Protein Stability

Incremental Contribution to Protein Stability from a β Hairpin "Finger": Limits on the Stability of Designed β Hairpin Peptides**

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Current models for protein folding point to a hierarchical process of assembly in which local interactions within the polypeptide chain play an important role in restricting the conformational search for the native state.[1] Peptide model systems seem to support this hypothesis since relatively short peptide sequences (< 20 residues) have been shown to fold autonomously into α helices and β sheets in aqueous solution.[2,3] A β hairpin (two antiparallel β strands linked by a reverse β turn) is the smallest increment of β sheet accessible as a model system for investigating the interplay of noncovalent, weak interactions that drive the folding process.[3] The biological significance and potential applications of β sheet motifs are more wide ranging. They play important roles in protein–DNA recognition, in pathological disease processes involving protein aggregation and amyloidosis, and have been used in creating novel peptide ligands aimed at protein targets, or as new peptide-based drugs with pre-organized structures carrying bioactive motifs for specific recognition.[4]

An evaluation of sequence-specific effects that stabilize these small increments of β sheet, and subsequent quantitative analysis of the effects of mutations on hairpin stability, is emerging to place the process of rational design on a firm thermodynamic footing.[5] However, quantitative evaluation of hairpin stability relies on reference states to define spectroscopic parameters with which to estimate folded populations.[3] To date, several approaches have emerged: cosolvents have been used to enhance secondary-structure stability by promoting amide–amide hydrogen-bond formation; alternatively, disulfide or backbone cyclization has been successful in providing the fully folded reference state from which Hα chemical shift values can be determined.[5,6] Herein, we report that we have inserted a structurally independent β hairpin "finger" into native ubiquitin to examine the incremental contribution of the hairpin to protein stability, and as a reference state for assessing the stability of an autonomously folding 16-residue β hairpin peptide sequence. To achieve this, the N-terminal β hairpin sequence of ubiquitin was extended into solution by replacing the solvent-exposed native LTG type I G-bulged β-turn with a 14-residue sequence corresponding to the greater portion of a short de novo designed 16-residue hairpin sequence (**β4**: KKYTVEINGKKITVEI; Figure 1).[7] To maintain the correct hydrogen-bonding register the β-finger was truncated by one residue at the N- and C-termini (Figure 1a). In subsequent studies, we plan to examine the role of this independently stable hairpin motif in nucleating protein folding.[8]

a)

WT MQIFVKT------**LTG**-----KTITLEV..
Uβ4 MQIFVKT**KYTVEINGKKITVE**KTITLEV..
β4 KKYTVEINGKKITVEI

b)

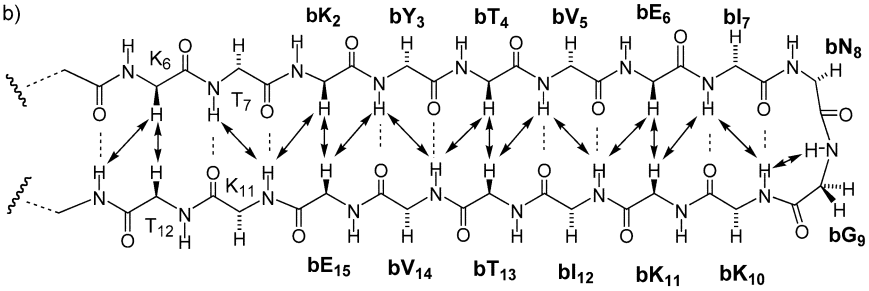

Figure 1. a) Sequence of the N-terminal hairpin of ubiquitin showing the **β4** hairpin insertion (**Uβ4**), and also the wild-type sequence (**WT**) and with the sequence of the isolated **β4** peptide (N-acetylated, free C-terminus); b) β-strand alignment of **β4** in the context of the extension of the N-terminal hairpin of ubiquitin, side chains have been removed for clarity. Residues within the hairpin are labeled $\mathbf{bK_2}$ through to $\mathbf{bE_{15}}$, with the flanking sequence following the native ubiquitin numbering. Arrows indicate main-chain (NH and Hα) NOEs observed between strands of **Uβ4**.

