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A Water Mediated Electrostatic Interaction Gives Thermal Stability to the “Tail” Region of the GrpE Protein from *E. coli*

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Abstract

The GrpE protein from *E. coli* is a homodimer with an unusual structure of two long paired α -helices from each monomer interacting in a parallel arrangement to form a “tail” at the N-terminal end. Using site-directed mutagenesis, we show that there is a key electrostatic interaction involving R57 (mediated by a water molecule) that provides thermal stability to this “tail” region. The R57A mutant showed a drop in T_m of 8.5°C and a smaller ΔH_u (unfolding) compared to wild-type for the first unfolding transition, but no significant decrease in dimer stability as shown through equilibrium analytical ultracentrifugation studies. Another mutant (E94A) at the dimer interface showed a decrease in ΔH_u but no drop in T_m for the second unfolding transition and a slight increase in dimer stability.

Keywords

Electrostatic interactions; GrpE; oligomerization; protein stability; salt bridge

1. INTRODUCTION

The GrpE protein from *E. coli* consists of a homodimeric structure (see Fig. 1) with a very unusual and unique feature of two long α -helices at the N-terminal end (one from each monomer) paired together in a non-coiled parallel arrangement (Harrison *et al.*, 1997). This “tail” region of the structure has been shown to act as a thermosensor in the temperature regulated action of the DnaK/DnaJ/GrpE heat shock system (Grimshaw *et al.*, 2003; Siegenthaler *et al.*, 2005). Structurally, there is also a four-helix bundle formed at the dimer interface with each monomer contributing two α -helices; previous work has shown that the bundle serves as a scaffold for the association of the long helices (Gelinas *et al.*, 2002; Mehl *et al.*, 2003). A beta sheet domain extends from each monomer at the C-terminal end and is not involved with the dimer interface (see Fig. 1).

Futhermore, an important ongoing area of protein research is the role of electrostatic interactions in protein stability, especially within oligomeric proteins. For example, hyperthermophilic proteins show increased oligomerization states compared with their counterparts found in mesophilic organisms and this leads to enhanced stability (Vieille and Zeikus, 2001). Additionally, electrostatic interactions involving surface residues and partially buried ones are of general interest in the design and development of more stable proteins via mutations (Luisi *et al.*, 2003; Bjork *et al.*, 2004). Here we probe the role of two surface

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electrostatic interactions at the dimer interface of GrpE, one water mediated interaction within the “tail” region and the other found near the surface of one end of the four-helix bundle motif. As shown in Fig. 2A the water mediated interaction occurs between an Arg57 from an α -helix from one monomer and a Glu58 residue from the α -helix from the other monomer. There is an identical corresponding interaction involving a water molecule between Glu58 and Arg57 in the reverse sense. The second electrostatic interaction (see Fig. 2B) is a salt bridge between Glu94 from one monomer and Lys136 from the other monomer. From the crystal structure the distance between these two residues in the reverse situation would not lead to an identical corresponding interaction (although, in solution an interaction could exist).

Two alanine mutants (E94A and R57A) were used to investigate the importance of these electrostatic interactions at the dimer interface of GrpE. The results show that the water mediated interaction involving Arg57 is an important electrostatic force in the thermal stability of the “tail” region but only marginally influences the overall dimeric structure. Interestingly, the E94A mutant showed a decrease in the enthalpy of unfolding of the four-helix bundle region and yet showed a slight stabilizing effect on the overall dimer stability.

2. MATERIALS AND METHODS

2.1. Site-specific Mutagenesis and Protein Purification

A slightly modified protocol from the Quick Change™ Site-directed mutagenesis method developed by Stratagene (La Jolla, CA, USA) was utilized to create the point mutants. Briefly, a PCR reaction containing appropriate primers (R57A: 5'-GCTGAAGCCCAGACCGCTGAACGTGACGGC-3'/ 5'-GCCGTCACGTTTCAGCGGTCTGGGCTTCAGC-3'; E94A: 5'-GAAATTCATCAACGCATTGCTGCCGGTGATTG-3'/ 5'-CAATCACCGGCAGCAATGCGTTGATGAATTTTC-3') from IDT, Inc. (Coralville, IA, USA) at 125 ng each, plasmid DNA (pRLM159) (Mehl *et al.*, 2001) at 50 ng, dNTP mix (Perkin Elmer, Branchburg, NJ, USA) at 0.01 mM, and Pfu DNA polymerase (New England Biolabs Inc., Beverly, MA, USA) at 2.5 units was run followed by addition of Dpn 1 (New England Biolabs Inc., Beverly, MA, USA) (40 units) at 37°C for 1.5 h. A 10 μ l aliquot was used to transform RLM569 cells (Mehl *et al.*, 2001). Plasmids were isolated and purified using Qiagen Midi Plasmid Kits (Valencia, CA, USA) and the correct plasmid DNA sequence was verified (Protein and Nucleic Acid Chemistry Laboratory, Washington University School of Medicine, St. Louis, MO, USA). Mutant genes were transformed and then over-expressed in DA262 cells (Wu *et al.*, 1994). The resulting plasmids were named pAFM49 for R57A and pAFM23 for E94A. The wild-type protein was purified as described previously (Mehl *et al.*, 2001) and both mutant proteins were purified using the same protocol utilized for that of the wild-type.

