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Electrostatic Interactions in the Denatured State Ensemble: Their Effect Upon Protein Folding and Protein Stability

Jae-Hyun Cho¹, Satoshi Sato², Jia-Cherng Horng³, Burcu Anil⁴, and Daniel P. Raleigh^{4,5}

¹ Department of Biochemistry and Molecular Biophysics, Columbia University, 650 West 168th Street, New York, NY 10032, USA, Jc2980@columbia.edu

² Okayama Research Park Incubation Center, 5303 Haga Okayama 701-1221 JAPAN, ssato@oric.ne.jp

³ Department of Chemistry, National Tsing Hua University, 101, Section 2, Kuang-Fu Road, Taiwan 30013 R.O.C, jchorng@mx.nthu.edu.tw

⁴ Department of Chemistry, State University of New York at Stony Brook, Stony Brook NY 11794-3400, USA, draleigh@notes.cc.suys.edu

⁵ Graduate Program in Biophysics, Graduate Program in Biochemistry and Structural Biology, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

Abstract

It is now recognized that the denatured state ensemble (DSE) of proteins can contain significant amounts of structure, particularly under native conditions. Well-studied examples include small units of hydrogen bonded secondary structure, particularly helices or turns as well hydrophobic clusters. Other types of interactions are less well characterized and it has often been assumed that electrostatic interactions play at most a minor role in the DSE. However, recent studies have shown that both favorable and unfavorable electrostatic interactions can be formed in the DSE. These can include surprisingly specific non-native interactions that can even persist in the transition state for protein folding. DSE electrostatic interactions can be energetically significant and their modulation either by mutation or by varying solution conditions can have a major impact upon protein stability. pH dependent stability studies have shown that electrostatic interactions can contribute up to 4 kcal mol⁻¹ to the stability of the DSE.

Keywords

Denatured State Ensemble; Unfolded State; Protein Stability; Protein Folding; Protein Engineering; Ribosomal Protein L9; Electrostatic Interactions; Protein Design; pH Titration; Thermodynamic Linkage

INTRODUCTION

Early work on protein folding lead to the view that the denatured state ensemble (DSE) of proteins could be treated as a random coil. Within this conceptual framework DSE interactions

Correspondence to: Jae-Hyun Cho; Daniel P. Raleigh.

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are usually considered to be limited to residues which are close in sequence. There has been a flurry of interest in the properties of the DSE in the last ten to fifteen years and it is clear that the DSE ensemble of real proteins is very different from the classic random coil especially under native or near native conditions. Considerable effort has been invested in characterizing residual structure in the DSE [1–10]. Well documented examples include hydrophobic clusters as well as small units of hydrogen bonded secondary structure, particularly helices or turns [1,2,4,5,7,9,10]. Other types of interactions are less well characterized. For example, it is often assumed that electrostatic interactions play a minor role in the DSE since charged residues are thought to be well solvated in the DSE and interactions amongst them are expected to be screened by the high dielectric medium. However a number of investigations have shown that both favorable and unfavorable electrostatic interactions can be formed in the DSE and importantly have demonstrated that they can make a significant contribution to the energetics of the DSE [1,6,7]. The focus of this article is on the role of electrostatic interactions in the DSE and upon the experimental methods that can be employed to characterize them. Of course, significant effort has been devoted to computational and theoretical studies of DSE electrostatic interactions but here we restrict ourselves to considering only experimental studies.

The stability of a protein, defined as ΔG^0_u of unfolding, is given by the difference between the Gibbs free energy of the denatured state ensemble (DSE) and the Gibbs free energy of the folded state; $\Delta G^0_u = G_{DSE} - G_F$. The stability of a protein represents a very small difference between two large numbers. Thus mutations or changes in solvent conditions which alter the energetics of the DSE even by just a Kcalmol^{-1} can have a significant effect upon protein stability. The vast majority of work on protein stability and folding has ignored potential DSE effects and instead has focused on native state effects. Thus a key implicit assumption is invoked when analyzing protein engineering experiments; namely that mutations exert their energetics effects upon the folded state and any effects upon G_{DSE} can be ignored. Surprisingly this assumption is sometimes employed even with proteins that are known to have a compact DSE with significant residual structure.

