J|A|C|S
ARTICLES
Published on Web 03/01/2002

Measurement of Electrostatic Interactions in Protein Folding with the Use of Protein Charge Ladders

Russell S. Negin and Jeffrey D. Carbeck*

Contribution from the Department of Chemical Engineering, Princeton University, Princeton, New Jersey 08544

Received August 28, 2001

Abstract: This paper describes a new method for the measurement of the role of interactions between charged groups on the energetics of protein folding. This method uses capillary electrophoresis (CE) and protein charge ladders (mixtures of protein derivatives that differ incrementally in number of charged groups) to measure, in a single set of electrophoresis experiments, the free energy of unfolding (ΔG_{D-N}) of α-lactalbumin (α-LA) as a function of net charge. These same data also yield the hydrodynamic radius, R_H, and net charge measured by CE, Z_{CE}, of the folded and denatured proteins. α-LA unfolds to a compact denatured state under mildly alkaline conditions; a small increase in R_H (11%, 2 Å) coincides with a large increase in Z_{CE} (71%, −4 charge units), relative to the folded state. The increase in Z_{CE}, in turn, predicts a large pH dependence of free energy of unfolding (−22 kJ/mol per unit increase in pH), due to differences in proton binding in the folded and denatured states. The free energy of unfolding correlates with the square of net charge of the members of the charge ladder. The differential dependence of ΔG_{D-N} on net charge for holo-α-LA, $\partial \Delta G_{D-N}/\partial Z = -0.14Z$ kJ/mol per unit of charge. This dependence of ΔG_{D-N} on net charge is a result of a net electrostatic repulsion among charge groups on the protein. These results, together with data from pH titrations, show that both the effects of electrostatic repulsion and differences in proton binding in the folded and denatured states can play an important role in the pH dependence of this protein; the relative magnitude of these effects varies with pH. The combination of charge ladders and CE is a rapid and efficient tool that measures the contributions of electrostatics to the energetics of protein folding, and the size and charge of proteins as they unfold. All this information is obtained from a single set of electrophoresis experiments.

Introduction

The importance of interactions between charged groups on protein stability is clear from the dependence of folding free energy (ΔG_{D-N}) on pH.[1] Our understanding of the role of electrostatics on protein stability has been limited because both long-range electrostatics and binding of protons contribute to ΔG_{D-N};[2] contributions of these two mechanisms to ΔG_{D-N} are difficult to measure independently. Furthermore, the ability of electrostatic theory[3] to predict contributions of these mechanisms to ΔG_{D-N} has been restricted by a lack of information on conformations of proteins in denatured states.[4−7] Structures of denatured proteins are not described by a single, well-defined conformation and are therefore difficult to measure[5] or predict by simulation.[8] In this work, we use capillary electrophoresis to monitor the thermally induced unfolding[9] of protein charge ladders[10]—collections of protein derivatives created by random partial neutralization of charged groups.[11] These data provide values of ΔG_{D-N} for the proteins that make up each rung of the charge ladder; analysis of these data also gives contributions of both long-range electrostatics and proton binding to the pH dependence of ΔG_{D-N}. These data also provide the hydrodynamic radius and net charge of proteins as they unfold. All this information is obtained from a single set of electrophoresis experiments.

Under mildly denaturing conditions, many small proteins form a partially structured, compact denatured state called a molten globule. Molten globules at equilibrium—described as "low-resolution solutions" to the problem of protein folding[12]—are good models for the structure and properties of kinetic intermediates in protein folding.[13,14] α-Lactalbumin (α-LA)—a component of lactose synthase complex—is a relatively small

* To whom correspondence should be addressed. Princeton University, Department of Chemical Engineering, Room A319, E-Quad, Princeton, NJ 08544. Phone: (609) 258-1331. Fax: (609) 258-0211. E-mail: jcarbeck@princeton.edu.

(1) Tanford, C. *Adv. Protein Chem.* **1970**, *25*, 1−95.
(2) Yang, A.-S.; Honig, B. *J. Mol. Biol.* **1993**, *231*, 459−474.
(3) Honig, B.; Nicholls, A. *Science* **1995**, *268*, 1144−1149.
(4) Stigter, D.; Alonso, D. O. V.; Dill, K. A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4176−4180.
(5) Shortle, D. *FASEB J.* **1996**, *10*, 27−34.
(6) Schaefer, M.; Sommer, M.; Karplus, M. *J. Phys. Chem. B* **1997**, *101*, 1663−1683.
(7) Elcock, A. H. *J. Mol. Biol.* **1999**, *294*, 1051−1062.
(8) Paci, E.; Smith, L. J.; Dobson, C. M.; Karplus, M. *J. Mol. Biol.* **2001**, *306*, 329−347.
(9) Hilser, V. J.; Freire, E. *Anal. Biochem.* **1995**, *224*, 465−485.
(10) Carbeck, J. D.; Colton, I. J.; Gao, J.; Whitesides, G. M. *Acc. Chem. Res.* **1998**, *31*, 343−350.
(11) Colton, I. J.; Anderson, J. R.; Gao, J.; Chapman, R. G.; Isaacs, L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1997**, *119*, 12701−12709.
(12) Wu, L. C.; Kim, P. S. *J. Mol. Biol.* **1998**, *280*, 175−182.
(13) Dobson, C. M. *Curr. Opin. Struct. Biol.* **1992**, *2*, 6−12.
(14) Creighton, T. E. *Trends Biochem. Sci.* **1997**, *22*, 6−10.

