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FOR THE RECORD

Charge–charge interactions influence the denatured state ensemble and contribute to protein stability

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Abstract: Several recent studies have shown that it is possible to increase protein stability by improving electrostatic interactions among charged groups on the surface of the folded protein. However, the stability increases are considerably smaller than predicted by a simple Coulomb's law calculation, and in some cases, a charge reversal on the surface leads to a decrease in stability when an increase was predicted. These results suggest that favorable charge–charge interactions are important in determining the denatured state ensemble, and that the free energy of the denatured state may be decreased more than that of the native state by reversing the charge of a side chain. We suggest that when the hydrophobic and hydrogen bonding interactions that stabilize the folded state are disrupted, the unfolded polypeptide chain rearranges to compact conformations with favorable long-range electrostatic interactions. These charge–charge interactions in the denatured state will reduce the net contribution of electrostatic interactions to protein stability and will help determine the denatured state ensemble. To support this idea, we show that the denatured state ensemble of ribonuclease Sa is considerably more compact at pH 7 where favorable charge–charge interactions are possible than at pH 3, where unfavorable electrostatic repulsion among the positive charges causes an expansion of the denatured state ensemble. Further support is provided by studies of the ionic strength dependence of the stability of charge–reversal mutants of ribonuclease Sa. These results may have important implications for the mechanism of protein folding.

Keywords: denatured states, electrostatic interactions; pH dependence; protein folding; protein stability

In a recent paper, we showed that reversing the charge on a side chain on the surface of a protein is a useful way of increasing stability (Grimsley et al., 1999). Here we show that this approach is not always successful. In an earlier paper, Dao-pin et al. (1991) had shown that the stability of T4 lysozyme was generally decreased by a charge–reversal mutations, even though an increase was expected, and concluded that long-range electrostatic interactions between the substituted amino acid residues and other charged groups on the surface of the molecule are weak or nonexistent. In this paper, we suggest that these results show that long-range electrostatic interactions are important in determining the denatured state ensemble and that charge–charge interactions can be more favorable in the denatured state than they are in the native state.

Some results from the studies cited above are summarized in Table 1. In both cases, an effort was made to choose side chains for charge reversal that do not form intramolecular hydrogen bonds in the wild-type protein and are not close enough to other charges to form ion pairs. For example, Dao-pin et al. (1991) state: "The charged amino acids that were altered in the present study . . . are all mobile and highly solvent exposed, and do not participate in any hydrogen bonding or salt-bridge interactions. . . . In the crystal structures of the three mutants R119E, K135E, and K147E the side-chain which is replaced in wild-type lysozyme is quite mobile, and the substituted side-chain remains mobile." For RNases T1 and Sa, structures of the mutants are not available, but, as shown in Table 1, the residues selected for charge reversal are even more accessible to solvent than those chosen for T4 lysozyme. For each protein, the distance from the charge to be replaced in the wild-type protein to the nearest positive and negative charges is given, and they are great enough that ion pairs should not be able to form in the mutants. In addition, the accessibility of the side chains will minimize the contribution of differences in conformational entropy and desolvation costs between the side chains. Four of the mutations occur in α-helices (D17K, D25K, R119E, and K147E) and differences in helix propensity will contribute to the $\Delta(\Delta G)$ values for these substitutions. However, these contributions will be small, ranging from -0.1 to $+0.4$ kcal/mol (Pace & Scholtz, 1998), and taking them into account would not change any of our conclusions. Consequently, the major contribution to the $\Delta(\Delta G)$ values for these charge reversal mutations is the change in the long-range electrostatic interactions with the other charged groups in the protein.

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Table 1. *Effect of charge reversal mutations on protein stability*[a]

Protein	Mutant	Accessibility[b] (%)	Nearest charges[c] − (Å)	Nearest charges[c] + (Å)	Coulomb's law[d] (kcal/mol)	$\Delta(\Delta G)$[e] (kcal/mol)
RNase T1	D49H	101	8.1	18.4	1.6 + ≈1.6 = 3.2	+1.1
RNase Sa	D17K	66	6.1	12.6	1.6 + ≈1.6 = 3.2	−1.1
RNase Sa	D25K	92	10.7	9.9	1.2 + ≈1.2 = 2.4	+0.9
RNase Sa	E41K	71	12.4	10.0	0.6 + ≈0.6 = 1.2	−1.2
RNase Sa	E74K	63	8.4	6.6	1.1 + ≈1.1 = 2.2	+1.1
T4 Lyso	R119E	53 & 61	10.5	8.2	1.7 + 1.7 = 3.4	0.0
T4 Lyso	K135E	64 & 76	11.1	12.3	1.4 + 1.5 = 2.9	−1.0
T4 Lyso	K147E	49 & 49	13.1	9.9	1.5 + 1.5 = 3.0	−0.7

[a]The data for RNases T1 and Sa (D25K and E74K) are from Grimsley et al. (1999). The data for RNase Sa (D17K and E41K) have not been published previously. The preparation of the mutants and stability studies were done as described in Grimsley et al. (1999). The data for T4 Lysozyme are from Dao-pin et al. (1991).

