Protein Models

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Engineering Enhanced Protein Stability through β-Turn Optimization: Insights for the Design of Stable Peptide β-Hairpin Systems**

*Emma R. Simpson, Jill K. Meldrum, Roger Bofill, Maria D. Crespo, Elizabeth Holmes, and Mark S. Searle**

β-Hairpins are a common component of protein structure and represent the smallest increments of protein β-sheets that are accessible through peptide model systems for studying sequence-dependent folding and stability.[1] A number of model hairpin systems are proving useful vehicles for quantitative studies of noncovalent interactions and for the analysis of the cooperative assembly of multistranded sheets. The choice of β-turn plays a key role in dictating β-strand alignment and stability, and these models have evolved around a few key turn sequences that are found to be statistically abundant in protein β-hairpins.[1] In more general terms, not only have β-turns and β-hairpins been implicated in nucleating protein folding and accelerating folding kinetics[2] but they also constitute binding epitopes in protein–protein and protein–nucleic acid recognition.[3,4] They have been used in the design of small peptide-based protease inhibitors, as recognition motifs in HIV therapeutics, as structured peptides that trigger a specific immune response as the basis for the design of vaccines,[5] and as novel minimalist peptide–DNA recognition scaffolds.[6] Protein engineering studies have shown that the rational redesign of, for example, protein secondary structure and surface charge distribution can lead to enhanced protein stability,[7] which may be a desirable feature for proteins of potential use in a range of biotechnological applications or in the large-scale use of enzymes in

[*] E. R. Simpson, J. K. Meldrum, Dr. R. Bofill,[+] Dr. M. D. Crespo,[++] E. Holmes, Prof. M. S. Searle
Centre for Biomolecular Sciences
University Park, University of Nottingham
Nottingham NG72RD (UK)
Fax: (+44) 115-951-3564
E-mail: mark.searle@nottingham.ac.uk

[+] Current address:
Unitat de Química Inorgànica
Departament de Química
Universitat Autònoma de Barcelona
08193 Bellaterra-Catalonia (Spain)

[++] Current address:
Biozentrum der Universitat Basel
Division of Biophysical Chemistry
Klingelbergstrasse 70, 4056 Basel (Switzerland)

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synthesis. Proteins have been shown to be tolerant to large sequence variability in turn regions, with stabilities modulated according to loop size and geometry.[8] Similarly, the population of folded β-hairpin peptides has been shown to be particularly sensitive to small changes in sequence, although quantitative analysis has proved more problematic.[1] With a view to improving the rational design of model peptide systems, we have determined the contribution to β-hairpin stability of the commonly occurring type I′ turn by using native ubiquitin as a "host" system. Herein, we present some energetic guidelines for the optimization of β-turn stability in peptide-based model systems.

High-resolution structures have shown that two-residue type I′ β-turns are particularly abundant in protein β-hairpins.[9] The turn geometry strongly complements the right-handed twist of the antiparallel β-sheet. Following the nomenclature of Sibanda and Thornton,[9] the turn is described by the sequence (−B1)-(L1)-(L2)-(+B1), where −B1 and +B1 are the β-strand residues preceding and following the turn, and L1 and L2 are the turn residues. The backbone ϕ and ψ angles of L1 and L2 both lie in the left-handed α-helical (L-α) region of the Ramachandran plot. In an analysis of 512 high-resolution structures we identified 408 two-residue type I′ turn sequences and were able to demonstrate clear residue preferences at the various sites,[10] in agreement with earlier studies that used a smaller database.[11] The data showed that Gly is by far the most abundant residue at position L2 (308 out of 408 cases) as the required backbone angles ($\phi \approx 90°$ and $\psi \approx 0°$) are disallowed for all residues other than Gly.[11] At position L1, the dihedral angles are accessible to other non-β-branched residues, with Asn, Asp, and Gly appearing in around 60% of cases, with Asn preferred. In contrast, Val, Ile, and Thr are rarely found in this position. On this basis the Asn-Gly type I′ turn has been widely used in model hairpin peptide systems for stability and folding studies and has proved highly effective at inducing folded structures.[1] In other cases, the use of D-Pro at position L1 to induce the correct L-α backbone geometry has also proved highly successful.[1]