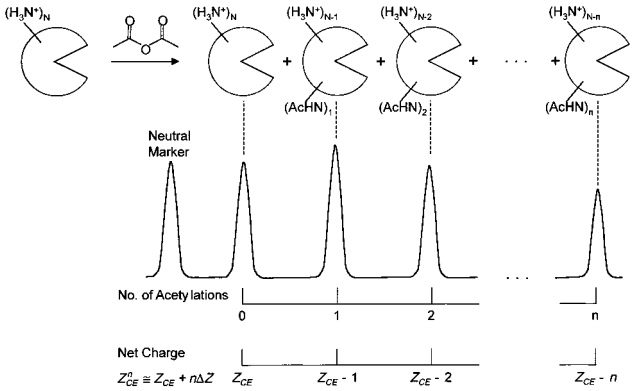

Figure 1. Formation of a charge ladder by the random partial acetylation of Lys ϵ-amino groups with acetic anhydride, and the separation of the protein derivatives into individual rungs by capillary electrophoresis. The unmodified protein has N Lys ϵ-NH_3^+ groups; the nth rung of the ladder is composed of proteins that have n neutral ϵ-$NHCOCH_3$ derivatives (AcHN). The protein derivatives that make up the nth rung of the charge ladder have approximately the same net charge, $Z_{CE}^n = Z_{CE} + n\Delta Z$: Z_{CE} is the net charge of the unmodified protein; ΔZ is the change in charge due to the conversion of a Lys ϵ-NH_3^+ group to its neutral ϵ-$NHCOCH_3$ derivative. A neutral marker is used to measure the rate of electroosmotic flow.

protein that denatures under mildly acidic[15] and alkaline[16] conditions. The acid denatured state of α-LA has served as a model system for the study of molten globules.[8,12,17,18] The compact denatured state of α-LA formed under alkaline conditions is less well studied. Our objective is to measure, independently, the contributions of proton binding and long-range electrostatic interactions to the pH dependence of unfolding of α-LA under mildly alkaline pH.

Charge ladders of proteins and capillary electrophoresis (CE) have been used to measure the net charge[19] and hydrodynamic size[20] of proteins and the role of long-range electrostatic interactions on the energetics of receptor−ligand binding.[21,22] In this paper we report the use of charge ladders and CE to evaluate quantitatively the contributions of electrostatics to ΔG_{D-N}, and to measure values of hydrodynamic radius and net charge of α-LA as it unfolds. We find that both proton linkage and long-range electrostatic interactions can contribute significantly to the pH dependence of ΔG_{D-N} for α-lactalbumin; the relative magnitude of these effects varies with pH. We demonstrate the generality of this approach by measuring changes in hydrodynamic radius and net charge for the urea denaturation of α-LA and the thermal denaturation of the proteins lysozyme and cytochrome *c*.

Results and Discussion

We synthesized charge ladders of bovine α-LA ($M_w = 14\,200$ Da, p$I = 4.8$) by partial acetylation of Lys ϵ-NH_3^+ groups with acetic anhydride (Figure 1). Acetylation produced a distribution of derivatives of this protein that differed in the number (n) of neutral ϵ-$NHCOCH_3$ groups. We measured the electrophoretic mobility (μ_{elec}, $m^2\ V^{-1}\ s^{-1}$) of charge ladders as a function of temperature of the capillary (Figure 2). The protein derivatives separated in free solution by CE into individual groups or rungs; each rung contained regio-isomeric derivatives with the same

