20 Mb per year in 1997 to >200 Mb per year in 1999.

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Hückel theory, and predicts a linear correlation between μ_{elec} and Z, consistent with eq 1: R_h is the hydrodynamic radius of the protein, κ is the inverse Debye length, η is the viscosity of the electrophoresis buffer, e is the fundamental unit of charge, and f_1 is a function of κR_h that describes the effect of the protein on the electric field.[11]

$$\mu_{elec} = \frac{eZ}{6\pi\eta R_h} \frac{f_1(\kappa R_h)}{(1 + \kappa R_h)} \quad (2)$$

Previous attempts to apply models of colloids[12] to μ_{elec} for macromolecules required knowledge of the hydrodynamic size of the molecules, and many were valid only when the electrostatic potential was less than ~25 mV. Menon applied eq 2 to estimate values of charge of bovine serum albumin from values of μ_{elec} measured by CE.[13] Allison applied the standard model of electrokinetics[5] to predict values of μ_{elec} for lysozyme at different values of pH using a detailed atomistic model of the protein.[14] Huang used CE to estimate the net charge of dendrimers terminated in carboxyl groups;[15] they found nonlinear correlations between the number of charged groups and values of μ_{elec} that they ascribed to ion condensation.

We fit μ_{elec} for the first seven rungs of the charge ladder of HCAII as a function $n\Delta Z$ to eq 2 using a linear least-squares analysis (Figure 1). In so doing, we assumed $Z = Z_o + n\Delta Z$: Z_o is the charge of the unmodified protein, and each acetylation results in an assumed increment of charge, $\Delta Z \approx -1$. The x-intercept of the best-fit line gives the value of Z_o; the slope provides R_h. In this way, both the charge and size of a protein may be determined in a single electrophoretic experiment.

From Figure 1 we observe that beyond the seventh rung the data are no longer described accurately by eq 2; that is, there is no longer a linear correlation between μ_{elec} and Z. Two assumptions yield the linear correlation between μ_{elec} and Z: (i) the electrostatic potential can be described by the linearized Poisson−Boltzmann equation (i.e., Debye−Hückel theory); (ii) the effects of polarization and relaxation of the diffuse cloud of ions surrounding the protein are negligible. Both assumptions are valid when the average potential at the surface of the particle is less than ~25 mV.

To estimate the effects of the first assumption, we calculated the mobility predicted by Henry's equation using the nonlinear Poisson−Boltzmann equation, eq 3.[16] In this expression, $\varphi(r)$ is the electrostatic potential at a distance, r, from the surface; φ_o is the potential at the surface of the protein. From Figure 1 we see that inclusion of nonlinear electrostatic effects results in a modest nonlinearity in the relation of μ_{elec} to Z.

$$\mu_{elec} = \frac{\epsilon\epsilon_o}{\eta} \varphi_o \left\{ 1 + \frac{R_h^3}{\varphi_o} \left(5R_h^2 \int_\infty^{R_H} \frac{\varphi(r)}{r^6}\, dr - 2 \int_\infty^{R_H} \frac{\varphi(r)}{r^4}\, dr \right) \right\} \quad (3)$$

To include effects of ion relaxation and polarization, in addition to nonlinear electrostatic effects, we applied the "standard model" of electrokinetics to predict the values of μ_{elec} of the members of protein charge ladders. This model provides numerical solutions of the coupled equations of fluid dynamics (the Stokes equation); electrostatics (the Poisson−Boltzmann equation) and ion mobilities that predict values of μ_{elec} as a function of Z and R_h.[5] From Figure 1, we see that this model accurately describes both the linear and nonlinear correlation of μ_{elec} with Z; Figure 2, shows that the standard model also describes the values of μ_{elec} of bovine carbonic anhydrase II (BCAII) at different concentrations of salt added to the electrophoresis buffer.

We draw three conclusions from this work. First, charge ladders provide a good model system for testing theories of electrophoresis of proteins; a single charge ladder can span regions of both linear and nonlinear electrophoretic behavior. The observed nonlinear behavior is the result of nonlinear electrostatic effects, and ion relaxation and polarization; these effects all become significant at electrostatic potentials greater than ~25 mV.

Second, the standard electrokinetic model of colloids accurately describes both the linear and nonlinear correlations of μ_{elec} with Z for protein charge ladders. The nonlinear dependence of μ_{elec} on Z is primarily a function of the physics of electrophoresis; changes in the physiochemical properties of proteins, such as shifts in values of pK_a,[4] association of ions[4] and ion condensation,[15] are not required to explain the dependence of μ_{elec} on Z.

Third, the combination of charge ladders and CE, together with models of the electrokinetic properties of colloids, provides an explicit measure of both the size and charge of proteins. Proteins with different combinations of charge and size may have similar values of μ_{elec}. The approach described here allows the size and charge of a protein to be measured directly in a single electrophoretic separation using complex solution conditions that mimic real biological environments.

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(7) 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, ionic strength 7.4 mM). Detection was by direct UV absorbance at 214 nm.

(8) For all calculations we assumed values 1×10^{-3} kg m^{-1} s^{-1} for the viscosity and 80 for the dielectric constant of the electrophoresis buffer; for calculations with the standard model we used values of ionic conductivity of 2.325×10^{-3} and 3.956×10^{-3} m^2 Ohm^{-1} equiv^{-1} for the ionic conductivity of Tris$^+$ and Gly$^-$, respectively.

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(11) $f_1 = 1$ when $\kappa R_h < 1$ (the Hückel limit) and $f_1 = {}^3/_2$ when $\kappa R_h > 10$ (the Helmholtz−Smoluchowski limit); between the two limiting cases, f_1 may be calculated from (see ref. 10):

$$f_1(\kappa R_h) = \left(1 + \frac{\kappa^2 {R_h}^2}{16} - \frac{5\kappa^3 {R_h}^3}{48} - \frac{\kappa^4 {R_h}^4}{96} + \frac{\kappa^5 {R_h}^5}{96} + -\frac{11}{96} e^{\kappa R_h} \int_\infty^{\kappa R_h} \frac{e^{-r}}{r}\, dr \right)$$

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(16) In solving eq 3, we first calculated the electrostatic potential as a function of the distance from the particle, $\varphi(r)$, from the nonlinearized Poisson−Boltzmann equation using the shooting method with a fifth-order Cash-Karp Runge Kutta method for solving the ordinary differential equations. We then evaluated the integrals in eq 3 numerically using Romberg integration on an open interval: see Press: W. H.; Teukolsky, S. A.; Vetterling, W. T.; Flannery, B. P. *Numerical Recipes in FORTRAN*, 2nd. ed.; Cambridge University Press: Cambridge, 1992 for details.