To examine the influence of β-turn sequences on hairpin stability, we used the N-terminal 17-residue β-hairpin of native ubiquitin as a "host" for mutational studies (Figure 1a).[12–14] By using a fluorescent ubiquitin construct with a background F45W mutation (which we subsequently term wild-type ubiquitin, **WT***),[15–17] we engineered a series of turn mutations into the ubiquitin hairpin to examine quantitatively the effects on protein stability (Figure 1b). In the X-ray crystal structure of native ubiquitin,[12] the first and last of the five residues of the TLTGK loop close the native 3:5 type I G-bulged turn through cross-strand hydrogen bonding. We initially mutated the native turn sequence to the more flexible TGGGK loop (**G3** mutant) without affecting these closing residues. Protein stability was determined through fluorescence-detected chemical denaturation methods using guanidinium chloride (GdmCl). Sigmoidal unfolding curves were fitted to a two-state model from which transition midpoints (c_m), denaturant m values, and free energies of folding were determined (see Table 1).[18] Using this approach, **WT*** was observed to unfold with a transition midpoint (c_m) of 2.68 M GdmCl at pH 5.0 in acetate buffer (25 mM) at 298 K (Figure 2) from which we estimate a free energy of folding of $-27.3(\pm 0.6)$ kJ mol^{-1} (see Table 1). Strikingly, the **G3** mutant appears to be marginally more stable ($\Delta\Delta G \approx -1.6$ kJ mol^{-1}) despite the enhanced flexibility of the Gly-rich turn sequence and loss of side-chain interactions across the loop sequence.

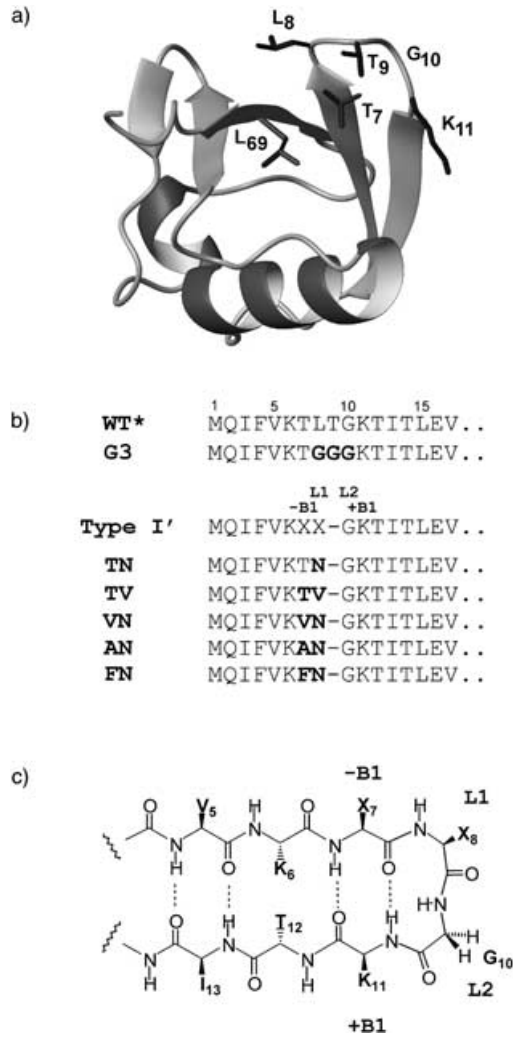

Figure 1. a) X-ray crystal structure of ubiquitin (1UBQ[12]) showing the solvent-exposed TLTGK type I G-bulged turn at the end of the N-terminal β-hairpin sequence. The position and orientation of the side chains of the β-turn residues T_7, L_8, T_9, and K_{11}, and also the L_{69} side chain on the C-terminal β-strand are also illustrated. b) Sequences of ubiquitin mutants containing type I′ β-turn substitutions. Only the N-terminal 17 residues that encompass the β-hairpin are illustrated. c) Two-residue type I′ β-turn conformation showing the mutated residues X_7 and X_8 and cross-strand hydrogen-bonding interactions. The residue numbering in (b) follows that in the **WT*** sequence (residue 9 has been deleted in the mutants). Residues X_7, X_8, G_{10}, and K_{11} represent turn residues −B1, L1, L2, and +B1, respectively, according to the nomenclature of Sibanda and Thornton (ref. [9]).

Subsequently, we replaced the native loop sequence (TLTGK) with the type I′ turn sequence (TXGK) by substituting LT with residue X at position L1 (Figure 1c).[9] We considered residues at position X which occur with either high frequency (X = Asn) or low frequency (X = Val) in protein β-

Table 1: Thermodynamic data for the ubiquitin mutants derived from chemical denaturation experiments.