The existence of residual interactions in the DSE formally gives rise to the possibility that some mutations might exert part of their effect by altering the energetics of the DSE. Our understanding of protein folding and stability depends in large part on mutational studies, thus careful assessment of the effects of mutations upon the DSE is essential. Indeed, the characterization of DSE interactions has emerged as a major research topic in protein engineering and protein folding [1–7]. A key challenge is to move beyond just characterizing the structural propensities of the DSE and to instead develop an understanding of how modulating DSE interactions can influence protein stability and protein folding [8].

Perhaps we should first make a distinction between the DSE populated under denaturing conditions such as elevated temperature or high concentrations of denaturants and the DSE populated under more native conditions. Both states are interesting, but characterization of the DSE under native conditions is the most relevant goal since it is the starting state for protein folding and the reference state for protein stability studies. Unfortunately the DSE formed under native conditions can be very difficult to study due to its low population and because of the cooperativity of protein folding. In contrast, the DSE induced by high concentrations of chemical denaturants or at high temperature can be highly populated under equilibrium conditions and hence is much easier to study. However, the DSE populated under strongly denaturing conditions generally does not coincide with the DSE populated under more native conditions and can differ considerably [5,9–12].

In a few cases conditions have been found where the DSE is well populated and in equilibrium with the native state under near native conditions. However, indirect methods normally need to be used to study the DSE under native conditions [13]. A wide range of approaches have

been used including, but not limited to, the study of peptide fragments, the analysis of destabilizing point mutants or truncation mutants, amide $^1\text{H}/^2\text{H}$ exchange experiments of the native state, changes in m-values upon mutation and the analysis of the pH dependence of protein stability [14–17]. Numerous investigations have provided compelling evidence for compact DSEs with significant amounts of residual structure under native conditions. These studies have provided less information about the energetics of DSE interactions, although there are examples which demonstrate that protein stability can be affected by altering DSE interactions [6,18–20].

Material and Methods

In this article we confine ourselves to reviewing published investigations. Experimental details are provided in the relevant references. The experimental data is taken from our studies of the N-terminal domain of L9 (NTL9) (figure-1)

Results and Discussion

The pH dependence of protein stability: An indirect probe of the DSE ensemble

Studies of the pH dependence of protein stability and folding kinetics offer important insight into the role of electrostatic interactions in protein stability and folding [21,22]. They also offer a relatively simple method for probing electrostatic interactions in the DSE [1,6,17,23–27]. The approach exploits the linkage relationship that connects pH dependent changes in ΔG° with the difference in the number of protons bound to the native state and to the DSE [23]:

$$\partial\Delta G^\circ/\partial\text{pH}=2.303RT\Delta Q \quad (1)$$

ΔQ in equation 1 is equal to the difference in the number of protons bound to the native state (Q_N) and to the DSE (Q_D) and is a function of pH. ΔG° is the apparent free energy of unfolding. Q_N can often be directly measured (see below) but the value of Q_D under native conditions is normally not accessible. Thus ΔQ under native conditions can not be determined. Nonetheless, information about the DSE can still be obtained. One can assume a model for the titration behavior of the DSE and calculate the expected values of ΔQ . Comparison with the experimentally determined pH vs. stability curve indicates if the model can account for the data. A discrepancy proves that there must be interactions in the DSE that perturbs Q_{DSE} from the values predicted by the model. Thus DSE electrostatic interactions can be probed and models tested.