After purification, the proteins were dialyzed against 25 mM potassium phosphate buffer pH 7.2 containing 5% (v/v) glycerol that had been filtered and degassed (for CD analysis), and against 25 mM potassium phosphate buffer pH 7.2 containing 100 mM NaCl, again filtered and degassed (for AUC analysis). Protein concentrations were determined using either the Bio-Rad protein assay based on the Bradford dye-binding procedure (Bradford, 1976) with bovine gamma globulin as the standard, or using an extinction coefficient of 0.059 A₂₈₀ mg⁻¹ ml⁻¹ (Gelinas *et al.*, 2002).

2.2. Circular Dichroism Spectroscopy and Thermal Unfolding Experiments

Circular dichroism (CD) spectra were recorded using an OLIS (Online Instruments Inc., Bargar, GA USA) RSM 1000 spectrometer configured with a CD module for dual beam CD. Initial spectra (Fig. 4) were obtained using the following conditions: T, 20°C; pathlength, 0.1 cm; protein concentrations, 0.5-0.7 mg/ml; scanned from 260-185 nm with a rate dependent

on amount of signal produced at each wavelength. Digital filtering (11 pt.) was applied to the average of four scans. Temperature was controlled using a Peltier-type temperature control system (OLIS and Quantum Northwest, Spokane, WA, USA).

Thermal unfolding studies (Fig. 5) were carried out using the same conditions described above except that the scan window was from 260 to 195 nm and no digital filtering was applied. A full scan was taken at each temperature from 6°C to 94°C (every 2°C) with an equilibration time of 2 min at each temperature. The entire data set was fitted to a three species model consisting of a folded protein proceeding to an intermediate species (thought to be the unfolded “tail” and folded four-helix bundle) and then on to the unfolded protein using the GlobalWorks™ (OLIS, Bargar, GA, USA) software. The software allows a singular value decomposition (SVD) factor analysis on the entire data set using all the points from each scan at each temperature (a three dimensional data matrix). The data presented in Table 1, namely the transition temperatures (T_m) and enthalpies of unfolding (ΔH_u) for each transition, were obtained from the fitting results. The error estimation procedure utilized is described in Matheson *et al.*, 2004.

2.3. Analytical Ultracentrifugation Equilibrium Experiments

All sedimentation experiments were performed with a Beckman Optima XL-A at the Keck Bio-physical Facility at Northwestern University. Sedimentation equilibrium and Monte Carlo analyses were performed with UltraScan (Demeler, 2006) version 7.3. Hydrodynamic corrections for buffer conditions were made from buffer tables included in UltraScan according to data published by Laue and coworkers (Laue *et al.*, 1992). The molar extinction coefficient at 280 nm was determined with the method by Gill and von Hippel (Gill and von Hippel, 1989). The partial specific volume for the wildtype and each mutant were estimated from peptide sequence according to the method by Durchschlag (Durchschlag, 1986), and found to be 0.7361 cc/g for the wildtype, 0.7365 cc/g for the E94A mutant, and 0.7364 cc/g for the R57A mutant. Data were fitted to multiple models. The most appropriate model was chosen based on visual inspection of the residual run patterns, and based on the best statistics. 95% confidence intervals were determined by Monte Carlo analysis. Each protein was measured at six loading concentrations (0.3, 0.5 and 0.7 OD at both 208 nm and 230 nm) covering a concentration range covering approximately three orders of magnitude (~40 nM-45 μ M). For each scan, extinctions above 0.5 OD for 208 nm and 0.9-1.0 OD at 230 nm were excluded from the analysis (buffer absorbance at 208 nm contributes about 0.4 OD - data not shown). Extinction coefficients for 208 and 230 nm were measured as described in (Demeler, 2005). Proteins were measured in a 25 mM potassium phosphate buffer containing 100 mM NaCl and sedimented to equilibrium at 4°C. Absorbance scans were collected at equilibrium at five speeds (20.0, 27.5, 35.0, 42.5, and 50 krpm). Protein solutions were spun in 6-channel titanium centerpieces (Nanolytics, Gesellschaft für Kolloidanalytik mbH Hauptstr. 20, 14624 Dallgow, Berlin, Germany) in the AN-50-TI rotor.

