

Probing the Folding Transition State Structure of the Villin Headpiece Subdomain via Side Chain and Backbone Mutagenesis

Michelle R. Bunagan,[†] Jianmin Gao,[‡] Jeffery W. Kelly,^{*,‡} and Feng Gai^{*,†}

Department of Chemistry, University of Pennsylvania, 231 South 34th Street, Philadelphia, Pennsylvania 19104, and Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, BCC265, La Jolla, California 92037

Received March 10, 2009; E-mail: gai@sas.upenn.edu; jkelly@scripps.edu

Abstract: Backbone–backbone hydrogen bonds are a common feature of native protein structures, yet their thermodynamic and kinetic influence on folding has long been debated. This is reflected by the disparity between current protein folding models, which place hydrogen bond formation at different stages along the folding trajectory. For example, previous studies have suggested that the denatured state of the villin headpiece subdomain contains a residual helical structure that may provide a bias toward the folded state by confining the conformational search associated with its folding. Although helical hydrogen bonds clearly stabilize the folded state, here we show, using an amide-to-ester mutation strategy, that the formation of backbone hydrogen bonds within helices is not rate-limiting in the folding of the subdomain, thereby suggesting that such hydrogen bonds are unlikely to be formed en route from the denatured to the transition state. On the other hand, elimination of hydrogen bonds within the turn region elicits a slower folding rate, consistent with the hypothesis that these residues are involved in the formation of a folding nucleus. While illustrating a potentially conserved aspect of helix-turn-helix folding, our results further underscore the inherent importance of turns in protein supersecondary structure formation.

Introduction

Backbone–backbone hydrogen bonds (H-bond) are common and apparently important features of native protein structures,^{1–4} yet their role in folding transition state structure remains unclear. One possibility is that H-bonds result as a consequence of the formation of native side chain contacts during folding.⁵ Another possibility is that they play a more deciding role by providing a framework that directs the flow of folding toward the native state.⁶ To provide a better understanding of the kinetic influence of backbone–backbone H-bonds in protein folding, we investigate the role of helical H-bonding in the folding kinetics of the villin headpiece subdomain (HP-35) using the Φ -value analysis method in conjunction with amide-to-ester (A-to-E) backbone mutagenesis.^{7–15}

Φ -Value analysis¹⁶ is commonly used to examine the critical features of the folding transition state. The Φ -value arising from a specific mutation, which is calculated according to $\Phi_F = \Delta\Delta G^\ddagger / \Delta\Delta G_F$, where $\Delta\Delta G^\ddagger$ is the change of the folding free energy barrier and $\Delta\Delta G_F$ is the change of the folding free energy, provides information regarding the extent to which the native contacts contribute to the stability of the folding transition state (Figure 1). However, using traditional side chain mutagenesis-based Φ -value analysis to definitively assess the kinetic role of backbone H-bonding in protein folding is difficult, if not impossible. Thus, the recently established A-to-E backbone mutagenesis approach^{7–15} was applied to eliminate specific backbone–backbone hydrogen bonds. While an ester lacks a H-bond donor, it is an ideal steric and electronic mimic of the amide group, exhibiting nearly identical bond lengths and angles, and strongly favoring a planar, trans-conformation, although the cis–trans barrier is significantly lower.¹²

[†] University of Pennsylvania.

[‡] The Scripps Research Institute.

- (1) Pauling, L.; Corey, R. B.; Branson, H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1951**, *37*, 205–211.
- (2) Pauling, L.; Corey, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1951**, *37*, 729–740.
- (3) Dill, K. A. *Biochemistry* **1990**, *29*, 7133–7155.
- (4) Baldwin, R. L. *J. Biol. Chem.* **2003**, *278*, 17581–17588.
- (5) Fersht, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10869–10873.
- (6) Kim, P. S.; Baldwin, R. L. *Annu. Rev. Biochem.* **1982**, *51*, 459–489.
- (7) Koh, J. T.; Cornish, V. W.; Schultz, P. G. *Biochemistry* **1997**, *36*, 11314–11322.
- (8) Belligere, G. S.; Dawson, P. E. *J. Am. Chem. Soc.* **2000**, *122*, 12079–12082.
- (9) Blankenship, J. W.; Balambika, R.; Dawson, P. E. *Biochemistry* **2002**, *41*, 15676–15684.

- (10) Deechongkit, S.; Dawson, P. E.; Kelly, J. W. *J. Am. Chem. Soc.* **2004**, *126*, 16762–16771.
- (11) Deechongkit, S.; Nguyen, H.; Powers, E. T.; Dawson, P. E.; Grubele, M.; Kelly, J. W. *Nature* **2004**, *430*, 101–105.
- (12) Powers, E. T.; Deechongkit, S.; Kelly, J. W. *Adv. Pro. Chem.* **2006**, *72*, 39–78.
- (13) Yang, X. Y.; Wang, M.; Fitzgerald, M. C. *J. Mol. Biol.* **2006**, *363*, 506–519.
- (14) Wang, M.; Wales, T. E.; Fitzgerald, M. C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 2600–2604.
- (15) Gao, J.; Kelly, J. W. *Protein Sci.* **2008**, *17*, 1096–1101.
- (16) Matouschek, A.; Kellis, J. T., Jr.; Serrano, L.; Fersht, A. R. *Nature* **1989**, *340*, 122–126.