Mutant	Transition midpoint (c_m) [M][a]	ΔG_{eq} [kJ mol^{-1}][b]	$\Delta\Delta G$ (mut–WT*) [kJ mol^{-1}]
WT*	2.68	-27.3 ± 0.6	0
G3	2.84	-28.9 ± 0.7	-1.6 ± 0.9
TN	2.83	-28.8 ± 0.6	-1.5 ± 0.9
TV	2.54	-25.9 ± 0.6	$+1.4 \pm 0.9$
VN	3.33	-33.9 ± 0.8	-6.6 ± 1.0
AN	2.58	-26.3 ± 0.6	$+1.0 \pm 0.9$
FN	3.78	-38.5 ± 0.9	-11.2 ± 1.1

[a] Transition midpoints (c_m, 50% unfolded protein relative to [GdmCl]) were determined by fluorescence measurements at 298 K on 1.0 μM solutions of protein in acetate buffer (25 mM) at pH 5.0; fitting errors are typically less than 0.02 M. [b] Equilibrium stabilities calculated using $m = 10.18$ kJ mol^{-1} M^{-1} (with a standard error of ± 0.23 kJ mol^{-1} M^{-1}).

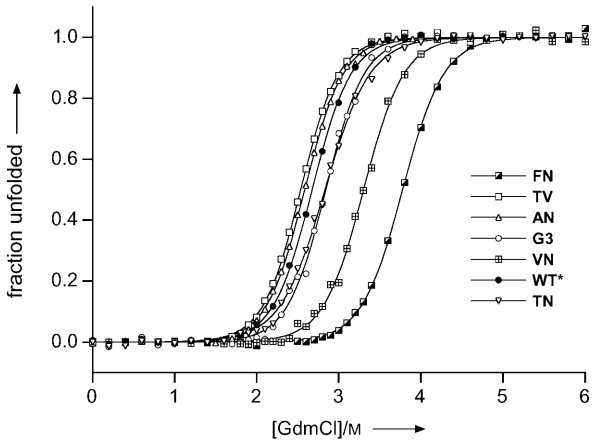

Figure 2. a) Guanidinium chloride (GdmCl) denaturation curves for the family of β-turn mutants measured at 298 K and pH 5.0 in acetate buffer (25 mM). Unfolding transitions were measured by fluorescence changes at $\lambda = 358$ nm, and the data were converted into the fraction unfolded versus the concentration of GdmCl. Stabilities were determined using the linear extrapolation method by fitting the curves to a two-state unfolding model to determine the midpoint denaturant concentration (c_m) and the slope (m value). Stability data are shown in Table 1.

hairpins (**TN** and **TV** mutants, respectively) while retaining the same β-strand residues present in the **WT*** sequence at positions −B1 and +B1.[10] The requirement for ϕ and ψ angles at position L1 which lie in the left-handed α-helical region highly disfavors Val at this position.[11] Despite the high abundance of type I′ turns in protein hairpins, the insertion of the Asn-Gly sequence produces only a small incremental increase in stability of -1.5 kJ mol^{-1} relative to that of the native G-bulged turn, suggesting that these are equally good turn sequences in this context. Substitution with the least favored L1 residue (TVGK) resulted in a modest reduction in stability of 2.9 kJ mol^{-1} relative to that of the most favored (TNGK) type I′ turn sequence. Although this is a relatively small overall effect on the stability of native ubiquitin, in the context of small hairpin peptides of only marginal stability ($\Delta G_{fold} \approx 0$), this equates to a significant change in the folded population (threefold change in equilibrium constant).[1]

To confirm that both the TNGK and TVGK turns are accommodated with the same turn conformation, rather than the stability difference reflecting grossly different backbone geometries, we examined in detail the **TN** and **TV** ubiquitin mutants by NMR spectroscopy. A complete assignment of the backbone NH and Hα protons enabled us to identify cross-strand NOEs and Hα chemical shift perturbations. By comparison with **WT***, Hα chemical shift perturbations from random coil values ($\Delta\delta$Hα [ppm]) for **TN** are shown in Figure 3a and demonstrate that the structural changes arising from the substitution of the turn sequence are largely confined to the region immediately adjacent to the mutated residues. Residues within the N and C termini of the β-strands (1–5 and 12–17) are perturbed by $\Delta\delta$Hα < 0.1 ppm. Intense cross-strand Hα–Hα NOEs, particularly between K_6 Hα and T_{12} Hα, confirm the nativelike strand alignment close to the type I′ turn. The ratio of NOE intensities at the T_7-N_8 step ($H\alpha_i$-NH_{i+1}/$H\alpha_i$-$NH_i \approx 1$) together with a small $^3J_{NH\text{-}H\alpha} < 5$ Hz for N_8 and a strong sequential NH_i-NH_{i+1} NOE between G_{10} and K_{11} identifies an abrupt change in direction of the peptide