The 14-residue extension was introduced into wild-type yeast ubiquitin containing the background F45W mutation (**WT***).[9] The partial burial of the indole side chain in a surface hydrophobic cleft results in a fourfold quenching of fluorescence on folding providing a useful probe for biophysical studies of protein stability. The F45W mutation has only a minor effect on local structure and protein stability ($\Delta\Delta G \sim 1$ kJ mol^{-1}).[10] 2D homonuclear NMR spectroscopy experiments have enabled a complete backbone assignment for **Uβ4** to be obtained allowing numerous (> 80) medium and long-range cross-strand NOEs to be identified within the β-finger extension (Figure 1b). We observe all predicted cross-strand Hα–Hα NOEs (Figure 2), and the majority of NH–NH and NH–Hα NOEs that establish that the two strands are aligned with the β-finger adopting the desired folded conformation as an extension of the existing N-terminal hairpin of ubiquitin. On the basis of this data a structural model was generated (Figure 2).[11] Native-like NOEs between $\mathbf{K_6}$NH–$\mathbf{H_{68}}$Hα and $\mathbf{T_7}$Hα–$\mathbf{L_{69}}$NH show that the alignment between β-strands 1 and 5 of ubiquitin are not significantly perturbed by the hairpin extension. Hα chemical

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[**] This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC), Engineering and Physical Sciences Research Council (EPSRC) of the UK, the EU Marie-Curie Fellowship Scheme and GlaxoSmithKline (UK).

 DOI: 10.1002/ange.200352955

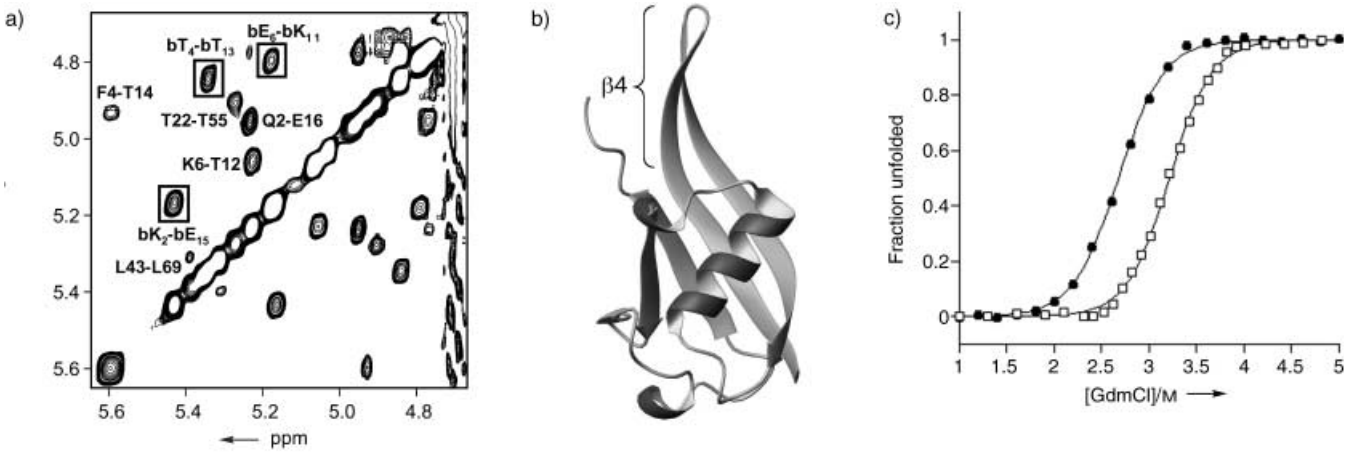

Figure 2. a) Portion of the 300 ms NOESY spectrum of **Uβ4** at 308 K showing well-resolved Hα–Hα NOE cross-peaks within the β hairpin extension, those in the β finger are shown in boxes; b) ribbon structure of **Uβ4** showing the protrusion of the β finger extension (displayed with MOLMOL[17]); c) guanidinium chloride (GdmCl) denaturation curves for **WT*** (●) and **Uβ4** (□) at 298 K showing fraction unfolded versus [GdmCl] in 25 mM acetate buffer at pH 5.5.