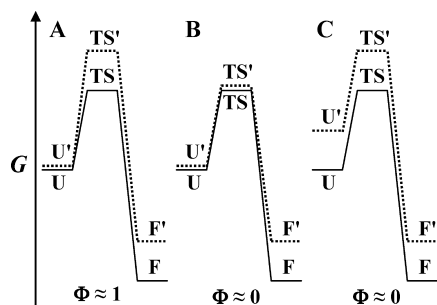


Figure 1. Schematic illustration of how an A-to-E mutation might change the free energy of the unfolded (U), transition (TS), and folded (F) states (solid lines, wild-type; dashed lines, mutants) in Φ -value analysis. (A) Elimination of a native H-bond that is also formed in the transition state, (B) elimination of a native H-bond that is not formed in the transition state, and (C) elimination of a native H-bond that is also formed in the unfolded state.

An A-to-E mutation within a residue that donated an NH to create a backbone–backbone H-bond is expected to increase the free energy of the folded state by 3 to 30 kJ/mol, the extent of destabilization increasing with the degree of desolvation of the perturbed H-bond in the folded state.¹² While an A-to-E mutation introduces a lone pair–lone pair repulsion between the noncarbonyl oxygen of the ester and what was the H-bond acceptor carbonyl oxygen of the amide, we have shown that this destabilization only amounts to ≈ 1 kJ/mol.¹⁵ The energetic preference for the transfer of an ester bond over an amide bond from the solvated unfolded ensemble to the folded state is determined by the extent to which the bond is desolvated in the folding process and the extent to which the proximal side chains contribute, although this difference is generally small, ~ 2 kJ/mol.¹⁵ Therefore, the $\Delta\Delta G_f^\ddagger$ resulting from an A-to-E mutation largely arises from removal of a native backbone–backbone H-bond that is absent or largely absent in the denatured state and the majority of this perturbation free energy does not arise from desolvation and O–O repulsion contributions, which are small in comparison. Thus, deletion of a H-bond that is formed in both the transition and folded states would be expected to have a large influence on the folding free energy barrier (ΔG^\ddagger) (Figure 1A). On the other hand, deletion of a H-bond that is only formed in the folded state would be expected to have little or no influence on ΔG^\ddagger (Figure 1B). Similarly, deletion of a H-bond that is also formed in the unfolded state and the transition state would be expected to have little or no influence on ΔG^\ddagger (Figure 1C). Therefore, A-to-E backbone mutagenesis in conjunction with Φ -value analysis should allow estimation of the extent of the formation of specific H-bonds within the folding transition state, thus providing a more comprehensive understanding of the folding mechanism of HP-35, as has been demonstrated for the Pin1 WW domain,^{10,11} the B domain of protein L and the P22 Arc repressor.¹³

There has been extensive study of the folding mechanism of HP-35 (Figure 2), a small helical protein¹⁷ whose folding is exceedingly fast.^{18–46} Several studies have suggested that the unfolded state retains residual native-like interactions and

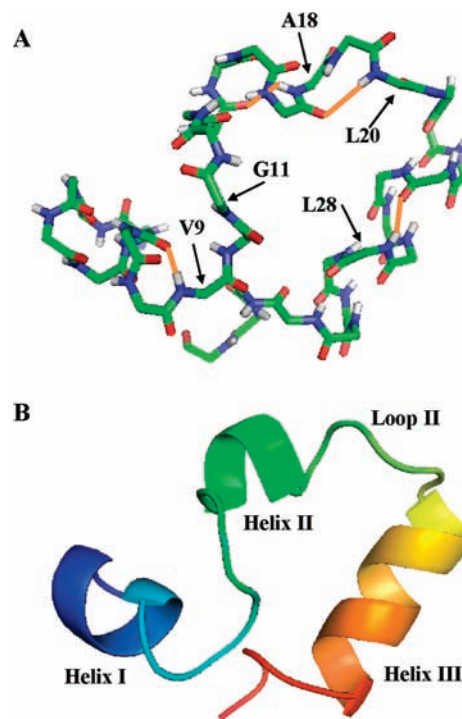


Figure 2. NMR structure of HP-35 (1 VII). The figure was generated using the program PyMol (<http://pymol.sourceforge.net/>).

structures, including tertiary contacts between residues forming the hydrophobic core and native helical segments.^{27,33,38,39} Although such features have been invoked to explain the ultrafast folding of HP-35 and other proteins,^{47–49} a recent kinetic study demonstrated that hydrophobic core formation is not rate-limiting for folding of HP-35.²⁸ In order to provide further insight into H-bond mediated helix and turn formation, herein we examine the folding thermodynamics and kinetics of the wild-type HP-35 and five single site A-to-E mutants (Figure

- (17) McKnight, C. J.; Doering, D. S.; Matsudaira, P. T.; Kim, P. S. *J. Mol. Biol.* **1996**, *260*, 126–134.
 (18) Duan, Y.; Wang, L.; Kollman, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9897–9902.
 (19) Frank, B. S.; Vardar, D.; Buckley, D. A.; McKnight, C. J. *Protein Sci.* **2002**, *11*, 680–687.
 (20) Islam, S. A.; Karplus, M.; Weaver, D. L. *J. Mol. Biol.* **2002**, *318*, 199–215.