If the titration behavior can be described using an independent sites model, the number of protons bound as a function of pH can be obtained from the degree of protonation, $\theta_{i,A}$, of the individual sites (i refers to the residue and A represents either the folded state or the DSE). $\theta_{i,A}$ can be determined by using the Henderson-Hasselbalch equation provided the individual pK_a values can be measured.

$$\theta_{i,A} = \frac{10^{(\text{pK}_i^A - \text{pH})}}{1 + 10^{(\text{pK}_i^A - \text{pH})}} \quad (2)$$

pK_i^A represents the pK_a of site i in a particular state (A is either N or D). The linkage relationship defined by equation 1 is valid even when equation 2 is not. Equation 2 applies if the titration behavior can be described as the sum of individually titrating sites. In this case the pH dependence of stability relative to the stability at a reference pH can be calculated by combining

equation 2 with the integrated form of equation 1 provided that the pK_a values for all the ionizable residues are known in the native state and in the DSE:

$$\Delta\Delta G^\circ(pH - pH6) = RT \sum_{i=1}^j \ln \left[\frac{(1+10^{(pH-pK_{i,N})})(1+10^{(6-pK_{i,D})})}{(1+10^{(6-pK_{i,N})})(1+10^{(pH-pK_{i,D})})} \right] \quad (3)$$

Here j is the number of titratable sites in the protein, i is an index which identifies the residue, $pK_{i,D}$ is the pK_a for the i th residue in the DSE and $pK_{i,N}$ is the pK_a for the i th residue in the native state. In the present example pH 6 has been selected as the reference pH. This choice is arbitrary and does not affect the analysis since it is changes in ΔG^0 that are of interest. Native state pK_a values can be often be measured by NMR, although the experiments are usually confined to the acidic branch of the titration curve because of the difficulty in determining the pK_a 's of Lys and ARG residues. If the native state pK_a values have been independently determined, then the only unknowns are the pK_a values for the DSE. The pH dependence of ΔG^0 can be calculated by assuming model compound values for DSE pK_a 's. The calculated result, $\Delta\Delta G^\circ_{cal}$ vs. pH, has no simple physical meaning, but comparison of the $\Delta\Delta G^\circ_{cal}$ vs pH curve with the experimental curve, $\Delta\Delta G^\circ_{exp}$ vs. pH, rigorously tests if the model DSE pK_a 's are reproduce the experimental result. If the experimental curve and the calculated curve differ than there must be interactions in the DSE that perturb the DSE pK_a 's away from the model values [6,27]. This forms the basis of the method. On the other hand, agreement between the calculated and experimental curves does not prove that the chosen model DSE pK_a 's provide an accurate description of the titration behavior of the DSE. The ambiguity arises because a negative deviation at one site can be masked by a positive deviation at another site. The key point is that a discrepancy between the calculated and experimental curves must be due to DSE effects if the titration behavior of the native state is known.

Figure-2 shows schematic diagrams of two proteins. In the first example (Figure 2A) there are no significant electrostatic interactions in the DSE that perturb the DSE pK_a 's from model compound values. In this case the experimental points fall on the calculated plot of $\Delta\Delta G^\circ$ vs. pH. In the second case (Figure-2B) there is a significant electrostatic interaction in the DSE that perturbs the titration behavior. In this case the model compound pK_a 's fail to account for the titration behavior of the DSE and the calculated curve deviates from the experimental points. The plots of $\Delta\Delta G^0$ vs. pH sometimes cause confusion and sometimes lead to the erroneous conclusion that acid denaturation is being studied. This is not the case. The plots require a choice of reference pH. The choice is arbitrary and the plots could have been constructed using pH 2 as a reference pH, in that case the experimental and calculated curves would always agree, by definition, at pH 2.0 and might deviate at higher pH. The analysis does assume that the unfolding equilibrium can be treated as two-state.

The linkage relationship can be exploited even when individual native state pK_a values can not be determined. An alternative experimental approach is to use potentiometric titrations to directly determine the number of protons bound to the folded state. Repeating the titration in the presence of high concentrations of denaturant measures the number of protons bound to a fully unfolded state. This data is combined to yield the $\Delta\Delta G^0$ vs. pH curve expected for the case of an unstructured DSE. The curve can compared to the one generated using experimentally determined ΔG^0 values. Again, any deviation between the two plots demonstrates the presence of electrostatic interactions in the DSE above and beyond those expected for a fully unfolded state [1].