[b]Accessibilities were calculated as described by Lee and Richards (1971). For the RNases, the side-chain accessibility in the wild-type protein is given: RNase T1 = 9RNT; RNase Sa = 1RGG. For the three T4 lysozyme entries, the first number is the side-chain accessibility in the wild-type protein (2LZM), and the second is the side-chain accessibility in the mutant protein: R119E = 1L44; K135E = 1L45; and K147E = 1L46.

[c]Nearest charges gives the distance from the charge on the designated side chain to the nearest negative (−) and positive (+) charges in the wild-type protein. The charge was placed on the following groups: α-amino, N; α-carboxyl, C; Asp, Cγ; Glu, Cδ; His, Cϵ1; Lys, Nζ; and Arg, Cζ.

[d]Coulomb's Law (Equation 1) with a dielectric constant of 80 was used to calculate the first number by summing the interactions of the charge on the designated side chain of the wild-type protein with all of the other charges on the protein. For T4 lysozyme, the second number was calculated by summing the interactions of the charge introduced by the mutation with all of the other charges on the protein. For RNases T1 and Sa, the second calculation could not be done because the mutant structures are not available. Consequently, we just doubled the first number given. The results with T4 lysozyme where both wild-type and mutant structures are available shows that this is a reasonable approach.

[e]The last column gives the observed $\Delta(\Delta G)$ value for the charge-reversal mutants. $\Delta(\Delta G)$ = ΔG(mutant) − ΔG(wild-type) so that a positive $\Delta(\Delta G)$ indicates that the mutant protein is more stable than the wild-type protein. The $\Delta(\Delta G)$ values were measured at pH 5.3, in the presence of 25 mM KCl and 20 mM potassium phosphate for T4 lysozyme, and at pH 7 in the presence of 30 mM MOPS for RNases Sa and T1.

Coulomb's Law gives the energy required to bring two unit charges, q_1 and q_2, from infinity to a distance r apart in a medium with a dielectric constant D:

$$E = q_1 q_2 / Dr. \quad (1)$$

We estimated the electrostatic stabilization expected for the charge reversal mutations using Coulomb's Law, and the results are given in Table 1. The $\Delta(\Delta G)$ values for the T4 lysozyme studies were measured at pH 5.3 where the molecule has a net charge of ≈+9. Consequently, removing a positive charge should generally stabilize the molecule, and replacing it with a negative charge should almost double the effect. This is seen to be the case for T4 lysozyme where both the wild-type and mutant structures are known and could be used for the calculations. The striking result is that Coulomb's Law predicts sizable increases in stability for the mutants, even using $D = 80$, but for two of the mutants the stability decreases. Dao-pin et al. (1991) used a more sophisticated calculation, but the results were the same: an increase in stability was predicted for all three mutants. They interpreted this as follows: "For charged residues that are far apart on the surface of the protein, the electrostatic interaction between them is attenuated by distance, by high dielectric constant of the solvent, and by the screening effect of counter-ions." We suggest instead that electrostatic interactions are important, but that the stability of the denatured state is lowered more than that of the native state for some of the charge–reversed mutants, and this is why a stability decrease is observed.

At pH 7, the net charge is ≈−8 for RNase T1 and ≈−7 for RNase Sa. Consequently, to stabilize these proteins by improving electrostatic interactions, we replaced a negative charge with a positive charge. For the Coulomb's law calculation, we assumed that the contribution of adding the positive charge in the mutant would be the same as removing the negative charge from the wild-type protein. Based on the results for T4 lysozyme, this is a good assumption. As shown in Table 1, the predicted increases in stability were more than twice as large as the measured increases in stability, and for two of the mutants the stability decreased. We interpreted this as follows (Grimsley et al., 1999): "This is most likely a reflection of the existence of electrostatic interactions in the denatured states as experimental (Oliveberg et al., 1995; Tan et al., 1995) and computational (Warwicker, 1999) studies have shown." What had not occurred to us was that in some cases electrostatic interactions are probably more favorable in the denatured state than in the native state.