- (21) Fernandez, A.; Shen, M.-Y.; Colubri, A.; Sosnick, T. R.; Berry, R. S.; Freed, K. F. *Biochemistry* **2003**, *42*, 664–671.
 (22) Jang, S. M.; Kim, E.; Shin, S.; Pak, Y. *J. Am. Chem. Soc.* **2003**, *125*, 14841–14846.
 (23) Kubelka, J.; Eaton, W. A.; Hofrichter, J. *J. Mol. Biol.* **2003**, *329*, 625–630.
 (24) Lin, C.-Y.; Hu, C.-K.; Hansmann, U. H. E. *Proteins* **2003**, *52*, 436–445.
 (25) Wang, M.; Tang, Y.; Sato, S.; Vugmeyster, L.; McKnight, C. J.; Raleigh, D. P. *J. Am. Chem. Soc.* **2003**, *125*, 6032–6033.
 (26) Ripoll, D. R.; Vila, J. A.; Scheraga, H. A. *J. Mol. Biol.* **2004**, *339*, 915–925.
 (27) Tang, Y.; Rigotti, D. J.; Fairman, R.; Raleigh, D. P. *Biochemistry* **2004**, *43*, 3264–3272.
 (28) Brewer, S. H.; Vu, D. M.; Tang, Y.; Li, Y.; Franzen, S.; Raleigh, D. P.; Dyer, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 16662–16667.
 (29) Buscaglia, M.; Kubelka, J.; Eaton, W. A.; Hofrichter, J. *J. Mol. Biol.* **2005**, *347*, 657–664.
 (30) Carr, J. M.; Wales, D. J. *J. Chem. Phys.* **2005**, *123*, 234901.
 (31) Chiu, T. K.; Kubelka, J.; Herbst-Irmer, R.; Eaton, W. A.; Hofrichter, J.; Davies, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7517–7522.
 (32) DeMori, G. M. S.; Colombo, G.; Micheletti, C. *Proteins* **2005**, *58*, 459–471.
 (33) Havlin, R. H.; Tycko, R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3284–3289.
 (34) Herges, T.; Wenzel, W. *Structure* **2005**, *13*, 661–668.
 (35) Bandyopadhyay, S.; Chakraborty, S.; Bagchi, B. *J. Chem. Phys.* **2006**, *125*, 084912.
 (36) Ensign, D. L.; Kasson, P. M.; Pande, V. S. *J. Mol. Biol.* **2007**, *374*, 806–816.
 (37) Kubelka, J.; Chiu, T. K.; Davies, D. R.; Eaton, W. A.; Hofrichter, J. *J. Mol. Biol.* **2006**, *359*, 546–553.

2). Mutations were chosen to perturb specific native-state H-bonds. Of the A-to-E mutations investigated, three eliminate helical hydrogen bonds, V9 ω in helix I, A18 α in helix II, and L28 λ in helix III, while G11 γ and L20 λ eliminate H-bonds formed in the loop between helices I and II and the turn between helices II and III, respectively. Additionally, side chain mutants T13A and Q25 nL (norleucine) remove side chain to backbone H-bonds, while mutant G11A reduces the flexibility of the loop and P21A reduces the rigidity of the turn in order to test the kinetic role of the conformational entropy in these two regions. Our results show no evidence of H-bond-mediated helix formation in the transition state, as determined via Φ -value analysis. Instead, our results suggest the loop and turn may act as nuclei for folding through a nucleation-condensation mechanism.

Materials and Methods

Peptide Synthesis and Purification. The wild-type and side chain variants of HP-35 were synthesized on an ABI 433A peptide synthesizer employing Fmoc/tBu chemistry. The ester mutants were prepared manually using Boc/benzyl strategy, during which the α -hydroxy acids were incorporated individually in place of the corresponding amino acids according to the previously published protocol.^{10–12} The α -hydroxy acids used in this study were purchased from commercial sources and used without further purification. All peptides were purified by reverse-phase HPLC, and the A-to-E HP-35 variants were further purified by size-exclusion chromatography employing a Superdex 30 column. Multiple rounds of lyophilization were performed against 0.1 M DCl to remove the trifluoroacetic acid from peptide purification and to allow for proton–deuterium exchange. Exchanged A-to-E samples were again characterized by mass spectroscopy to ensure that the acid had not hydrolyzed the ester bond. For circular dichroism (CD) and IR experiments, the samples were prepared by directly dissolving the lyophilized peptides in either 20 mM (CD) or 40 mM (IR) 2-(*N*-morpholino)ethanesulfonic acid (MES) D₂O buffers (pH* 5.5). The concentration of the sample was determined by absorbance at 280 nm, using an extinction coefficient of 5500 cm⁻¹ M⁻¹. The final concentration was ~40 μ M and ~1.5 mM for CD and IR, respectively.

CD Spectroscopy. Both CD wavelength scans and thermal melting curves were collected on an Aviv model 202SF circular dichroism spectrometer equipped with a cell holder with a Peltier temperature controller (Hellma, Forest Hills, NY). Far-UV CD spectra were recorded from 200 to 250 nm at 2 °C. The peptide

sample was dissolved in 20 mM MES (pH 5.5). Thermal denaturation was monitored at 222 nm over the temperature range of 2–98 °C with a 2 °C step size and a 90 s equilibration time. The signal was averaged for 30 s at each temperature. After the highest temperature was reached, the sample was cooled to 2 °C and another full CD spectrum was measured to ensure that folding was reversible. The folding-unfolding thermodynamics of each peptide were obtained by fitting its CD thermal melting transition obtained at 222 nm to the following two-state model:

$$\theta(T) = \frac{\theta_F(T) + K_{eq}(T) \times \theta_U(T)}{1 + K_{eq}(T)} \quad (1)$$

$$K_{eq}(T) = \exp(-\Delta G(T)/RT) \quad (2)$$

$$\Delta G(T) = \Delta H_m + \Delta C_p \cdot (T - T_m) - T[\Delta S_m + \Delta C_p \cdot \ln(T/T_m)] \quad (3)$$