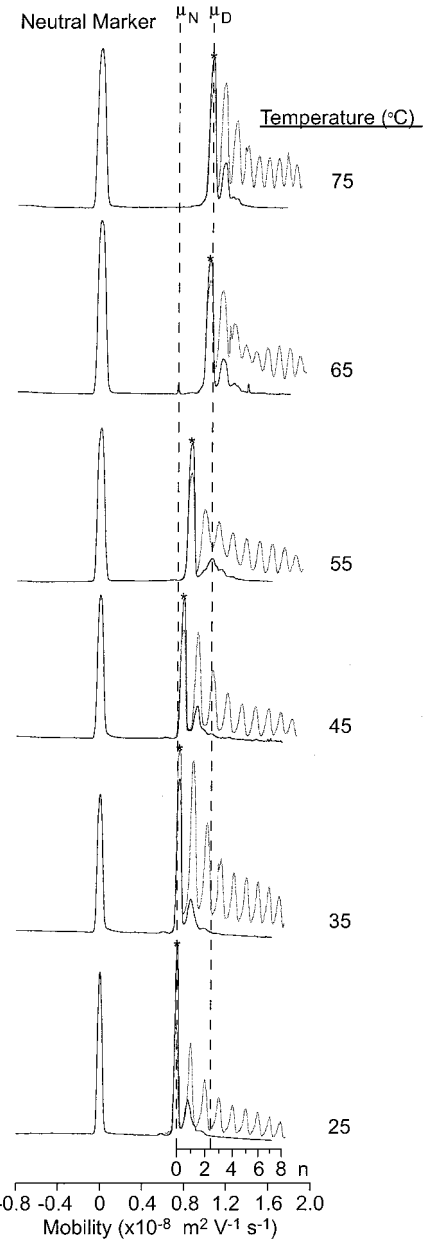

Figure 2. Electropherograms of the thermal denaturation of the charge ladder of holo-α-LA (in grey) superimposed on those of the unmodified protein (in black). Separations were done in a buffer composed of 25 mM Tris, 192 mM Gly (pH 8.4), 20 mM NaCl, and 35 μM $CaCl_2$. The UV absorbance at 220 nm is plotted as a function of electrophoretic mobility. Values of mobility were corrected for changes in viscosity due to changes in temperature of the electrophoresis buffer.[26] The peak marked with $*$ corresponds to unmodified protein; the number of acetylated Lys ϵ-NH_3^+ groups, n, is indicated below the corresponding peak. The dashed lines indicate the electrophoretic mobility of unmodified α-LA in the native (μ_N) and denatured (μ_D) states. The rungs of the charge ladder, as well as the peak that corresponds to the unmodified protein, show broadening near the middle of the transition (i.e., near the melting temperature of holo-α-LA, ~56 °C). This broadening may reflect the finite rate of conversion between the folded and denatured states. Broadening of the rungs of the charge ladder may also reflect some heterogeneity in the free energies of unfolding of different derivatives of α-LA that have the same number of acetylated Lys ϵ-NH_3^+ groups.

(15) Robbins, F. M.; Raymond, E. A.; Holmes, L. G.; Kronman, M. J. *Biochim. Biophys. Acta* **1967**, *133*, 33−45.
(16) Kronman, M. J.; Holmes, L. G.; Robbins, F. M. *Biochim. Biophys. Acta* **1967**, *133*, 46−55.
(17) Kim, S.; Baum, J. *Protein Sci.* **1998**, *7*, 1930−1938.
(18) Redfield, C.; Schulman, B. A.; Milhollen, M. A.; Kim, P. K.; Dobson, C. M. *Nature Struct. Biol.* **1999**, *6*, 948−952.
(19) Gao, J.; Gomez, F. A.; Härter, R.; Whitesides, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12027−12030.
(20) Carbeck, J. D.; Negin, R. S. *J. Am. Chem. Soc.* **2001**, *123*, 1252−1253.
(21) Caravella, J. A.; Carbeck, J. D.; Duffy, D. C.; Whitesides, G. M.; Tidor, B. *J. Am. Chem. Soc.* **1999**, *121*, 4340.
(22) Gao, J.; Mammen, M.; Whitesides, G. M. *Science* **1996**, *272*, 535.

Table 1. Net Charge (Z_{CE}) and Hydrodynamic Radius (R_H) of Proteins in Folded and Denatured States[a]

	folded		denatured			
protein	Z_{CE}	R_H (Å)	Z_{CE}	R_H (Å)	$\Delta Z_{CE,D-N}$	$\Delta R_{H,D-N}$ (Å)
α-lactalbumin (holo)						
thermal denatured[b]	−5.6	20.1	−9.6	22.3	−4.0 (71%)	2.2 (11%)
urea denatured[c]			−9.0	25.5	−3.4 (61%)	5.4 (27%)
published values		20.2[d]		22.2[e]		2.0 (10%)
lysozyme						
thermal denatured[f]	7.1	19.3	6.6	22.0	−0.5 (7.0%)	2.7 (14%)
published values	7.2[g]	19.7[h]	6.5[i]	21.6[j]	−0.7 (9.7%)	1.9 (9.2%)
cytochrome *c*						
thermal denatured[f]	6.4	19.0	5.2	21.5	−1.2 (−18.7%)	2.5 (13.2%)
published values		18.7[k]		22.4[l]		3.7 (19.8%)

[a] $\Delta Z_{CE,D-N}$ and $\Delta R_{H,D-N}$ are the changes in net charge and hydrodynamic radius between native and denatured states. Quantities in parentheses are percent changes in these quantities, relative to the native state. [b] This work: 25 mM Tris, 192 mM Gly (pH 8.4), 20 mM NaCl, and 35 μM $CaCl_2$. [c] This work: 25 mM Tris, 192 mM Gly (pH 8.4), 20 mM NaCl, 35 μM $CaCl_2$, and 8 M urea. [d] 20 °C, 20 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$. Kataoka et al., *Protein Sci.* **1997**, *6*, 422. [e] 20 °C, 10 mM HCl (pH 2.0). Kataoka et al., *Protein Sci.* **1997**, *6*, 422. [f] This work: 25 mM Tris, 192 mM Gly (pH 8.4). [g] 25 °C, 0.1 M KCl. Tanford and Roxby, *Biochemistry* **1972**, *11*, 2192. [h] 20 °C, 0.6 M GdnHCl, 50 mM NaOAc (pH 5.2). Segel et al. *J. Mol. Biol*. **1999**, *288*, 489. [i] 25 °C, 6 M GdnHCl. Tanford and Roxby, *Biochemistry* **1972**, *11*, 2192. [j] Transient folding intermediate. Segel et al., *J. Mol. Biol*. **1999**, *288*, 489.[k] 15 °C (pH 3.2). Hagihara et al., *Folding Des.* **1998**, *3*, 195. [l] 20 °C, 0.5M NaCl (pH 2.0). Hagihara et al., *Folding Des.* **1998**, *3*, 195.