Electrostatic interactions are found in the denatured state ensemble of a wide range of proteins

These methods have been used to investigate a number of proteins. In most of the cases examined there are clear deviations between the observed pH dependence of ΔG^0 and the behavior predicted based upon a fully unfolded DSE (Table-1). Examples include CI2, barnase, Staphylococcal Nuclease (SNase), the turkey ovomucoid third domain, the N-terminal domain of L9 and a mutant of the B1 domain of protein G [1,17,24–26,28]. Most of these proteins lack disulfides thus the DSE interactions are not simply a consequence of the presence of covalent cross links. For barnase and CI-2 the data could be accounted for by assuming that the pK_a values of the acidic residues in the DSE were depressed by 0.3 to 0.4 pK_a units relative to model compound values. SNase exhibited a very large discrepancy, 4 kcal mol⁻¹, between the actual pH dependence of stability and that predicted by potentiometric titrations conducted under native and strongly denaturing conditions. This is an important observation since it shows that DSE electrostatic interactions can make large contributions to protein stability. Significant DSE electrostatic interactions have also been observed in RNase H and ribonuclease Sa [29–31]. The case of RNase H is particularly interesting because it provides an example of the possible functional consequences of DSE structure. The heat capacity change for the unfolding of thermophilic RNase H, ΔC_p^0 , is smaller than the value obtained for the mesophilic protein. The smaller value of ΔC_p^0 leads to a flatter ΔG^0 vs. pH profile for the thermophilic protein and a significantly higher midpoint temperature for unfolding. Calorimetric studies show that the difference is due to DSE effects. The reduced ΔC_p^0 for thermophilic RNase H is due a pH-dependent hydrophobic cluster in the DSE [29]. The detailed studies of the Pace group on ribonuclease Sa has shown that mutation of a single surface exposed Asp significantly stabilized the protein by disrupting non-native DSE electrostatic interactions. Furthermore they demonstrated that the non-native interactions persisted in the transition state for folding [18, 31]. DSE effects involving charged residues have also been detected in the drkN SH3 domain [16], although they are believed to be due interactions involving adjacent residues. Work on T4 lysozyme, RNase A and RNase T1 have shown that mutations that are predicted to increase protein stability by altering native state electrostatic interactions often have much smaller effects than expected. These results have been interpreted to indicate the presence of favorable electrostatic interactions in the DSE [32,33]. Not all proteins display significant DSE electrostatic effects. For example the pH dependent stability of the C-terminal domain of L9 can be well accounted for by assuming unperturbed DES pK_a values [34]. In addition the $\Delta\Delta G^0$ values for a range of mutations in a number of proteins have been well predicted by considering only native state electrostatic interactions suggesting that DSE electrostatic interactions may not be critical in these systems [35].

Mutational analysis of electrostatic interactions in the denatured state ensemble

The approaches outline above can be extended to obtain site specific information by analyzing sets of mutants. If a charged residue makes an important denatured state interaction then its removal should reduce the deviation between the calculated and experimental $\Delta\Delta G^0$ vs. pH curves. On the other hand, mutation of a residue that does not take participate in the DSE interactions will not affect the deviation between the two curves. This leads to a simple mutational strategy for probing the role of specific residues. Mutations are made and their effect upon the $\Delta\Delta G^0$ vs. pH curve analyzed. In favorable cases it is even possible to fit the curves to obtain the difference in the native state and DSE pK_a of the mutated residue [26]. It is important to note that changes in the native state pK_a 's caused by the mutation can be rigorously taken into account by using native state pK_a 's values measured for each mutant. An important, but largely untestable, assumption is that mutation of a charged residue does not change significantly the structure of the DSE and thus indirectly alter the pK_a 's of other sites in the DSE. We believe that the approach is best applied in a semi- quantitative way because of this potential concern.

The N-terminal domain of L9 (NTL9) is particularly well suited for such studies. It is a small α - β protein that remains folded from at least pH 1 to 11. Folding is reversible over the entire range and the native state titration behavior can be well described as a sum of individual titrating groups over the pH range of interest. [36]. The six acidic residues in NTL9 are all located on the surface and are all well distributed throughout the structure (Figure 1).