where $K_{eq}(T)$ is the equilibrium constant for unfolding, $T_m = \Delta H_m / \Delta S_m$ is the thermal melting temperature, ΔH_m and ΔS_m are the enthalpy and entropy changes at T_m , respectively, and ΔC_p is the heat capacity change associated with unfolding, which has been assumed here to be temperature independent. Since all backbone and side chain mutations studied here are not expected to significantly change the hydrophobicity of the protein, ΔC_p was treated as a global fitting parameter. In addition, the folded and unfolded CD baselines $\theta_F(T)$ and $\theta_U(T)$ are assumed to be linear functions of temperature, as follows:

$$\theta_F(T) = m + n \times T \quad (4)$$

$$\theta_U(T) = p + q \times T \quad (5)$$

During fitting, n and q were treated as global parameters.

FT-IR Measurements. FT-IR spectra were collected on a Magna-IR 860 spectrometer (Nicolet, WI) equipped with a HgCdTe detector using a spectral resolution of 2 cm⁻¹. A homemade, two-compartment, 52 μ m CaF₂ sample cell mounted on a programmable translation stage was used to allow separate and alternate measurements of the single-beam spectra of the sample and reference under identical conditions. Temperature control with ± 0.2 °C precision was achieved through a thermostatted copper block and a temperature bath. The reported spectra correspond to the average of 256 scans.

Infrared T-Jump Kinetic Measurements. The time-resolved *T*-jump IR apparatus used in the current study has been described in detail elsewhere.⁵⁰ Briefly, a 3 ns laser pulse centered at about 1.9 μ m was used to generate a *T*-jump in the laser interaction volume, and the *T*-jump induced transient absorbance change of the sample was measured by a continuous wave (CW) IR diode laser in conjunction with a 50 MHz MCT detector. In all cases, the observed relaxation kinetics were well described by a double exponential. The fast component, which was too fast to be resolved, was attributed to imperfect background subtraction and temperature-dependent spectral shifting.⁵¹ The slower component was attributed to the conformational redistribution kinetics of the peptide. By assuming that the *T*-jump induced relaxation of HP-35 and the mutants occurs between two states, the corresponding folding (k_f) and unfolding (k_u) rate constants were further evaluated using the relaxation rate constant (k_R) and the equilibrium constant (K_{eq}):

$$k_R = k_f + k_u \quad (6)$$

$$K_{eq} = k_f/k_u \quad (7)$$

Φ -value Analysis. The Φ -values were calculated using the following equation:

- (38) Tang, Y.; Goger, M. J.; Raleigh, D. P. *Biochemistry* **2006**, *45*, 6940–6946.
 (39) Wickstrom, L.; Okur, A.; Song, K.; Hornak, V.; Raleigh, D. P.; Simmerling, C. L. *J. Mol. Biol.* **2006**, *360*, 1094–1107.
 (40) Brewer, S. H.; Song, B.; Raleigh, D. P.; Dyer, R. B. *Biochemistry* **2007**, *46*, 3279–3285.
 (41) Lei, H.; Duan, Y. *J. Mol. Biol.* **2007**, *370*, 196–206.
 (42) Lei, H.; Wu, C.; Liu, H.; Duan, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 4925–4930.
 (43) Chen, J.; Brooks, C. L. *Phys. Chem. Chem. Phys.* **2008**, *10*, 471–481.
 (44) Godoy-Ruiz, R.; Henry, E. R.; Kubelka, J.; Hofrichter, J.; Munoz, V.; Sanchez-Ruiz, J. M.; Eaton, W. A. *J. Phys. Chem. B* **2008**, *112*, 5938–5949.
 (45) Kubelka, J.; Henry, E. R.; Cellmer, T.; Hofrichter, J.; Eaton, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 18655–18662.
 (46) Yang, J. S.; Wallin, S.; Shakhnovich, E. I. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 895–900.
 (47) Mayor, U.; Guydosh, N. R.; Johnson, C. M.; Grossman, J. G.; Sato, S.; Jas, G. S.; Freund, S. M. V.; Alonso, D. O. V.; Daggett, V.; Fersht, A. R. *Nature* **2003**, *421*, 863–867.
 (48) Zhu, Y.; Alonso, D. O. V.; Maki, K.; Huang, C.-Y.; Lahr, S. J.; Daggett, V.; Roder, H.; DeGrado, W. F.; Gai, F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15486–15491.
 (49) Mok, K. H.; Kuhn, L. T.; Goetz, M.; Day, I. J.; Lin, J. C.; Andersen, N. H.; Hore, P. J. *Nature* **2007**, *447*, 106–109.

- (50) Du, D. G.; Bunagan, M. R.; Gai, F. *Biophys. J.* **2007**, *93*, 4076–4082.
 (51) Mukherjee, S.; Chowdhury, P.; Gai, F. *J. Phys. Chem. B* **2007**, *111*, 4596–4602.

$$\Phi_F = \frac{RT \ln \left(\frac{\tau_{f,\text{mut}}}{\tau_{f,\text{wt}}} \right)}{\Delta \Delta G_{\text{mut-wt}}} \quad (8)$$

where τ_f is the folding time constant, the subscripts “mut” and “wt” represent the mutant and wild-type, respectively, R is the gas constant, T is the temperature, and $\Delta \Delta G_{\text{mut-wt}} = \Delta G_{\text{mut}} - \Delta G_{\text{wt}}$.