shift deviations from random coil values (Figure 3) are consistent with contiguous well-ordered β-sheet secondary structure along the full length of the 28 residue hairpin sequence with no evidence to suggest nonregular secondary structure or significant backbone flexibility at the junction point. Although NH chemical shift data are more difficult to interpret because of their greater sensitivity to solvation effects and pH value, alternating large and small shift perturbations along the β-strands reflect differences between residues in hydrogen-bonded versus solvent exposed sites (Figure 3). Many hydrophobic contacts within the hairpin extension are evident from side chain NOEs (for example, $\mathbf{bY_3}$ to $\mathbf{bI_{12}}$ and $\mathbf{bV_{14}}$), but no NOEs are detected from residue side chains within the β-finger extension to any other part of the structure, ruling out the possibility of new non-native tertiary contacts being introduced by the mutations. A global analysis of Hα chemical shift perturbations ($\Delta\delta H\alpha = \delta\mathbf{WT}^* - \delta\mathbf{U\beta4}$) confirms that the largest effects ($\Delta\delta H\alpha < 0.15$ ppm) are confined to regions of the structure close to where the β-finger has been introduced. Thus, the 14-residue β-finger represents an autonomously folding motif that does not require tertiary contacts to define its conformation making it a structurally independent extension of the rest of the protein.

The equilibrium stabilities of **WT*** and **Uβ4** were determined from guanidinium chloride (GdmCl) denaturation experiments by monitoring the change in fluorescence at 358 nm as a function of denaturant concentration (Figure 2).[12] The mid-point of the transition shifts from 2.67 M GdmCl (**WT***) to 3.21 M for **Uβ4** corresponding to an increase in stability of $-5.6\ \mathrm{kJ\,mol^{-1}}$.[13] Thus, an estimate of the incremental free-energy contribution of the β-finger to protein stability suggests a value of approximately $6\ \mathrm{kJ\,mol^{-1}}$ when this motif is fully constrained as part of the global cooperative folding event. The cooperative nature of the folding transition implies that any increase in stability should reflect the stability of the whole system and not just interactions within the hairpin extension. Global perturba-

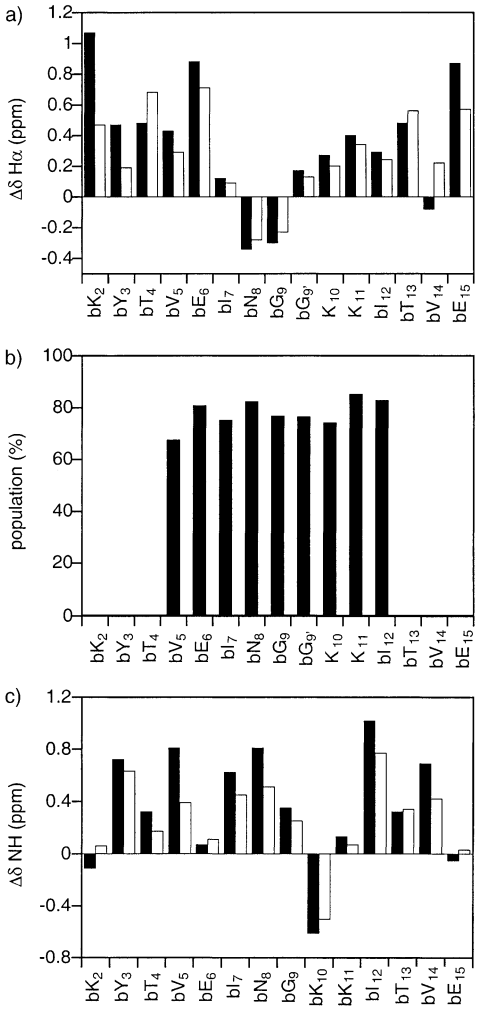

Figure 3. a) Hα chemical shift deviations from random-coil values at pH 5.5, 298 K for **Uβ4** (black bars) and **β4** peptide (white bars); b) estimated folded populations (percentage, $\phi_F \times 100$) for individual residues using the **Uβ4** reference state; c) comparison of NH chemical shift deviations for **Uβ4** and **β4** as in (a).