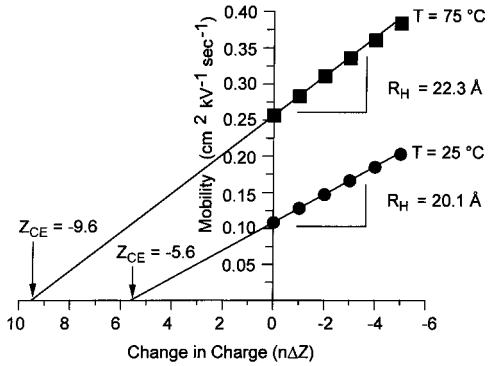

Figure 3. Values of electrophoretic mobility (μ_{elec}) of the rungs of the charge ladder of holo-α-LA determined from analysis of data in Figure 2, plotted as a function of $n\Delta Z$: n is the number of acetylated lysine ϵ-amino groups; each acetylation results in an assumed increment of charge, ΔZ, 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). These data have not been corrected for changes in viscosity of the electrophoresis buffer with temperature. (●) Native protein at 25 °C; (■) compact denatured state at 75 °C. The data are fit to eqs 3 and 4 using linear least-squares analysis: the x-intercept gives the net charge of the unmodified protein, Z_{CE}; the hydrodynamic radius, R_H, is determined from the slope of the line.

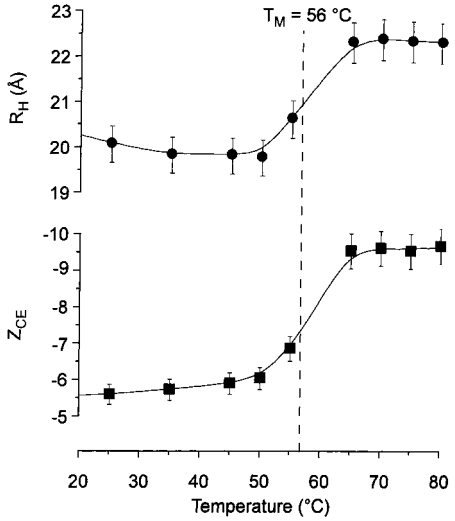

Figure 4. Values of hydrodynamic radius, R_H, and net charge, Z_{CE}, of holo-α-LA determined from analysis of data in Figure 2, plotted as a function of temperature. The dashed line indicates the melting temperature of 56 °C.

number of modifications[23] and approximately the same value of net charge.[11,19] In the folded state, each rung is composed of *constitutional* isomers (i.e., regio-isomeric derivatives that have the same number of acetylated Lys ϵ-amino groups). Rungs of the ladder in the denatured state were as well resolved as in the folded state. In the denatured state, collections of constitutional isomers that make up each rung of the ladder also exist as an ensemble of *conformational* isomers.

Separation of α-LA by CE at different temperatures showed that μ_{elec} of this protein increased as it denatured. Mobility is directly proportional to charge and inversely proportional to hydrodynamic size. To quantify changes in charge and size, we determined the effective hydrodynamic radius (R_H) and net charge (Z_{CE}) of α-LA as it unfolded using a combination of Debye−Hückel theory and Henry's model of electrophoresis (Figure 3).[20] The magnitude of both Z_{CE} and R_H increased as the protein denatured (Figure 4). Effects on mobility of changes in charge outweighed effects of changes in hydrodynamic size, and the mobility of α-LA increased as the protein unfolded.

The change in R_H of α-LA upon thermal denaturation at pH 8.4 was 11% (Table 1), similar to the formation of the compact denatured state of α-LA by acid denaturation.[18] Thermal denaturation was accompanied by a change in charge of ∼70% or −4 charge units, relative to the folded state (Table 1). This large change in Z_{CE} upon denaturation ($\Delta Z_{CE,D-N}$) reflects a significant difference in the number of charged species (protons or other ions) bound to the protein between the native and denatured states.

Measurements of Z_{CE} for the apo protein, in the absence of a bound Ca^{2+} ion, gave values of $\Delta Z_{CE,D-N} = -3.3$, in comparison to the value of −4.0 measured for the holo protein in the presence of a bound Ca^{2+} ion. We infer that changes in the binding of Ca^{2+} ions contribute less than 20% to values of $\Delta Z_{CE,D-N}$. Values of $\Delta Z_{CE,D-N}$ therefore likely reflect significant differences in affinity of groups on the protein for protons between the folded and denatured states, as measured by shifts in pK_a, the log of the proton dissociation constant.