Results and Discussion

Wild-type HP-35 and nine single site variants were synthesized using standard solid-phase peptide synthesis protocols. In the case of the A-to-E mutants, specific α -amino acid residues were replaced by their α -hydroxy acid counterparts, which are commercially available.^{10–12} Following previously used nomenclature,^{10–12} all α -hydroxy acid residues are denoted by lowercase Greek letters that correspond to the single letter code of the corresponding α -amino acid residues. A 1D ^1H NMR spectrum was collected at 10 °C for each HP-35 sequence studied. In every case, the ^1H amide NH and aromatic regions of the NMR spectra exhibit well dispersed resonances similar to those of wild-type HP-35 (Figure S1 in Supporting Information), providing evidence that A-to-E mutations do not alter the integrity of the HP-35 fold.

Equilibrium Study. The far-UV CD spectra of wild type HP-35 and its mutants all exhibit double minima at 208 and 222 nm, characteristic of α -helical proteins.¹⁷ Temperature dependent CD measurements further indicate that all of the HP-35 sequences undergo a cooperative thermal unfolding transition, as determined by monitoring the increase in ellipticity at 222 nm (Figure S2 in Supporting Information). The thermodynamic folding parameters were extracted by globally fitting all CD thermal denaturation curves to a two-state model (Table S1 in Supporting Information). All of the A-to-E mutations are destabilizing relative to wild-type HP-35, which is consistent with these H-bonds being formed in the folded state, but not the denatured state. For example, L28 λ eliminates a H-bond and weakens an additional H-bond owing to the ester carbonyl being a weaker acceptor. The L20 λ HP-35 variant, which eliminates a H-bond between the amide NH of L20 and the backbone carbonyl of F17 in wild-type HP-35, is the least stable A-to-E variant (Table S1 in Supporting Information), underscoring the thermodynamic importance of the H-bond-mediated loop sandwiched between these two residues. A collective look at the perturbation free energies reveals that all H-bonds do not contribute equally to the native state stability, as observed previously.^{9–11,14}

The thermal unfolding transition of HP-35 and its single site variants was also studied using FT-IR spectroscopy. The amide I' band of folded HP-35, which arises mainly from the backbone C=O stretching vibrations, is centered at $\sim 1645\text{ cm}^{-1}$ at 5.6 °C (Figure S3 in Supporting Information). Increasing temperature shifts the peak position toward higher wavenumbers. Consequently, the FT-IR difference spectra of HP-35, calculated by subtracting the 5.6 °C spectrum from those obtained at higher temperatures, exhibits a negative feature centered around 1632 cm^{-1} and a broad positive feature centered at $\sim 1675\text{ cm}^{-1}$. The former results primarily from loss of α -helical conformations with increasing temperature, whereas the latter is due to the concurrent formation of a nonhelical conformational ensemble.⁵² Since temperature-induced variations in backbone solvation also shift the amide I' band toward higher frequency and thus

contribute to the aforementioned spectral changes,⁵¹ we did not attempt to quantify the thermal denaturation of HP-35 or the mutants using the amide I' band. However, these spectral features are useful conformational markers and have been used in the subsequent kinetic studies to monitor the conformational relaxation kinetics of HP-35 and its mutants in response to a T -jump. In addition, the temperature dependent FT-IR spectra of all the peptides except P21A, indicate that under the current experimental conditions (for both equilibrium and kinetic studies), no detectable aggregates are formed. Antiparallel β -sheet aggregates are known to give rise to a pair of characteristic sharp bands, centered at ~ 1620 and $\sim 1680\text{ cm}^{-1}$.⁵³

T-Jump Kinetics. The folding-unfolding (or relaxation) kinetics of these peptides were studied by the T -jump IR technique, which has been described in detail elsewhere.⁵⁰ As shown (Figure S4 in Supporting Information), the relaxation (or global folding-unfolding) kinetics of HP-35 in response to a T -jump of 54.5–59.9 °C, probed at 1632 cm^{-1} , indicate that the single-exponential conformational re-equilibration of HP-35 occurs on the microsecond time scale, in agreement with observations of previous NMR line shape experiments,²⁵ T -jump IR,^{28,40} T -jump fluorescence,^{23,31,37} and fluorescence quenching²⁹ studies of this system. While a three-state model has been invoked to describe the folding dynamics of HP-35,^{40,45} Eaton and co-workers have shown that the intermediate state is located on the folded side of the major free-energy barrier and its relaxation takes place on a much faster time scale than the global folding-unfolding transition, and thus a simple two-state treatment is adequate for Φ -value analysis.⁴⁵ As shown (Figure 3), the folding rate constant of HP-35, obtained using eqs 6 and 7 (methods section), is relatively insensitive to temperature over the temperature range investigated (48–85 °C), which is also consistent with previous studies.^{23,28,31,37,40} The T -jump induced relaxation kinetics of the A-to-E backbone and side chain mutants are similar to those of the wild-type. In spite of their decreased conformational stability, most of these mutants exhibit a folding rate that is either similar to or only slightly slower than that of wild-type HP-35 (Figure 3). Interestingly, the slowest folder is mutant L20 λ , which is also the most destabilized relative to the wild-type, and it has a folding time constant that is more than three times that of HP-35 at 50 °C (i.e., 23.9 versus 7.4 μs).