3. RESULTS AND DISCUSSION

3.1. Protein Purification and General Characterization

Both mutant proteins, E94A and R57A, were produced in normal amounts by the heat inducible over-expression system (Mehl *et al.*, 2003) and could be purified with the standard protocol developed for the wild-type protein. Figure 3 shows the final purified proteins (greater than 95% purity). Figure 4 shows the CD spectra of the two mutants compared to the wild-type protein. The CD results show that there are no major structural changes occurring with the mutant proteins as one would expect with introducing a small aliphatic alanine side chain into the polypeptide backbone.

3.2. Thermal Stability and Oligomerization State

Shown in Fig. 5 are the thermal unfolding profiles as monitored by CD for the wild-type and the two mutant proteins. All three proteins showed nearly complete reversibility (greater than 90%) in refolding upon lowering the temperature (data not shown). Unfolding of the wild-type protein showed two transitions with midpoints at 50°C and 77°C (see Table 1); these results agree with previous research on the unfolding of GrpE (Gelinas *et al.*, 2002; Grimshaw *et al.*, 2001) in that the first transition is thought to be due to the unfolding of the long paired N-terminal helices or “tail” thermosensor region and the second transition is due mainly to the four-helix bundle. Additionally, the enthalpies of unfolding (ΔH_u) for each transition (see Table 1) are also in agreement with previously reported values (Gelinas *et al.*, 2002).

The E94A mutant gave almost identical transition midpoint temperatures as that of the wild-type (see Table 1). However the ΔH_{u1} for the first transition was higher by 10 kcal/mol and the second transition (ΔH_{u2}) was lower by 20-30 kcal/mol. The lowering of the second transition might have been predicted since less energy is needed to unfold the protein in the four-helix bundle region due to the absence of a salt-bridge between monomers. Additionally, with this mutant there could be the loss of a potential intra-helical electrostatic interaction between the Glu94 residue and Lys90 residue (see Fig. 2B), although the distance between these two residues is greater. The increase of the enthalpy for the first transition perhaps could be explained by a potential new interaction occurring between the lysine at position 136 and some residue within in the “tail” or the beta sheet domain. There is some evidence that the stability of the “tail” is linked to the stability of the beta-sheet domain (Gelinas *et al.*, 2002).

The R57A mutant, concerned with the water mediated interaction within the “tail” region, showed a significant decrease in T_{m1} ($\Delta T_{m1} = -8.5^\circ\text{C}$) and in the enthalpy of unfolding ($\Delta\Delta H = -17.5$ kcal/mol) for the first transition. Additionally, from the unfolding profiles (see Fig. 5), one can see a broadening of the first transition. Clearly, these results indicate that the water mediated electrostatic interaction between the two long paired α -helices is important for the thermal stability of this unusual structural region. Ikura and coworkers have shown that there are important water-mediated interactions at the barnase-barstar protein-protein interface (Ikura *et al.*, 2004). Additional research into the significance of water mediated interactions have shown that engineered ion pairs from a protein found in a thermophile when introduced into an homologous protein from a mesophilic bacterium lead to additional water mediated interactions and enhanced stability (Bogin *et al.*, 2002). Another study showed that a new water mediated interaction was present for a protein at lower temperature suggesting the plasticity of ion pairs in proteins (Natesh *et al.*, 2003).

From the AUC results (see Table 1) both mutant proteins still formed dimeric structures. The R57A mutant gave essentially (within the error limits) the same dimer association constant as that for the wild-type. This was expected since the “tail” region does not contribute significantly to the dimerization of GrpE (Gelinas *et al.*, 2002; Mehl *et al.*, 2003), and thus the majority of conformational stability resides in the four-helix bundle region. The E94A mutant showed a small increase in the dimer association constant indicating slight stabilizing effect for the folded dimeric species. There are examples in the literature of alanine mutants involved in surface salt bridges for native proteins showing both stabilizing and destabilizing effects for the folded protein. For example, Mayo and Strop showed that an alanine mutant of hyperthermophilic rubredoxin gave only a small destabilizing effect (Strop and Mayo, 2000). Considering the folding of GrpE essentially involves a pathway of unfolded polypeptide to a folded dimeric species (a reasonable scenario given the four-helix bundle at the interface between monomers and the amount of monomer species at equilibrium is extremely small), this could be a case where a favorable electrostatic interaction in the folded dimeric state does not outweigh the cost of desolvation of the charged residues in the unfolded state (Hendsch and Tidor, 1994; Honig and Nicholls, 1995).