If unfolding involves shifts in pK_a, then ΔG_{D-N} will depend on pH.[1,24,25] A thermodynamic relationship, shown in eq 1,[1] describes the contribution of proton binding to the pH dependence of the free energy of unfolding $(\partial \Delta G_{D-N}/\partial \mathrm{pH})_{\Delta \mathrm{p}K_a}$, where

(23) Carbeck, J. D.; Severs, J. C.; Gao, J.; Wu, Q.; Smith, R. D.; Whitesides, G. M. *J. Phys. Chem. B* **1998**, *102*, 10596.

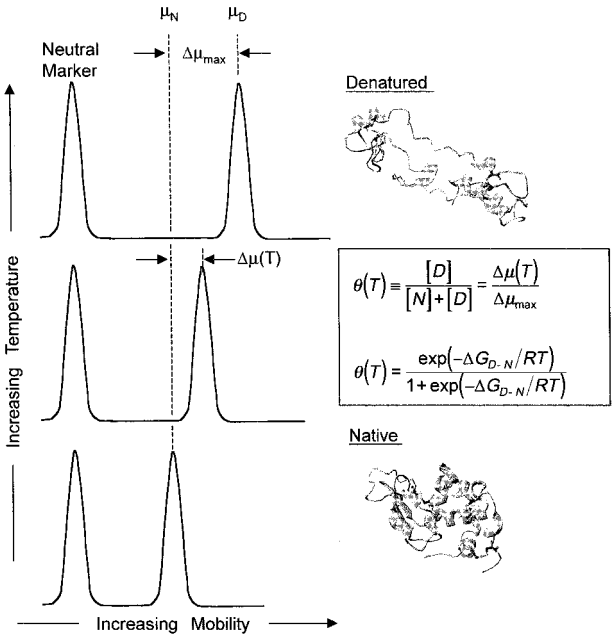

Figure 5. Illustration of how CE measures the fractional unfolding (θ) of a protein due to thermal denaturation. The protein is assumed to equilibrate rapidly between the folded and denatured states, relative to the time scale of the CE separation (typically 5−10 min). In this way, we assume a two-state model, and the value of mobility at some temperature, *T*, represents the concentration-averaged mobility of the native and denatured states, $\mu(T) = \theta\mu_D + (1 - \theta)\mu_N$. The difference between the mobility at this temperature and the mobility of the native protein, $\Delta\mu(T) = \mu(T) - \mu_N$, divided by the difference in mobility between the denatured and native states, $\Delta\mu_{max} = \mu_D - \mu_N$, provides a measure of θ. Values of θ measured at different temperatures are used to determine ΔG_{D-N} at 25 °C. Ribbon diagrams of α-LA in the native and denatured states are adapted from ref 8.

Q_N and Q_D are numbers of bound protons in native and denatured states, respectively.

$$\left(\frac{\partial \Delta G_{D-N}}{\partial pH}\right)_{\Delta pK_a} = 2.303RT(Q_N - Q_D) \qquad (1)$$

Changes in μ_{elec} with temperature, when corrected for changes in viscosity,[26] are proportional to the equilibrium distribution of proteins between folded and denatured states (Figure 5).[9] We used CE to measure values of ΔG_{D-N} for unmodified holo-α-LA at pH 8.0, 8.4, and 9.0; from these data we estimated a value of $\partial\Delta G_{D-N}/\partial pH = -22.35$ kJ/mol per unit of pH. From eq 1, this value corresponds to a $\Delta Q_{D-N} = -3.9$, similar to the value of $\Delta Z_{CE,D-N} = -4.0$ measured for holo-α-LA. We conclude that, at pH 8.4, values of $\Delta Z_{CE,D-N}$ reflect primarily a difference in number of bound protons between folded and denatured states of holo-α-LA.

The reason α-LA binds approximately four more protons in the folded state at pH 8.4 relative to the compact denatured state is not known. Crystallographic data show there are no acidic groups buried within the protein interior of α-LA in the folded state.[27] Small changes in side-chain conformation can have large effects on values of pK_a of ionizable groups: for example, continuum electrostatic theory showed that predicted

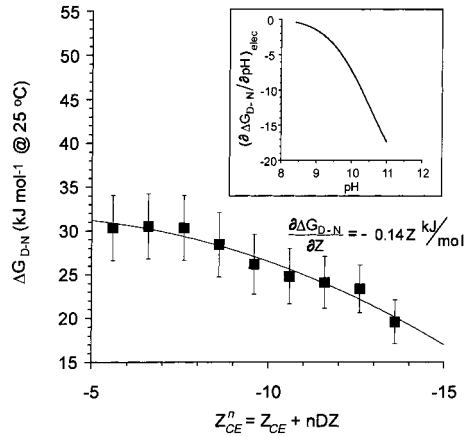

Figure 6. Dependence of free energy of unfolding (ΔG_{D-N}) at 25 °C on net charge, $Z_{CE}^n = Z_{CE} + n\Delta Z$, of proteins that make up rungs of charge ladders of α-LA. The curve is a fit of the data to a quadratic equation; the first derivative of this equation gives the differential dependence of ΔG_{D-N} on net charge: $\partial\Delta G_{D-N}/\partial Z = -0.14Z$ kJ/mol. (Inset) Contributions of long-range electrostatic interactions to the pH dependence of the free energy of unfolding, $(\partial\Delta G_{D-N}/\partial pH)_{elec}$, of α-LA. The partial derivative was determined from a combination of data from Figure 3 ($\partial\Delta G_{D-N}/\partial Z$) and pH titrations ($\partial Z/\partial pH$)[15] using the chain rule, eq 2.