Implications for the Folding Mechanism of HP-35. To assess whether specific native-state interactions are also formed in the folding transition state, we have further determined the Φ -value for each mutant at 50 °C. As shown (Table 1), none of the mutations lead to a Φ -value >0.5 , suggesting that most of the native interactions perturbed by these mutations do not contribute significantly to the transition state ensemble structure of HP-35. In particular, each of the A-to-E mutations made in the helical regions, namely V9 ω A18 α , and L28 λ , exhibit a Φ -value of essentially zero, indicating that the helical H-bonds perturbed by these mutations are most likely formed after the folding transition state of HP-35. A zero or near-zero Φ -value could also result from A-to-E elimination of a helical H-bond that exists in both the unfolded and transition states (Figure 1C). However, such a scenario would not elicit the observed destabilization of the folded state (removing the H-bond donor would destabilize both the unfolded and folded states leading to little change in ΔG_f). In fact, the magnitude of $\Delta \Delta G_f$ for

(52) Barth, A.; Zscherp, C. *Q. Rev. Biophys.* **2002**, *35*, 369–430.

(53) Miyazawa, T.; Blout, E. R. *J. Am. Chem. Soc.* **1961**, *83*, 712–719.

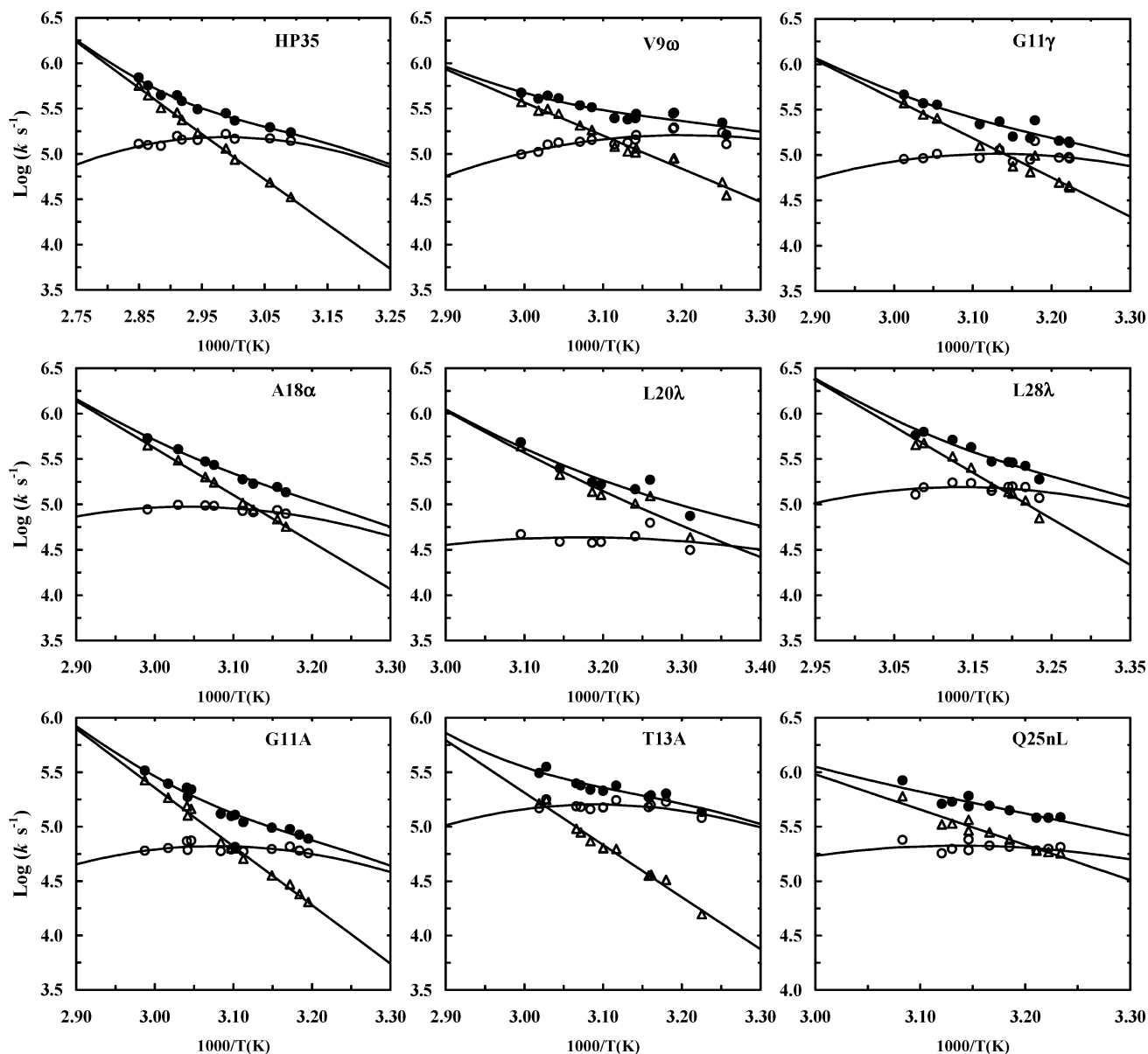


Figure 3. Arrhenius plots of the observed relaxation rate constant (solid circles) as well as the folding (open circles) and unfolding (open triangles) rate constants for wild-type HP-35 and mutants, as indicated. Lines are fits to the Eyring equation.