There is a large deviation between the experimental plot of $\Delta\Delta G^0$ vs. pH and the one calculated assuming non-perturbed DSE pK_a values (Figure-3). This proves that there are DSE effects in NTL9 which are not captured by the model pK_a values. The sign of the deviation indicated that there are attractive electrostatic interactions in the DSE. Their removal by mutation will destabilize the DSE and is expected to lead to an increase in ΔG^0 of unfolding. pK_a 's can be perturbed by local sequence effects even in unstructured polypeptides hence we used pK_a 's measured for a set of peptide fragments derived from the sequence of NTL9 as model compound values. The goal of the experiment was not to model the DSE as a sum of peptides but rather to see if there are interesting DSE interactions above and beyond those that can be accounted for by local sequence effects.

A systematic study of the contribution of surface charge residues to the stability of NTL9 provided key insight into the origins of the DSE effects. Somewhat surprisingly, mutation of the surface exposed residue K12 to M stabilized the domain by $1.8 \text{ kcal mol}^{-1}$. $1.8 \text{ kcal mol}^{-1}$ is a very large increase in stability for a single point mutant, particularly for a surface exposed residue. Detailed analysis showed that the energetic consequences of the mutation were due to its effect upon the DSE. The mutation does not alter the structure of the native state nor did it perturb the native state pK_a 's of the acidic residues. NTL9 is a basic protein but analysis of the pH dependent stability data rules out diminished electrostatic repulsion in the native state as an explanation for the increase in stability. As noted above, mutation that abolishes the DSE interactions will make the deviation between the calculated and experimental pH stability profile much smaller [17]. This leads to an experimentally testable prediction; namely that the K12M mutation should lead to better agreement between the calculated and experimental $\Delta\Delta G^0$ curves. This is exactly what was observed (Figure-3). The calculated and experimental pH stability profiles for K12M NTL9 are in good agreement in sharp contrast to the case for wildtype NTL9. The results demonstrates that K12 participates in important interactions in the DSE and show that the mutation shifts the pK_a 's of one or more of the acidic residues in the DSE.

These initial observations were extended by analysis of a large set of points mutants. We individually mutated all of the Lys residues in the loop that contains K12 to M (K7, K10, K12, K14 and K15). With the single exception of K12M, none of the mutations appreciably changed the stability of the protein at pH 5.5. Control experiments showed that the Lys to Met mutations did not change the structure of the protein or alter significantly the energetics of the native state electrostatic interactions involving the acidic residues. With the exception of K12M, the observed pH dependent stability profiles of all of the mutants still showed significant deviations from the calculated stability curves (Figure 4). Only the K12M mutant showed a significantly improved correlation over the entire pH range. This is a striking result and shows that K12 plays a unique role in the DSE. The K7M and K15M mutants are notable since they are closest in primary sequence to an acidic residue (D8 or E17). Therefore one might have expected that mutation of K7 or K15 would have caused the largest effects among the five mutants; however these two mutants showed very little change from wild type, indicating that K7 and K15 do not playing a major role in determining the DSE electrostatic interactions.

The effects of mutating each acidic residue were also examined. Again the appropriate control experiments showed that the mutations did not alter the native state. The D8N and E17Q mutants were of special interest, since E17 and D8 are located at the start and end of the lysine-

rich loop (Figure 2). The two mutants behaved very differently even though both are close to K12 in the primary sequence. The D8N mutation had a large effect on the $\Delta\Delta G^\circ$ vs. pH plot, reducing the discrepancy while the E17Q mutation had little effect. This strongly suggests that D8 is involved in significant DSE interactions, almost certainly with K12 [27]. The DSE interaction between D8 and K12 in the DSE is non-native since the two residues do not interact in the folded state [6]. Quantitative analysis of the data argues that the D8-K12 DSE interaction contributes on the order of $1.2 \text{ kcal mol}^{-1}$. The additional stabilization caused by the K12M mutation likely arises from the disruption of other DSE interactions.