4. CONCLUSION

Water mediated electrostatic interactions are important for both the thermal stability and stability of oligomeric structures for numerous proteins. Here we have shown that the thermal stability of the unusual “tail” structure found within the GrpE protein from *E. coli* is achieved, in part, via a crucial water mediated electrostatic interaction between the two α -helices. Although this electrostatic interaction is important for stability of the long paired helices, it does not contribute significantly to the overall dimer (oligomeric) stability. A second electrostatic interaction (a specific salt-bridge) at the dimer interface appears to fall in to the class of interactions that do not have significant stabilizing influence to the overall oligomeric protein stability, perhaps, due to entropic costs of desolvation of ionic residues.

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Abbreviations

Arg, arginine; Glu, glutamic acid; Lys, lysine; E, glutamic acid; R, arginine; A, alanine; CD, circular dichroism; AUC, analytical ultracentrifugation; UV, ultraviolet.

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Fig. 1. Ribbon diagram of the GrpE dimer (amino acids 34-197). The structure is based on the X-ray crystallographic data determined for a deletion mutant of GrpE missing the first 33 amino acids on the N-terminal end and is in a complex with the ATPase domain of DnaK (Harrison *et al.*, 1997). The three main regions are: the N-terminal α -helical "tail" portion composed of amino acids 34-88 from each monomer, the four-helix bundle motif composed of amino acids 89-138 from each monomer, and the C-terminal β -sheet domain composed of amino acids 139-197 from each monomer. The Figure was produced using MOLMOL (Koradi *et al.*, 1996).

Fig. 2. Details of the electrostatic interactions at the dimer interface of GrpE. (A) The water-mediated interaction in the “tail” region involving an Arg57 side chain from one monomer and a Glu58 side chain from the other monomer (some atoms have been removed to highlight this interaction); (B) and a salt-bridge between Glu94 on one monomer and Lys136 of the other monomer. Distances between atoms are given in Å. Drawn with Weblab Viewer Pro (MSI Inc).

Fig. 3. Electrophoretic analysis of purified GrpE, GrpE(E94A), and GrpE(R57A) proteins. A 13% SDS-polyacrylamide gel showing purified GrpE and the two point mutants used in this study. Approximately 5 μ g of protein was used in each lane. Polypeptide bands were visualized by staining with Coomassie brilliant Blue R250. Lane 1, wild-type GrpE; lane 2, GrpE(E94A); lane 3, GrpE(R57A). Molecular weight markers are (from top to bottom) phosphorylase b (94,000), albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α -lactalbumin (14,400).

Fig. 4. Far-UV CD spectra of GrpE (●), GrpE(E94A) (■), and GrpE(R57A) (□). Conditions are described in the Materials and Methods section.

Fig. 5. Thermal unfolding of GrpE (●), GrpE(E94A) (■), and GrpE(R57A) (□). Conditions are described in the Materials and Methods section.

Transition Midpoint Temperatures (T_m), Enthalpies of Unfolding (ΔH_u) and Monomer-dimer Association Constants (K_a) for GrpE (WT), E94A, and R57A

Protein	T_{m1} ($^{\circ}\text{C}$)	ΔH_{u1} (kcal mol $^{-1}$)	T_{m2} ($^{\circ}\text{C}$)	ΔH_{u2} (kcal mol $^{-1}$)	a Association constant (K_a) (M^{-1})
WT	50.1 $\pm$.13	43.5 $\pm$.12	77.1 $\pm$.14	62.4 $\pm$.16	3.25 $\times 10^6$ (2.63 $\times 10^5$ -4.02 $\times 10^6$)
E94A	49.9 $\pm$.04	53.0 $\pm$.40	78.5 $\pm$.06	30.0 $\pm$.10	1.17 $\times 10^7$ (7.76 $\times 10^6$ -1.75 $\times 10^7$)
R57A	41.6 $\pm$.20	26.0 $\pm$ 5	76.5 $\pm$.10	63.0 $\pm$ 3.0	6.89 $\times 10^6$ (2.37 $\times 10^6$ -2.00 $\times 10^7$)

^a Determined by analytical ultracentrifugation (AUC) equilibrium sedimentation studies. Values in brackets refer to the 95% confidence intervals determined by Monte Carlo analysis.