Spector et al. (2000) have shown that a charge reversal mutation in a small peripheral subunit-binding domain leads to a 1.1 kcal/mol increase in the stability, but that an increase in stability of 5.3 kcal/mol is predicted by a modified version of DELPHI (Gilson & Honig, 1988). Using the same Coulomb's law calculation shown in Table 1 leads to a predicted stability increase of 2.4 kcal/mol. Our experience is that predictions based on Coulomb's law will generally be smaller than predictions based on DELPHI that use a dielectric constant <80 for the protein. In another recent paper, Loladze et al. (1999) show that charge reversal mutations can be

used to increase the stability of ubiquitin in agreement with the theoretical predictions based on the solvent-accessibility-corrected Tanford–Kirkwood model. A similar approach was used earlier by Garcia-Moreno et al. (1985) to predict the contribution of electrostatic interactions to myoglobin stability. Ramos et al. (1999) studied some of the specific electrostatic interactions in myoglobin and concluded: "These results suggest that the accessibility-modified Tanford-Kirkwood model overestimates the actual electrostatic interactions in proteins, for a reason that remains to be determined." We think the reason is primarily the neglect of electrostatic interactions in the denatured state.

If the Coulomb's Law calculations in Table 1 give a rough estimate of the expected increase in stability, then a comparison with the experimental results leads to some interesting conclusions about the denatured states. For two of the RNase Sa mutants and for two of the T4 lysozyme mutants, the results suggest that electrostatic interactions among the charges in the denatured state ensemble must be more favorable than in the native state. This might be possible. In the denatured state, the hydrophobic core and many of the intramolecular hydrogen bonds will be disrupted so that the polypeptide chain can rearrange itself to optimize the electrostatic interactions among the charged groups. If the denatured state is quite compact, as both experiment (Smith et al., 1996; Gillespie & Shortle, 1997; Dyson & Wright, 1998) and theory (Stigter & Dill, 1990; Elcock, 1999; Warwicker, 1999) suggest, the rearrangement might lead to distances among the charges that are shorter than those in the native state, but with an effective dielectric constant that is still less than bulk solvent. This may be easier to do in a protein like T4 lysozyme with no disulfide bonds than in RNases Sa and T1 where the denatured states will be more restricted by disulfide bonds. Thus, the results in Table 1 suggest that electrostatic interactions among the charged groups in the denatured state are improved in the charge–reversed mutants, in some cases more so than in the native state.

For the charge–reversal mutants of RNase Sa, the stability is increased for two of the mutants and decreased for two. As suggested above, we think electrostatic interactions are more favorable in the denatured state ensembles than in the native state for the two mutants with decreased stability, and that the reverse is true for the mutants with increased stability. It seems likely that electrostatic interactions in the denatured state ensemble will be more sensitive to Debye–Hückel screening by salts than in the native state. If so, increasing ionic strength should exert a greater effect on the stability of the mutants with decreased stability than on the mutants with increased stability. This is what we observe (Fig. 1), and this offers further support for an important contribution of charge–charge interactions to the denatured state ensemble.

Solvent denaturation curves are generally analyzed using the linear extrapolation method (Greene & Pace, 1974; Pace, 1986):

$$\Delta G = \Delta G(H_2O) - m[\text{denaturant}] \quad (2)$$

where ΔG is the free energy change for folding, $\Delta G(H_2O)$ is the free energy change for folding in the absence of denaturant, and m measures the dependence of ΔG on denaturant. Tanford (1970) showed that m depends on the groups in a protein that are buried in the native state but exposed to solvent in the denatured state. An analysis of m values using model compound data shows convincingly that proteins do not unfold completely and unfold to different extents (Pace et al., 1990). We have shown that for RNases A, T1, and Ba (barnase), the m value for urea denaturation increases markedly as the pH is lowered from 7 to 3 (Pace et al., 1992). The most reasonable explanation is that the denatured state ensemble expands at low pH due to electrostatic repulsion among the excess positive charges, and this increases the accessibility of the denatured state to urea. In support of this interpretation, Privalov et al. (1989) have shown that the intrinsic viscosity of unfolded proteins increases at low pH. We now describe results with RNase Sa that provide further support for this idea.

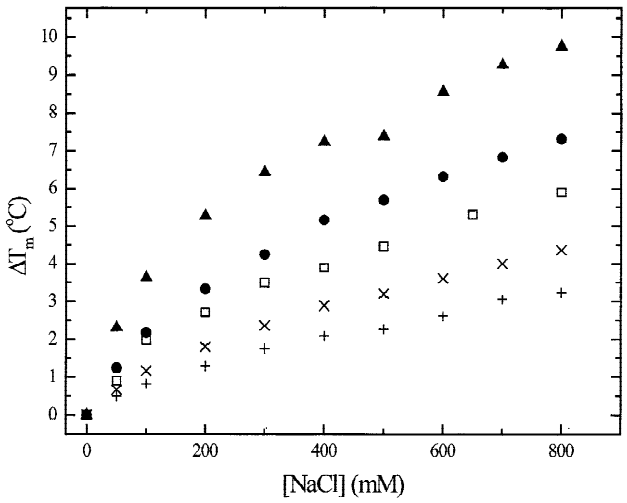

Fig. 1. Change in T_m as a function of NaCl concentration at pH 7 in 30 mM MOPS buffer. RNase Sa (□), E41K (▲), D17K (●), E74K (×), D25K (+). $\Delta T_m = T_m$ (NaCl) $- T_m$ (0 mM NaCl). The T_m values were measured as described in Grimsley et al. (1999).