The origins of the relatively specific interactions in the DSE of NTL9 are difficult to determine but some possibilities can be eliminated. Peptide fragment studies rule out the possibility that the effects arise from a propensity for the local sequence to encode a preferred conformation which leads to a strong interaction between D8 and K12. A peptide fragment consisting of the first 22 residues of NTL9, which included the β -strand -1, loop, β strand - 2 unit, was not able to fold in isolation [37] even when the N and C-termini were constrained by a Gly-Cys-Cys-Gly linker. Constraining the fragment had only modest effects on the pK_a of D8 or E17. These studies indicate that DSE interactions involving K12 are not caused by locally encoded structural propensities. Instead they need to be viewed in the context of the overall structural propensities of the DSE ensemble. The most likely scenario is that clustering of hydrophobic residues helps to bring D8 and K12 in close proximity (Figure-5).

Denatured state interactions as a target for protein design

Attempts to rationally manipulated protein stability by targeting the DSE have a long, if not always successful, history. Early efforts attempted to increased stability by decreasing the entropy of the DSE. These approaches typically involved the insertion of disulfides or the use of Gly to Ala and X to Pro substitution [38–40]. Problems arose if strain was introduced into the native state or if the modification inadvertently stabilized a compact denatured state. Mutation of glycines provides an example of the former. Glycines have often been the target of mutations designed to increase protein stability by reducing the entropy of the unfolded state. However glycine is commonly found in conformations with positive ϕ -angles and substitution by an L-amino acid at such a site can introduce strain into the folded state. We have demonstrated an alternative strategy that consists of replacing glycine residues that have positive values of ϕ with D-amino acids. Advances in peptide synthesis and native protein ligation make this a practical approach [41]. Such a substitution can enhance protein stability provided no steric clashes are introduced and provided no adverse solvation effects are introduced [42,43].

The work reviewed here shows that targeting DSE electrostatic interactions provides another possibility for rationally manipulating protein stability. It is impossible to tell if electrostatic interactions stabilize the DSE simply via inspection of the native structure or primary sequence but pH dependent stability studies are relatively straight forward and the methods outlined here can be used to test for suitable DSE interactions to target. Alternatively, potentiometric titrations in native buffer and under strongly denaturing conditions can be combined to measure ΔQ for the transition from the folded state to a fully unfolded state. The curve can compared to one generated from stability measurements that measure the difference in free energy between the folded state and DSE which is populated under native conditions. Comparison of the two experiments indicates if there are significant DSE interactions. If so single mutants can be screened to find stabilizing variants. The approach avoids the tedium of having to determine a set of pK_a values and avoids having to make assumption about single site titration behavior but requires that the protein be soluble enough for the potentiometric approach.

Recent work on NTL9 has combined the Gly to D-Ala substitution approach with mutations that target the DSE. A designed double mutant of NTL9 was so thermally stable that it did not

melt in the absence of denaturant. In the presence of 6 M urea the T_m of the mutant was 83°C [42].

The role of non-native DSE electrostatic interactions in folding

Do the non-native DSE interactions persist in the transition state for folding or are they broken as folding progresses from the DSE to the transition state? The question is important since it dictates their effect upon folding. DSE interactions will not influence the folding rate if they are present in the transition state for folding and have the same strength in the both the DSE and transition state, since will not have to be broken to cross the transition state barrier. If they are not present in the transition state than they must have been broken as folding progressed from the DSE to the top of the free energy barrier. In this case their removal by mutation will have a significant effect upon the folding rate. Kinetic studies show that the nonnative DSE electrostatic interactions found in NTL9 still exist in the folding transition state. The folding rate of K12M NTL9 was increased only 2.3-fold relative to wild type but the unfolding rate was about 11-fold less. The small change in the folding rate and large change in the unfolding rate indicates that the non-native DSE interactions are present although somewhat weaker in the transition state for folding. The ϕ -value for the K12M mutation is 0.27. The standard interpretation of small ϕ -values is that, in the absence of DSE effects, they represent the partial formation of native interactions in the transition state for folding [44]. Here DSE effects are important and the small ϕ -value indicates just the opposite, namely that the non-native interaction probed by the mutation is present in the transition state but is slightly weakened relative to the DSE. Similar effects have been observed by the Texas A&M group with their studies of the role of surface acidic residues in the folding ribonuclease Sa [30].