Table 1. Summary of Thermodynamic and Kinetic Data for Wild-Type HP-35 and Mutants Obtained at 50 °C

	HP35	V9 ω	G11 γ	A18 α	L20 λ	L28 λ	G11A	T13A	Q25 nL
ΔG_f (kJ/mol)	-3.9	0.4	1.2	1.0	6.0	2.8	0.2	-2.1	2.2
$\Delta\Delta G_f$ (kJ/mol)*	0	4.3	5.1	4.9	9.9	6.7	4.1	1.8	6.1
τ_f (μ s)	7.4	7.0	9.8	10.9	23.9	6.6	15.1	6.2	4.8
Φ_f		-0.04	0.15	0.20	0.32	-0.05	0.47	-0.26	-0.19

* $\Delta\Delta G_f = \Delta G_{f, \text{mutant}} - \Delta G_{f, \text{HP-35}}$.

each A-to-E mutation is consistent with previous estimates of H-bond energies (Table 1).^{7–12}

Previous studies have shown that the unfolded or denatured state of HP-35 contains native-like interactions and structural elements,^{27,33,38,39} including hydrophobic contacts²⁷ between Phe residues that are critical to the native stability and helical structures.¹⁹ As has been suggested in the ultrafast folding of other small proteins,^{47–49} the presence of such residual structure might prompt one to assume that the denatured state of HP-35

is structurally biased toward the native state, thus leading to very fast folding. However, the low Φ -values of the A-to-E variants indicate that residual H-bonded helical content in the denatured state of HP-35, which has been estimated to be as high as 25% for helix II,³³ has little, if any, effect on its folding rate. Consistent with this picture, the recent isotope-edited *T*-jump IR study of Brewer et al.⁴⁰ indicates that helix II may unfold prior to the global unfolding of the protein. In other words, this helix is formed on the native side of the major folding free energy barrier. Their findings are consistent with our data, which suggest that most of the helical H-bonds of HP-35 have not yet been formed in the transition state. Brewer et al.²⁸ also demonstrate that modulating hydrophobic interactions in the denatured state of HP-35 also yields little change in its folding kinetics.

Taken together, these results suggest a folding mechanism for HP-35 wherein the formation of the secondary structures and the hydrophobic core is not rate-limiting. Instead, folding is likely initiated by assembling one of the two loops, as

observed in the folding of other proteins.^{11,54–57} The strongest experimental evidence supporting this hypothesis derives from the folding energetics exhibited by the L20 λ variant, which lacks the native (*i*, *i*+3) H-bond formed between the backbone carbonyl of F17 and the backbone NH of L20.¹⁸ This A-to-E variant folds more than three times slower than the wild-type. It is highly unlikely that the *i*, *i*+3 H-bond, which aids in defining the turn between helices II and III in the wild-type protein, is formed in the denatured state, as it is very important to the stability of the native state, as is evident from the large perturbation free energy of the L20 λ variant (Table 1). If this turn is indeed important in initiating folding of HP-35, one would expect P21 to also play a critical role by restricting movement of the interhelical region, as suggested by a recent folding simulation study.⁴¹ However, the FT-IR data of P21A (Figure S3 in Supporting Information) show that it is prone to aggregate, indicating that its folding free energy landscape is drastically different from that of the wild-type, as well as the other mutants of HP-35. While the aggregation propensity of P21A renders Φ -value analysis inconclusive, that P21 is an important residue in the folding of chicken HP-35 demonstrates the importance of this turn. In fact, NMR studies^{58,59} have shown that the human villin headpiece subdomain is unable to fold to its native structure when P21, which is conserved in the headpiece sequences of many F-actin binding cytoskeletal proteins,⁶⁰ is substituted by alanine.

The Q25 nL variant exhibits a negative Φ -value (Table 1). In the native state of HP-35, Q25 forms side chain to backbone H-bonds with the carbonyls of L20 and A18, which facilitate the productive docking of helices II and III. Thus, it is not surprising that eliminating these native H-bonds decreases the stability of the folded state. What is interesting, however, is that the resultant mutant folds faster than the parent protein (Table 1), in consequence yielding a negative Φ -value. Although the interpretation of negative Φ -values is often not straightforward and is in fact debated,⁶¹ we interpret the faster folding rate of Q25 nL as most likely arising from mutation and hence disruption of certain non-native H-bonds in the unfolded state. Destabilization of the unfolded state would consequently decrease the folding free energy barrier of this mutant. Eaton and co-workers^{31,37} have similarly shown that mutants K24 nL and K24 nL/K29 nL also fold faster than the wild-type protein.

Excluding G11A, mutations in the first loop (i.e., G11 γ , and T13A) do not change the folding rate significantly (the Φ -value of T13A is expected to have a large uncertainty because of the small $\Delta\Delta G_f$), indicating that this region is relatively unstructured in the folding transition state. The exception is G11A which exhibits a folding time which is twice that of the wild-type peptide. Position 11 is largely solvent exposed, and the mutation from Gly to Ala is unlikely to remove or modulate any

interactions of the side chain. However, since Gly is the most flexible amino acid, it is likely that mutation to Ala does reduce the conformational freedom of the loop, as indicated by the disparity between these two residues in their preferences for the native dihedral angles at position 11.⁶² However, it may not be the difficulty of attaining the native state conformation which restricts and slows folding, but rather the loss of loop flexibility throughout the folding process. The Gly flexibility may be important in allowing the loop to be dynamic, giving helix I the ability to dock against helices II and III without conformational restraints from the backbone. This idea is consistent with the results of *ab initio* folding of HP-35 by Lei and Duan.⁴¹ A similar scenario has also been suggested by Fersht and co-workers⁶³ in their description of the mechanistic role of the turn in the folding of the WW domain.