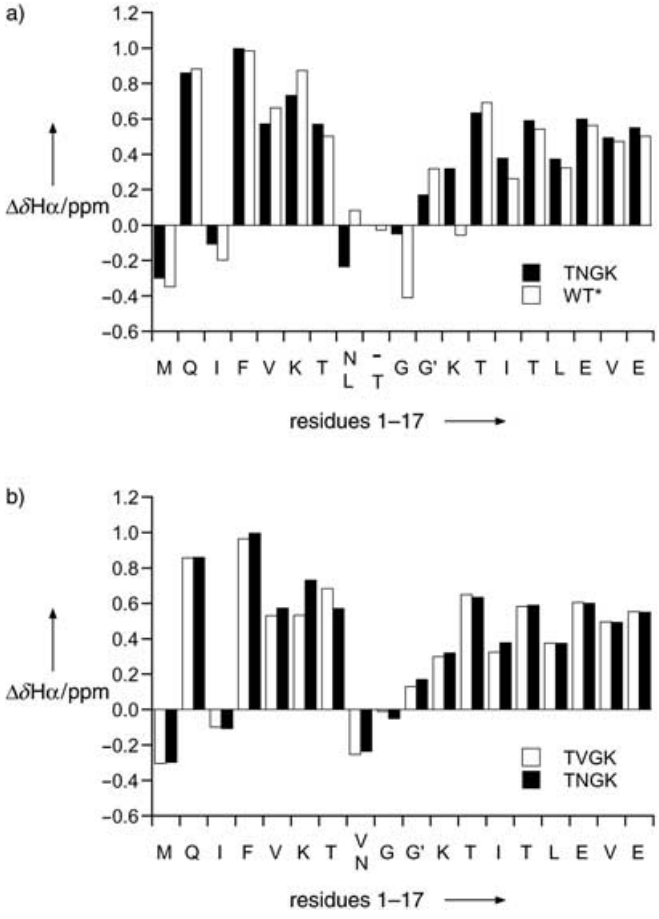

Figure 3. Perturbations to chemical shifts of Hα (measured values versus random coil values, $\Delta\delta$Hα [ppm]) of residues 1–17 in the N-terminal β-hairpin. NMR data were collected at 600 MHz at 298 K and pH 5.0. a) NMR data for **WT*** are compared to the **TN** mutant (TNGK turn sequence) and show that perturbations are largely confined to residues immediately adjacent to the mutated turn residues. b) Comparison of $\Delta\delta$Hα values for the **TN** and **TV** mutants, representing the most favored (Asn-Gly) and the least favored (Val-Gly) substitutions at position L1 in type I′ turns.

chain at the T_7-N_8 and G_{10}-K_{11} steps which is consistent with N_8 and G_{10} occupying the L-α region of ϕ,ψ space. In Figure 3b, the Hα chemical shift data for mutants **TN** and **TV** show similar perturbations that are consistent with the two turns adopting the same conformation. Analogous patterns of NOEs and $^3J_{NH\text{-}H\alpha}$ values (not shown) confirm these conclusions. Thus, the two turns appear to be readily accommodated in the desired geometry with only modest local disruption to secondary structure, despite the differences in steric demand and frequency of occurrence of Val and Asn at the L1 position.

The earlier statistical analysis of type I′ turns also showed some preference for residues at the −B1 and +B1 positions flanking the turn sequence.[10] For example, Lys occurs with high frequency at position +B1 and, consequently, this residue has been retained in all constructs as well as being present in the native sequence. At position −B1 there is a clear preference for the β-branched residue Val,[10] however, there was little apparent discrimination between remaining side chains on the basis of size or hydrophobicity. Introduction of Val at this position (**VN** mutant) resulted in a noticeable increase in stability, with a shift in the denaturant-induced transition midpoint from 2.83 M GdmCl observed for **TN** to 3.33 M for **VN** ($\Delta\Delta G = -5.1$ kJ mol^{-1}). NMR data for the **TN** and **VN** mutants show that the isosteric Thr and Val residues at the −B1 position are accommodated with the same side-chain rotamer conformation. The substitution of the β-OH group of T_7 with the V_7 methyl affects the contacts largely within the β-turn, as evident from cross-strand NOEs from the V_7 CH_3 to protons of the aliphatic side-chain of K_{11} (β-CH_2/γ-CH_2). On the basis of intrinsic β-sheet-forming propensities, Thr and Val were anticipated to promote hairpin folding, whereas Ala has a high helical propensity and was expected to be destabilizing in the context of the β-hairpin. Accordingly, the ANGK turn (**AN** mutant) resulted in a loss of stability of +2.5 kJ mol^{-1} compared to TNGK (see Table 1). Overall, the relative turn stabilities appear to reflect the intrinsic β-sheet-forming propensity of the residue at the −B1 position at the end of the β-strand. We conclude that optimization of residues at positions −B1 and L1 in the context of the type I′ turn can lead to substantial net changes in protein stability in a manner that appears to correlate well with backbone ϕ,ψ structural propensities.[10]