RNase Sa is an acidic protein with a pI = 3.5 that contains no lysine residues. We have prepared a triple mutant that we call 3K (D1K, D17K, E41K) with a pI = 6.4, and a quintuple mutant that we call 5K (D1K, D17K, D25K, E41K, E74K) with a pI > 9. At pH 3, the estimated net charges are +8 for wild-type Sa, +11 for 3K, and +13 for 5K. The dependence of the m values for urea denaturation on pH for the three proteins is shown in Figure 2. For all three proteins, the m value increases with decreasing pH and the increase is greatest for 5K. This is consistent with an increase in accessibility to urea caused by an expansion of the denatured state due to electrostatic repulsion among the positive charges. As the pH increases to 7, the carboxyl groups are titrated and both negative and positive charges are present. Now attractive charge–charge interactions are possible, and the decrease in the m values suggests that the denatured state ensemble becomes more compact because of these favorable Coulombic interactions.

The results in Table 1 and Figures 1 and 2 all suggest that electrostatic interactions among charged groups influence the denatured state ensemble. Obviously, the electrostatic interactions will be more important the more compact the denatured state. How compact might a denatured state be? Gillespie and Shortle (1997) concluded the following: "The global topology of this denatured form of staphylococcal nuclease, as described by an ensemble of conformations consistent with the data, is strikingly similar to that of the native state . . ." Elcock (1999) has shown that when "native-like" unfolded states are used in place of a fully extended conformation ". . . dramatic improvements in the description of pH effects on protein stability are obtained." Thus, these and many other experimental and theoretical studies support the idea that the denatured state ensemble might be quite compact. What we suggest

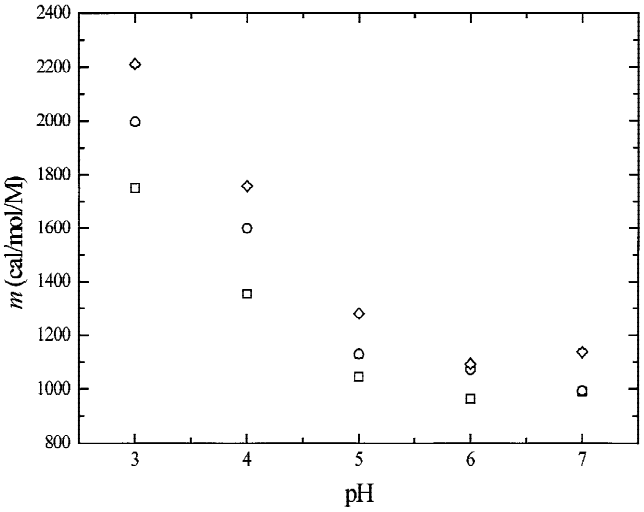

Fig. 2. The *m* value for urea denaturation as a function of pH for the folding of RNase Sa (□) and the 3K (○) and 5K (◇) mutants described in the text. The 3K and 5K mutants were prepared as described in Grimsley et al. (1999) and in more detail by Shaw (2000). The *m* values are defined by Equation 2, and they were measured as described in Pace et al. (1998).

is that favorable electrostatic interactions among the charged groups make an important contribution in determining the conformations that make up the denatured state ensemble. They will be most important for the more compact conformations.

We know much more about the conformations of folded proteins than we do about the many conformations of unfolded proteins. As a consequence, Spassov et al. (1994) were able to do an interesting comparison of electrostatic interactions in 141 folded proteins. It now seems clear that denatured proteins do not approach a randomly coiled conformation as closely as the early studies by Tanford (1968) suggested. Instead, on average, the denatured states appear to be quite compact with both elements of secondary structure and hydrophobic pockets present (Smith et al., 1996; Dyson & Wright, 1998). The two major forces stabilizing proteins are the hydrophobic effect and hydrogen bonding (Dill, 1990; Pace et al., 1996). In contrast, long-range electrostatic interactions make a smaller contribution to protein stability. We suggest that charge–charge interactions do indeed stabilize the folded state, but that they also stabilize the unfolded states so that the net contribution to stability is small. Nevertheless, if long-range electrostatic interactions are important in determining the makeup of the denatured state ensemble, then they might play an important role in the mechanism of protein folding.

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