values of pK_a for over half the ionizable groups in lysozyme varied from 1 to 3.3 p*K* units, depending on which crystal structure was used as the basis for calculations.[28] These results suggest that values of $\Delta Z_{CE,D-N}$ for α-LA are due to shifts in pK_a that reflect changes in specific, near-neighbor interactions between side chains. These changes result from small rearrangements of backbone and side-chain conformations that accompany the transition to the compact denatured state.

Site-directed mutagenesis has been useful in identifying specific interactions that contribute to differences in values of pK_a in the folded and denatured states. Anderson et al.[29] used site-directed mutagenesis to identify a single salt bridge that contributed as much as 20 kJ/mol to the stability of T4 lysozyme and played a key role in acid-induced denaturation of this protein. By measuring the change in charge between the native and denatured states, charge ladders and CE quantitatively measure effects of the linkage of proton binding and folding—site-directed mutagenesis may be useful in identifying the key interactions involved.

To quantify the effects of long-range electrostatics on the energetics of folding, we determined ΔG_{D-N} as a function of charge. From the data in Figure 2, we determined the fraction of unfolded protein as a function of temperature for members of charge ladders of α-LA. Analysis of these data yielded the melting temperature (T_M) and ΔG_{D-N} for the members of the charge ladders.[30] A value of T_M of 56 °C for the unmodified holo protein was in good agreement with the value of 58 °C previously published.[31] Values of T_M for members of the charge ladder of α-LA were approximately independent of the number of acetylated Lys ϵ-NH_3^+ groups.

Figure 6 compares values of ΔG_{D-N} for the members of ladders of holo-α-LA as a function of charge of the folded state.

(24) Wyman, J.; Gill, S. J. *Binding and Linkage: The Functional Chemistry of Biological Macromolecules*; University Science Books: Mill Valley, CA, 1990.
(25) Freire, E. *Adv. Protein Chem.* **1998**, *51*, 255−279.
(26) Ishihama, Y.; Oda, Y.; Asakawa, N.; Iwakura, M. *Anal. Sci.* **1997**, *13*, 931−938.
(27) Chrysina, E. D.; Brew, K.; Acharya, K. R. *J. Biol. Chem.* **2000**, *275*, 37021−37029.
(28) Bashford, D.; Karplus, M. *Biochemistry* **1990**, *29*, 10219−10225.
(29) Anderson, D. E.; Becktel, W. J.; Dahlquist, F. W. *J. Am. Chem. Soc.* **1990**, *106*, 765−784.
(30) Fersht, A. J. *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*; W. H. Freeman: New York, 1999.
(31) Permyakov, E. A.; Morozova, L. A.; Burstein, E. A. *Biophys. Chem.* **1985**, *21*, 21−31.

ΔG_{D-N} correlated with the square of net charge of the members of the charge ladder. This quadratic dependence of ΔG_{D-N} on charge is consistent with electrostatic interactions that act between charges on the protein. A simple estimate of contributions of electrostatic interactions to the energetics of protein folding is the energy to distribute the net charge evenly on the surface of a sphere; this charging free energy is proportional to the square of net charge, consistent with the data for holo-α-LA. The differential dependence of ΔG_{D-N} on net charge for holo-α-LA, $\partial\Delta G_{D-N}/\partial Z = -0.14Z$ kJ/mol per unit of charge, was determined from the first derivative of curves fit to ΔG_{D-N} vs Z (Figure 6).

Contributions of long-range electrostatic interactions to the pH dependence of the free energy of unfolding $(\partial\Delta G_{D-N}/\partial pH)_{elec}$—shown in the inset of Figure 6—were determined using the chain rule (eq 2) and a combination of data from charge ladders ($(\partial\Delta G_{D-N}/\partial Z)$, from Figure 6) and pH titrations ($(\partial Z/\partial pH)$.[15] For holo-α-LA at pH 8.4, $(\partial\Delta G_{D-N}/\partial pH)_{elec} \cong -0.5$

$$\left(\frac{\partial\Delta G_{D-N}}{\partial pH}\right)_{elec} = \frac{\partial\Delta G_{D-N}}{\partial Z}\frac{\partial Z}{\partial pH} \qquad (2)$$

kJ/mol is small, relative to the effects of proton binding, $(\partial\Delta G_{D-N}/\partial pH)_{\Delta pK_a} = -22.35$ kJ/mol. Effects of long-range electrostatic interactions on $(\partial\Delta G_{D-N}/\partial pH)_{elec}$ are predicted to increase with pH due to changes in both $(\partial\Delta G_{D-N}/\partial Z)$ and $(\partial Z/\partial pH)$. At pH 10, $(\partial\Delta G_{D-N}/\partial pH)_{elec} \cong -10.0$ kJ/mol for holo-α-LA is of the same order of magnitude as $(\partial\Delta G_{D-N}/\partial pH)_{\Delta pK_a}$ at pH 8.4. These results support our conclusion that the pH dependence of unfolding of α-LA at pH 8.4 is dominated by differences in proton binding between folded and denatured states. As pH increases, effects of long-range electrostatic interactions become more important. At pH > 10, both effects make a similar contribution to the overall pH dependence of ΔG_{D-N}. The combination of charge ladders and CE provides a unique tool to measure quantitatively these two effects of pH on protein stability.