We also modeled a number of other nonpolar residues at the −B1 position to investigate the possibility of engineering new stabilizing tertiary interactions at this site. The models suggested that the introduction of bulky aromatics such as Phe at the −B1 position should lead to very favorable context-dependent hydrophobic contacts with L_{69} and L_{71} on the adjacent C-terminal β-strand, even though Phe does not have a particularly high frequency of occurrence at position −B1 in type I′ turns.[10] As predicted from the model, the FNGK turn (**FN** mutant) showed a substantial further increase in stability over TNGK of −9.7 kJ mol^{-1}. As confirmation of the engineered F_7–L_{69}/L_{71} interactions, we observed significant upfield ring-current effects of between 0.6–0.9 ppm on the H_β, H_γ, and $C_\delta H_3$ resonances of L_{69}, placing the latter in a well-resolved region of the spectrum at $\delta = 0.03$ ppm. Upfield shifts (≈ 0.4 ppm) are observed for both $C_\delta H_3$ resonances of L_{71} (Figure 4a). We were readily able to identify NOEs from the side chain of F_7 to L_{69} and L_{71} which confirm the hydrophobic contacts. Local refinement of our structural model using the NOE data and restrained molecular dynamics enabled these local interactions to be visualized (Figure 4b). Thus, the high apparent β-sheet propensity of Phe at position −B1 appears to be rationalized largely on the basis of engineered tertiary interactions rather than intrinsic secondary structure propensity.

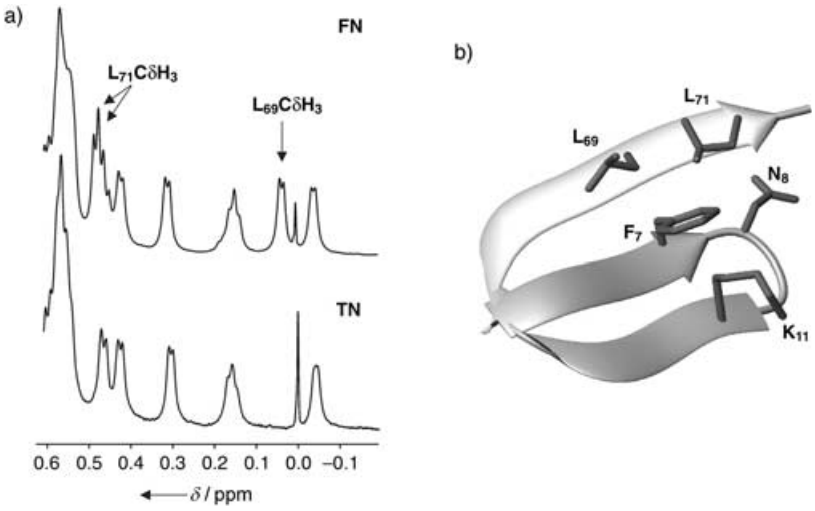

Figure 4. a) 1D ^{1}H NMR spectra of the **TN** and **FN** mutants showing the highfield-shifted methyl region ($\delta = -0.1$ to 0.6 ppm) at 298 K (pH 5.0). The spectra are similar except for the appearance of the two ring-current-shifted $C_\delta H_3$ resonances of L_{71} at $\delta = \approx 0.47$ ppm and that of L_{69} at $\delta = 0.03$ ppm owing to hydrophobic contacts with F_7 at position −B1 in the turn. b) Model of the **FN** mutant showing hydrophobic contacts between F_7 and L_{69}/L_{71} which account for the stability enhancement of the **FN** mutant and observed ring-current shifts to L_{69} and L_{71}. Other side chains of the turn residues N_8 and K_{11} are also shown.

In conclusion, we set out to examine quantitatively the effects of β-turn mutations on protein stability using the N-terminal hairpin sequence of ubiquitin as a "host" system. As demonstrated from a statistical analysis of β-hairpins in high-resolution protein structures and from work on model hairpin peptide systems, type I′ turns are effective promoters of hairpin folding, although the energetic contributions have not been systematically studied.[1,9–11,19] We have shown that optimization of residues at positions −B1 and L1 lead to substantial increases in stability that correlate well with backbone ϕ,ψ structural propensities. In the context of a weakly folded peptide model system of only marginal stability ($\Delta G \approx 0$), it is clear that the choice of type I′ turn sequence can be a critical factor in determining whether a significant population of the folded conformer can be detected.[1,11] The

work of Gellman has shown that D-Pro at position L1 is also highly effective in nucleating hairpin folding in model peptide hairpin systems because the preferred ϕ,ψ backbone angles, together with the entropic constraints imposed by the backbone-to-side-chain cyclization, match the right-handed twist of the β-sheet.[1a–c] Substituting with the mirror image L-Pro switches the turn geometry from right-handed (type I′ or II′) to left-handed (type I or II) and makes the latter incompatible with the twisted alignment of the two β-strands which leads to a completely unfolded state.[20] However, to our knowledge a quantitative estimate of the difference in stability between the pair of diastereomeric hairpin peptides has not been reported.