CONCLUSIONS

Examination of the existing body of literature unequivocally shows that electrostatic interactions can play an important role in the DSE. They have been observed in the DSE of a wide range of proteins and they can be energetically significant. NTL9 and ribonuclease Sa provide two clear examples of how protein stability can be significantly increased by targeting DSE interactions while the studies of ribonuclease H provide an interesting example of the functional significance of DSE interactions. Clearly unfolded proteins are not random coils and clearly their interactions are not restricted to local secondary structure or clusters of hydrophobic side chains. Key issues include the development of methods for predicting the presence of DSE electrostatic interactions, a by no means trivial undertaking, and the development of methods to correct standard approaches such as double mutant cycle analysis and ϕ -value analysis for DSE effects.

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Figure 1.

Diagram of the N-terminal domain of the ribosomal protein L9 (NTL9). Experimental data taken from our studies of NTL9 is used to illustrate how pH dependent stability studies can be used to obtain information about the DSE which is in equilibrium with the native state under native conditions.

Figure 2.

The use of pH dependent stability measurements to probe DSE interactions. (A) The case where the titration behavior of the DSE is accounted for by model pK_a values. The calculated $\Delta\Delta G^\circ$ versus pH curve (solid line) agrees with the experimental points (closed circles). (B) A protein in which there is a significant electrostatic interaction in the DSE. The calculated $\Delta\Delta G^\circ$ versus pH curve (solid line) deviates from the experimental points (closed circles).

Figure 3.

Plots of $\Delta\Delta G^0$ vs. pH. (a) wild type NTL9 and (b) K12M NTL9. $\Delta\Delta G^0$ is the difference between ΔG^0 of unfolding at a given pH and the value at pH 6.0 (i.e pH 6 was chosen as the reference pH). The curves are the predicted stabilities calculated assuming no perturbation of DSE pK_a 's beyond the peptide fragment values. Experimentally measured native state pK_a 's of the K12M mutant were used in the calculation and this provides a direct control for any native state effects. Reprinted from Journal of Molecular Biology, 338, Cho J.-H., Sato and Raleigh D.P. "Thermodynamics and Kinetics of Non-native Interactions in Protein Folding: A Single Point Mutant Significantly Stabilizes the N-Terminal Domain of L9 by Modulating Non-native

Interactions in the Denatured State” pages 827–837, Copyright (2004), with permission from Elsevier.

Figure 4.

$\Delta\Delta G^\circ$ vs. pH plots for Lys to Met mutants. (a) Wildtype (b) K7M (c) K10M (d) K12M (e) K14M (f) K15M. Experimental values are depicted as closed circles. The dashed lines represent the predicted stability change calculated using native state pK_a values and values of 4.4 and 4.0 for the Glu and Asp DSE pK_a 's respectively. The solid lines are the predicted curves calculated using measured native state pK_a values and peptide model values for the DSE. pH 6 is chosen as the reference pH for the plots. Any native state effects, which are expected to be small, are fully accounted for since the directly measured native pK_a 's were used every calculation. Reprinted from Journal of Molecular Biology, 353, Cho J.-H. and Raleigh D.P. "Mutational Analysis Demonstrates that Specific Electrostatic Interactions can Play a Key Role in the Denatured State Ensemble of Proteins" pages 174–185, Copyright (2005), with permission from Elsevier.

Figure 5.

A schematic representation of how the clustering of hydrophobic residues (dark circles) in the DSE of NTL9 could to bring D8 and K12 into close proximity. The diagram is meant to be illustrative and information on which hydrophobic residues make dominate contributes to the cluster is necessarily limited.

Table 1

Proteins that have been examined for the presence of DSE electrostatic interactions.

Protein	Electrostatic interactions are present that can not be accounted for by model compound pK_a 's	Reference
NTL9	Yes	[6,17]
CTL9	No	[34]
RNase H	Yes	[29]
SNase	Yes	[1]
CI2	Yes	[26]
Barnase	Yes	[24]
RNase Sa	Yes	[30,33]
Mutant of PGB1	Yes	[28,45]
drkN SH3	Yes	[16,46]
OMTKY3	Yes	[25]
E. coli	Yes	[47]
Thioredoxin		
CspB	No	[48]
Leucine zipper	Yes	[49]