Previous efforts to quantify effects of long-range electrostatic interactions on the energetics of protein folding have generally found this effect to be small. Hollecker and Creighton used charge ladders and urea-gradient gel electrophoresis to examine effects of reversing the charge of amino groups on protein stability.[32] Modification of the first several Lys ϵ-NH_3^+ groups produced little effect on the stability of cytochrome *c*; some of the later groups modified appeared to be more important to stability. Reversal of these groups caused unfolding in the absence of urea. The lower resolution of gel electrophoresis relative to CE prevented quantitative measurement of the effects of incremental changes in net charge on ΔG_{D-N}. Dao-Pin et al.[33] measured ΔG_{D-N} of 13 different single, double, triple, and quadruple mutants of T4 lysozyme produced by site-directed mutagenesis and concluded that long-range electrostatic interactions contributed little to the stability. The work of Dao-Pin, and others,[34] may have lacked sufficient numbers of mutants to accurately measure average effects of electrostatic interactions on protein stability.

Our measurements of long-range electrostatic interactions represent averages over a large number of different protein derivatives. The contribution of electrostatic interactions between charged groups to the free energy of unfolding, as measured by the slope of the curves in Figure 6, represents an average over ~3700 protein derivatives.[35] The accuracy of these estimates reflects the ability of CE to group these derivatives on the basis of charge into individual, well-resolved peaks. Previous work with charge ladders of the enzyme carbonic anhydrase II (CAII)[21] showed that examination of several thousand charge mutants was necessary to obtain precise quantitative information on how long-range electrostatic interactions affect free energies of binding (ΔG_b). Charge ladders and CE gave average interaction energies between lysine residues on CAII and charges on bound ligands in good agreement with predictions from continuum electrostatic theory and Monte Carlo simulations.[21] Averaging over such large numbers of mutants likely masks more complex distributions of interaction energies between specific groups. We conclude that examination of effects of many charge mutants is necessary to obtain quantitative information on the average effects of long-range electrostatic interactions to molecular recognition events involving proteins.

To demonstrate the generality of our approach, we measured values $\Delta Z_{CE,D-N}$ and $\Delta R_{H,D-N}$ for urea denaturation of holo-α-LA and thermal denaturation of lysozyme and cytochrome *c*—both of these proteins are known to form compact molten globule states—and compared these results to published values (Table 1). Values of $\Delta Z_{CE,D-N}$ for holo-α-LA were similar for both thermal and urea denaturation. Values of $\Delta R_{H,D-N}$ were somewhat larger for urea denaturation, relative to thermal denaturation, consistent with the observation that urea acts to denature proteins primarily through favorable interactions with the peptide backbone.[5] The results for lysozyme are particularly interesting because the structure of this protein is similar to that of α-LA. The relative change in R_H of lysozyme is similar to that of α-LA. Changes in Z_{CE} are different for the two proteins. Lysozyme at pH 8.4 transitions from a folded to a compact denatured state, but this transition results in a decrease in net charge of only ~3%, in good agreement with earlier results from pH titrations.[36]

Conclusions

We conclude that both proton linkage and long-range electrostatic interactions can contribute significantly to the pH dependence of ΔG_{D-N} for α-lactalbumin; their relative magnitudes vary with pH. The combination of protein charge ladders and CE is a convenient and efficient tool to measure these effects. Furthermore, values of hydrodynamic radius of denatured proteins measured using CE and charge ladders can provide constraints for the generation of ensembles of denatured proteins via molecular simulations.[8] Such ensembles may be required for accurate theoretical predictions of the pH dependence of ΔG_{D-N}.[2,6,7] An advantage of our approach is that all of this information is obtained under identical solution conditions from a single set of CE experiments using straightforward protein modifications—no genetic manipulations are required. Great effort would be required to produce equivalent numbers

(32) Hollecker, M.; Creighton, T. E. *Biochim. Biophys. Acta* **1982**, *701*, 395−404.
(33) Dao-Pin, S.; Söderlind, E.; Baase, W. A.; Wozniak, J. A.; Sauer, U.; Matthews, B. W. *J. Mol. Biol.* **1991**, *221*, 873−887.
(34) Sali, D.; Bycroft, M.; Fersht, A. R. *J. Mol. Biol.* **1991**, *220*, 779−788.
(35) Assuming equal reactivities of all N ϵ-amino groups on a protein, the number of derivatives in the nth rung of a charge ladder is given by the binomial coefficient, $N!/n!(N-n)!$.
(36) Tanford, C.; Roxby, R. *Biochemistry* **1972**, *11*, 2192−2198.