We have presented some energetic guidelines for the stability contributions of type I′ turns to hairpin stability. These estimates probably represent an upper limit for model peptide systems because we have measured the incremental free energy contribution of the various turn sequences in the context of the cooperative folding transition of native ubiquitin. These limiting values may not be fully realized in the context of a more dynamic folded peptide system, although enthalpy/entropy compensations may play some part in achieving these stability differences.[21] Such compensatory effects may also be evident in the case of the TGGGK turn sequence for which intrinsic flexibility potentially compensates for any reduction in enthalpic benefits from poorer side-chain interactions or locally weaker hydrogen bonds. With regard to the design of model peptide systems that contain β-hairpin motifs, we have presented a quantitative analysis of the energetic contribution of the type I′ β-turn to β-hairpin stability in ubiquitin. The N-terminal β-hairpin sequence has previously been studied in isolation as a possible protein folding nucleation site and has been used as the basis of a number of other model systems for folding studies.[1a,14,22] However, the results presented here should be of more general use in peptide and protein design and in understanding aspects of the energetics of molecular recognition and self-assembly.

Experimental Section

A pKK223–3 plasmid construct containing the yeast ubiquitin gene was used to express the wildtype protein in *Escherichia coli* strain BL21(DE3) under the control of the IPTG-inducible *tac* promoter. The F45W mutant gene was cloned by overlap PCR methodology using the wild-type yeast ubiquitin gene in pKK223-3 (Pharmacia Biotech) as a template. Competent *Escherichia coli* cells were transformed with this construct. Expression and purification of **WT*** yielded typically 10–15 mg L^{-1} of ubiquitin. All other mutations using the **WT*** background were introduced using the QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA) and confirmed by DNA sequence analysis (School of Biomedical Sciences, Nottingham) and by NMR studies.

Protein stabilities were determined using methods previously described in references [15] and [17a] and by using established protocols.[18a,b] Protein stability was determined by fluorescence measurements on 1.0-μM solutions of protein in acetate buffer (25 mM) at pH 5.0 and 298 K. The change in fluorescence at $\lambda =$ 358 nm was monitored as a function of the concentration of guanidinium chloride (GdmCl). The linear extrapolation method was employed assuming that the stability varies with the concentration of denaturant, [D], according to the expression $\Delta G^{D} = \Delta G_{eq} + m[D]$, where ΔG^{D} is the stability at a given [D], m is the constant of proportionality, and ΔG_{eq} is the stability in water alone. The data were plotted as the fraction of unfolded protein versus [D]. The midpoint of the unfolding transition c_m for each mutant was determined by nonlinear least-squares fitting (see references [16] and [18a]). The equilibrium stability ΔG_{eq} was determined from the expression $\Delta G_{eq} = -m\,c_m$. For the mutants described we observed only a small variation in m values, 10.18 ± 0.23 kJ mol^{-1} M^{-1} (± standard error), and the expression $\Delta\Delta G_{eq} = -m(c_m^{mut} - c_m^{WT})$ was used to calculated changes in stability. This approach is justified by NMR spectroscopic analysis of all mutants which shows that they all fold to a nativelike structure with only minor localized chemical shift perturbations and changes in the hydrophobic surface area buried.[18c,d]

All NMR spectroscopy experiments were performed on a Bruker Avance600 spectrometer. Total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments were used as previously described on 1-mM protein samples at pH 5.0.[15] Spectra were referenced internally to trimethylsilylpropionate (TSP), and random coil peptide shifts were used as reported.[23] Data were processed and assigned using Bruker XWINNMR and ANSIG software,[24] and structural models were visualized using MOLMOL.[25] The structure of the **FN** mutant was modeled from random unfolded starting structures using 345 NOE-derived distance restraints and 63 torsion angle restraints. 100 structures were refined using a combination of distance geometry and simulated annealing using the XPLOR-NIH program.[26] Acceptance criteria of no NOE violations greater than 0.5 Å and no angle restraint violations of greater than 5° were applied. Root-mean-square deviations for bonds and angles from ideality were less than 0.01 Å and 2°, respectively, for all structures. From this, 72 structures were accepted, the lowest energy of which is shown in Figure 4.

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[1] a) S. H. Gellman, *Curr. Opin. Chem. Biol.* **1998**, *2*, 717; b) M. S. Searle, *Biopolymers* **2004**, *76*, 185; c) M. S. Searle, B. Ciani, *Curr. Opin. Struct. Biol.* **2004**, *14*, 458; d) E. De Alba, M. A. Jimenez, M. Rico, *J. Am. Chem. Soc.* **1997**, *119*, 175.