Taken together, our results suggest that HP-35 folds via an early transition state wherein very few native contacts have been formed. While the current study alone does not rule out other possibilities, for example a small Φ -value could arise from a heterogeneous transition state ensemble the population of which shifts in response to a destabilizing mutation, the above picture is consistent with the fast folding behavior of HP-35 as well as the results of other mutational studies.⁴⁵ For example, Dyer and co-workers²⁸ have shown that the folding rate of mutant F6L/F10L is similar to that of the wild-type, although these mutations destabilize the hydrophobic core. Similarly, Eaton and co-workers^{23,31} have shown that the destabilized mutants F35A, A18S, and A18 V also fold with a rate that is similar to that of the wild-type and the Φ -values are unusually low at 310 K.⁴⁵ Besides L20 λ and G11A, other known mutants exhibiting significantly different folding times relative to wild-type HP-35 are K24 nL and K24 nL/K29 nL.^{31,37} Thus, these results indicate that neither hydrophobic core nor secondary structure formation provide a framework for fast folding of HP-35. Instead, folding is most likely initiated by the formation of a folding nucleus involving a turn between helices II and III.

The small size and fast folding kinetics of HP-35 have also made this subdomain an attractive model system for computational studies.^{18,20–22,24,26,30,32,34–36,39,41–43,46} Interestingly, however, those computer simulations have revealed somewhat different folding mechanisms. For instance, some have identified specific tertiary interactions as being critical for folding, while others have observed early helix formation. While our experimental results are in part consistent with the molecular dynamics simulations of Duan and co-workers,^{41,42} which identified formation of the turn between helices II and III as rate-limiting for HP-35 folding, additional mutational studies, especially those involving residues in the turn or loop regions, as well as experiments capable of revealing the role of the solvent, are warranted in order to provide a more complete view of the folding mechanism of HP-35. Moreover, analysis of the relaxation kinetics of wild-type HP-35 and HP-35 variants using other theoretical models, such as those based on Langevin dynamics simulations,^{64,65} may provide further insights into the folding energy landscape of this protein. Finally, in light of the

(54) Du, D.; Zhu, Y.; Huang, C.-Y.; Gai, F. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15915–15920.

(55) Du, D.; Gai, F. *Biochemistry* **2006**, *45*, 13131–13139.

(56) Du, D.; Tucker, M. J.; Gai, F. *Biochemistry* **2006**, *45*, 2668–2678.

(57) Li, H.; Frieden, C. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 11993–11998.

(58) Vermeulen, W.; Van Troys, M.; Bourry, D.; Dewitte, D.; Rossenu, S.; Goethals, M.; Borremans, F. A. M.; Vanderkerckhove, J.; Martins, J. C.; Ampe, C. *J. Mol. Biol.* **2006**, *359*, 1277–1292.

(59) Piana, S.; Laio, A.; Marinelli, F.; Van Troys, M.; Bourry, D.; Ampe, C.; Martins, J. C. *J. Mol. Biol.* **2008**, *375*, 460–470.

(60) Vermeulen, W.; Vanhaesebrouck, P.; Van Troys, M.; Verschueren, M.; Fant, F.; Goethals, M.; Ampe, C.; Martins, J. C.; Borremans, F. A. M. *Protein Sci.* **2004**, *13*, 1276–1287.

(61) Merlo, C.; Dill, K. A.; Weikl, T. R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 10171–10175.

(62) Scott, K. A.; Alonso, D. O. V.; Sato, S.; Fersht, A. R.; Daggett, V. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 2661–2666.

(63) Sharpe, T.; Jonsson, A. L.; Rutherford, T. J.; Daggett, V.; Fersht, A. R. *Protein Sci.* **2007**, *16*, 2233–2239.

(64) Xu, Y.; Purkayastha, P.; Gai, F. *J. Am. Chem. Soc.* **2006**, *128*, 15836–15842.

(65) Liu, F.; Dumont, C.; Zhu, Y. J.; DeGrado, W. F.; Gai, F.; Gruebele, M. *J. Chem. Phys.* **2009**, *130*, 061101.

backbone-based theories of protein folding,⁶⁶ it also would be interesting to investigate whether the formation of helical H-bonds in other proteins are rate-limiting for folding.

Conclusions

Using a small helical protein, the villin headpiece subdomain (HP-35), as a model, we demonstrated that amide-to-ester backbone mutagenesis is capable of revealing the role of individual backbone H-bonds in the folding transition state of a helical protein. This approach, when used in conjunction with traditional Φ -value analysis, is capable of providing new insight into the microscopic details of protein folding. For HP-35, our backbone mutagenesis studies show that removal of native helical H-bonds does not appreciably change the folding rate, but does significantly alter stability, suggesting that H-bond-mediated helix formation largely occurs after the transition state is surmounted. Interestingly, backbone mutagenesis of L20, eliminating an (*i*, *i*+3) H-bond located in the second loop,

dramatically slows the folding rate, which implies that the turn between helices II and III is involved in the formation of a folding nucleus. Integrating our data with that of others indicates that the folding transition state of HP-35 does not contain a significant amount of native tertiary contacts and/or H-bonded helices, but may contain a H-bonded loop, which is consistent with the relatively small folding free energy barrier⁴⁴ and thus the ultrafast folding kinetics of HP-35.

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Supporting Information Available: Thermodynamic unfolding parameters, ¹H NMR spectra, and CD melting curves for all of the mutants, as well as FT-IR data for wild-type HP-35 and P21A and representative *T*-jump relaxation kinetics for wild-type HP-35 and A18 α . This material is available free of charge via the Internet at <http://pubs.acs.org>.

(66) Rose, G. D.; Fleming, P. J.; Banavar, J. R.; Maritan, A. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 16623–16633.

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