of different combinations of lysine mutants via site-directed mutagenesis. This approach is general and may be applied to proteins that (i) form charge ladders that are resolvable by CE and (ii) do not aggregate or precipitate upon transition to the denatured state. This technique is applicable to solution conditions that accurately reflect real biological solutions: that is, high concentrations of dissolved inorganic and organic species—including non-interacting macromolecules—that produce molecularly crowded environments in cells.[37]

Experimental Section

Materials. α-Lactalbumin (type I, from bovine milk, calcium saturated), cytochrome c (from bovine heart) and lysozyme (from hen egg white) were purchased from Sigma (St. Louis, MO). Fused silica capillaries with an internal diameter of 50 μm were purchased from Polymicro Technologies (Phoenix, AZ). All other chemicals were obtained from commercial suppliers at analytical grade or higher.

Synthesis of Charge Ladders. 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 equivalents 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 prior to analysis. We assumed that the change in charge due to acetylation, ΔZ, of Lys ϵ-NH_3^+ groups ($pK_a \approx 10.4$) is ~ -1 at the pH of our experiments (pH 8.4). There are a total of 12 Lys residues on bovine α-LA, and charge ladders of this protein could consist of up to 13 rungs; we characterized charge ladders containing only the first eight rungs. Later rungs were excluded due to lower concentrations and larger degrees of peak broadening, relative to earlier rungs of the ladder.

Capillary Electrophoresis. Electrophoretic mobilities were measured using a modified Beckman PACE/5000 CE instrument. The intrinsic capillary cooling system was redirected through an external water bath that was operated between 4 and 95 °C. Proteins were denatured by increasing the temperature of the capillary, which contained a buffer composed of 25 mM Tris, 192 mM Gly (pH 8.4), 20 mM NaCl, and 35 μM $CaCl_2$. The total protein concentration in the injected plug was approximately 20 μM. Values of electrophoretic mobility were determined from $\mu_{elec} = ll_o/V(1/t_{nm} - 1/t)$: t is the instantaneous time, t_{nm} is the time of emergence of the neutral marker (4-methoxybenzyl alcohol), l is the total length of the capillary (37 cm), l_o is the length to the detector (30 cm), and V is the applied voltage (7.5 kV). Values of mobility were corrected for changes in viscosity due to changes in temperature of the electrophoresis buffer.[26]

(37) van den Berg, B.; Ellis, R. J.; Dobson, C. M. *EMBO J.* **1999**, *24*, 6927−6933.

Determination of Effective Hydrodynamic Radius and Net Charge. The effective hydrodynamic radius of a protein, R_H, is defined as the radius of a sphere that has the same translational coefficient of friction as the protein. Z_{CE} is the net effective charge that gives rise to electrophoretic motion of the protein. It differs from the net charge measured by proton titration, Z_{H^+}, in that Z_{CE} also includes the effects of other charged species associated with the protein; these species include ions bound specifically (e.g., Ca^{2+} ions in the case of α-LA) or nonspecifically to the protein. Z_{CE} may also include effects related to the environment specific to the CE experiment: for example, an induced asymmetry in the distribution of counterions surrounding the protein that reduces the effective electric field acting on the protein. From data in Figure 1, plots of μ_{elec} of the rungs of the charge ladder, μ^n_{elec}, vs $n\Delta Z$ were fit using a combination of Debye−Hückel theory and Henry's model of electrophoresis[38] (eq 3); κ is the inverse Debye length, η is the viscosity of the buffer, e is the fundamental unit of charge, and f_1 is a function of κR_H that describes effects of the protein on the local electric field; $f_1 = 1$ 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 is calculated from eq 4. The x-intercept of the best-fit line gave Z_{CE}, and the slope provided R_H for the unmodified protein.

$$\mu^n_{elec} = \frac{e(Z_{CE} + n\Delta Z)}{6\pi\eta R_H}\frac{f_1(\kappa R_H)}{(1+\kappa R_H)} \tag{3}$$

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

Determination of the pH Dependence of Z. To calculate the contributions of long-range electrostatic interactions to the pH dependence of the free energy of unfolding using eq 2, we determined the pH dependence of the net charge of α-LA, $\partial Z/\partial$pH, from proton titration data of Robbins et al.[15] We extracted the data from their published figure using DataThief II (www.nikhef.nl/~keeshu/datathief) and fit a plot of number of bound protons vs pH to a fifth-order polynomial. The analytical first derivative of this polynomial provided our estimate of $\partial Z/\partial$pH.

Acknowledgment. We thank George M. Whitesides, Frederick M. Hughson, Michael H. Hecht, Pablo G. Debenedetti, and Jannette L. Carey for their useful comments on this work and careful reading of the manuscript. We thank the National Science Foundation (Contract No. CTS-0134429), and the Camille and Henry Dreyfus Foundation for financial support.

JA0169567

(38) Henry, D. C. *Proc. Royal Soc. London, A* **1931**, *123*, 106−129.