[2] a) H. J. Dyson, M. Rance, R. A. Houghton, R. Lerner, *J. Mol. Biol.* **1988**, *201*, 161; b) D. K. Klimov, D. Thiumalai, *J. Mol. Biol.* **2002**, *315*, 721; c) J. Kim, S. R. Brych, J. Lee, T. M. Logan, M. Blaber, *J. Mol. Biol.* **2003**, *328*, 951; d) S. Nauli, B. Kuhlman, D. Baker, *Nat. Struct. Biol.* **2001**, *8*, 602; e) E. L. McCallister, E. Alm, D. Baker, *Nat. Struct. Biol.* **2000**, *7*, 669.

[3] a) M. A. Schumacher, B. K. Hurlburt, R. G. Brennan, *Nature* **2001**, *409*, 215; b) B. J. Hillier, K. S. Christopherson, K. E. Prehoda, D. S. Bredt, W. A. Lim, *Science* **1999**, *284*, 812; c) N. M. Stano, S. S. Patel, *J. Mol. Biol.* **2002**, *315*, 1009.

[4] a) H. C. Wong, J. H. Mao, J. T. Nguyen, S. Srinivas, W. X. Zhang, B. Liu, L. Li, D. Q. Wu, J. Zheng, *Nat. Struct. Biol.* **2000**, *7*, 1178; b) R. Berisio, F. Schluenzen, J. Harms, A. Bashan, T. Auerbach, D. Baram, A. Yonath, *Nat. Struct. Biol.* **2003**, *10*, 366; c) Z. Zavala-Ruiz, I. Strug, B. D. Walker, P. J. Norris, L. J. Stern, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13279.

[5] a) M. L. J. Korsinczky, H. J. Schirra, K. J. Rosengren, J. West, B. A. Condie, M. A. Anderson, D. J. Craik, *J. Mol. Biol.* **2001**, *311*, 579; b) A. Descours, K. Moehle, A. Renard, J. A. Robinson, *ChemBioChem* **2002**, *3*, 318; c) J. A. Robinson, *Synlett* **2000**, *4*, 429; d) R. Fasan, R. L. A. Dias, K. Moehle, O. Zerbe, J. W. Vrijbloed, D. Obrecht, J. A. Robinson, *Angew. Chem.* **2004**, *116*, 2161; *Angew. Chem. Int. Ed.* **2004**, *43*, 2109; e) Z. Athanassiou, R. L. A. Dias, K. Moehle, N. Dobson, G. Varani, J. A. Robinson,

J. Am. Chem. Soc. **2004**, *126*, 6906; f) R. L. Stanfield, M. K. Gorny, C. Williams, S. Zolla-Pazner, I. A. Wilson, *Structure* **2004**, *12*, 193; g) E. Drakopoulou, S. Zinn-Justin, M. Guenneuges, B. Gilquin, A. Menez, C. Vita, *J. Biol. Chem.* **1996**, *271*, 11979; h) C. E. Stotz, E. M. Topp, *J. Pharm. Sci.* **2004**, *93*, 2881.

[6] a) S. M. Butterfield, M. L. Waters, *J. Am. Chem. Soc.* **2003**, *125*, 9580; b) S. M. Butterfield, W. J. Cooper, M. L. Waters, *J. Am. Chem. Soc.* **2005**, *127*, 24.

[7] a) V. Villegas, A. R. Viguera, F. X. Aviles, L. Serrano, *Folding Des.* **1996**, *1*, 29; b) A. R. Viguera, V. Villegas, F. X. Aviles, L. Serrano, *Folding Des.* **1996**, *2*, 23; c) E. Lopez-Hernandez, P. Cronet, L. Serrano, V. Munoz, *J. Mol. Biol.* **1997**, *266*, 610; d) D. Perl, U. Mueller, U. Heineman, F. X. Schmid, *Nat. Struct. Biol.* **2000**, *7*, 380; e) A. Karshikoff, R. Ladenstein, *Trends Biochem. Sci.* **2001**, *26*, 550; f) S. Kumar, R. Nussinov, *Cell. Mol. Life Sci.* **2001**, *58*, 1216; g) C. Vetriani, D. L. Maeder, N. Tolliday, K. S. Yip, T. J. Stillman, K. L. Britton, D. W. Rice, H. H. Klump, F. T. Robb, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 12300; h) H. X. Zhou, R. H. Hoess, W. F. DeGrado, *Nat. Struct. Biol.* **1996**, *3*, 446.

[8] a) K. Takano, Y. Yamagata, K. Yutani, *Biochemistry* **2000**, *39*, 8655; b) T. R. Hynes, R. A. Krautz, Goodman, J. F. Gill, R. O. Fox, *Nature* **1989**, *339*, 73; c) P. F. Predki, V. Agrawal, A. T. Brunger, L. Regan, *Nat. Struct. Biol.* **1996**, *3*, 54; d) E. C. Ohage, W. Grami, M. M. Walter, S. Steinbacher, B. Steipe, *Protein Sci.* **1997**, *6*, 233; e) H. X. Zhou, R. H. Hoess, W. F. DeGrado, *Nat. Struct. Biol.* **1996**, *3*, 446.

[9] B. L. Sibanda, J. M. Thornton, *Nature* **1985**, *316*, 170.

[10] S. R. Griffiths-Jones, A. J. Maynard, M. S. Searle, *J. Mol. Biol.* **1999**, *292*, 1051.

[11] M. Ramirez-Alvarado, F. J. Blanco, H. Niemann, L. Serrano, *J. Mol. Biol.* **1997**, *273*, 898.

[12] S. Vijay-Kumar, C. E. Bugg, K. D. Wilkinson, R. D. Vierstra, P. M. Hatfield, W. J. Cook, *J. Biol. Chem.* **1987**, *262*, 6396.

[13] P.-Y. Chen, B. G. Gopalacushina, C.-C. Yang, S. I. Chan, P. A. Evans, *Protein Sci.* **2001**, *10*, 2063.

[14] a) M. S. Searle, D. H. Williams, L. C. Packman, *Nat. Struct. Biol.* **1995**, *2*, 999; b) M. Jourdan, S. R. Griffiths-Jones, M. S. Searle, *Eur. J. Biochem.* **2000**, *267*, 3539.

[15] G. W. Platt, S. A. Simpson, R. Layfield, M. S. Searle, *Biochemistry* **2003**, *42*, 13762.

[16] S. Khorasanizadeh, I. D. Peters, H. Roder, *Biochemistry* **1993**, *32*, 7054.

[17] a) M. D. Crespo, G. W. Platt, R. Bofill, M. S. Searle, *Eur. J. Biochem.* **2004**, *271*, 4474; b) H. Went, C. G. Benitez-Cardoza, S. E. Jackson, *FEBS Lett.* **2004**, *567*, 333; c) B. A. Krantz, T. R. Sosnick, *Biochemistry* **2000**, *39*, 11696.

[18] a) M. M. Santoro, D. W. Bolen, *Biochemistry* **1988**, *27*, 8063–8068; b) N. C. Pace, J. M. Scholtz, *Protein Structure—A Practical Approach* (Ed.: T. E. Creighton), *2nd ed.*, IRL, New York, **1997**; c) E. R. G. Main, S. E. Jackson, *J. Mol. Biol.* **1999**, *291*, 429; d) N. Ferguson, A. P. Capaldi, R. James, C. Kleanthous, S. E. Radford, *J. Mol. Biol.* **1999**, *286*, 1597.

[19] a) A. J. Maynard, G. J. Sharman, M. S. Searle, *J. Am. Chem. Soc.* **1998**, *120*, 1996; b) B. Ciani, M. Jourdan, M. S. Searle, *J. Am. Chem. Soc.* **2003**, *125*, 9038.

[20] a) T. S. Haque, S. H. Gellman, *J. Am. Chem. Soc.* **1997**, *119*, 2303; b) H. E. Stanger, S. H. Gellman, *J. Am. Chem. Soc.* **1998**, *120*, 4236; c) H. L. Schenck, S. H. Gellman, *J. Am. Chem. Soc.* **1998**, *120*, 4869.

[21] M. S. Searle, G. W. Platt, R. Bofill, S. A. Simpson, B. Ciani, *Angew. Chem.* **2004**, *116*, 2025; *Angew. Chem. Int. Ed.* **2004**, *43*, 1991.

[22] a) J. P. L. Cox, P. A. Evans, L. C. Packman, D. H. Williams, D. N. Woolfson, *J. Mol. Biol.* **1993**, *234*, 483; b) R. Zerella, P. A. Evans, J. M. C. Ionides, L. C. Packman, B. W. Trotter, J. P. Mackay, D. H. Williams, *Protein Sci.* **1999**, *8*, 1320; c) R. Zerella, P. Y. Chen, P. A. Evans, D. H. Williams, *Protein Sci.* **2000**, *9*, 2142.

[23] K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, **1986**.

[24] P. J. Kraulis, *J. Magn. Reson.* **1989**, *84*, 627.

[25] R. Koradi, M. Billeter, K. Wuthrich, *J. Mol. Graphics* **1996**, *14*, 51.

[26] C. D. Schwieters, J. J. Kuszewski, N. Tjandra, G. M. Clore, *J. Magn. Reson.* **2003**, *160*, 65.