tions to Hα shifts of <0.05 ppm are apparent and are suggestive of only very small long-range structural effects propagated from the hairpin extension. Thus, as an approximation, we could attribute all of the observed 6 kJ mol^{-1} free-energy increase to the interactions within the hairpin extension of **Uβ4**, with this representing an upper limit to the stability increase. Consequently, if this free-energy change were realized for the same set of interactions in the isolated hairpin peptide this would translate to an equilibrium constant for folding of the isolated hairpin peptide **β4** of approximately 10 and a maximum attainable folded population of around 90%.

The isolated 16-residue peptide **β4** shows all of the characteristics of a highly folded β hairpin in terms of long range NOEs, perturbations to Hα chemical shifts, and backbone torsion-angle preferences evident from $^3J_{NH\text{-}H\alpha}$ values.[7] A detailed structural characterization of **β4** confirms the formation of a compact fold, although the N- and C-terminal residues are more dynamic. We see a similar pattern of hydrophobic contacts to those described for **Uβ4**, particularly involving **bY$_3$**. The structural ensemble shows the characteristic twisted relationship between β strands although the effect is less pronounced than in protein β sheets. Subsequently, we have used the Hα chemical shift data for **Uβ4** as a reference state for the fully folded state of the isolated hairpin peptide **β4** (Figure 3a). It is evident that residues close to the N- and C-termini of the hairpin sequence experience different environments to those residues in **Uβ4** because of end-effects associated with fusion to the ubiquitin template. This situation is apparent for **bK$_2$** and **bE$_{15}$** whose shifts are much smaller in the isolated peptide reflecting disorder in the N- and C-termini, as evident from the structural modeling.[7] Residues **bY$_3$** and **bV$_{14}$** also show less pronounced Hα shifts in the context of the isolated hairpin **β4** which could, in part, arise from dynamic end-effects or from differences in ring current effects from the aromatic side chain. The following pair of residues **bT$_4$** and **bT$_{13}$**, in contrast, has greater downfield shifts in the **β4** peptide than in **Uβ4**. This difference becomes significantly more pronounced when peptide **β4** is examined in 50% (v/v) methanol, which suggests that the **bT$_4$–bT$_{13}$** pair is more "folded" in the isolated hairpin. This observation may have its origins in structural differences relating to the twisted conformation of the β-strands in the two contexts. When inserted as an extension of the N-terminal hairpin of ubiquitin, the twist of the β-finger is strongly dictated by the geometry of the β-sheet template, whereas flexibility in the isolated hairpin may allow the **bT$_4$–bT$_{13}$** pair to minimize side-chain steric interactions. The more exposed residues of the β-finger (**bV$_5$→bI$_{12}$**) are likely to have more geometrical flexibility, and indeed, a more uniform relationship between **β4** Hα shifts in the two contexts is evident. Thus, we have focused our quantitative analysis on this group of residues.

We have analyzed the Hα shifts for the isolated **β4** in terms of a two-state model, supported by temperature-jump IR kinetic data on a mutant of **β4**,[5a] which has a single activation barrier separating the folded and unfolded states.[14] Thus, the folded population of **β4** (ϕ_F) in aqueous solution can be estimated on an individual residue basis from the Hα shifts (δ_{obs}) of **bV$_5$→bI$_{12}$** using $\phi_F = (\delta_{obs}-\delta_U)/(\delta_F-\delta_U)$, where δ_F is the Hα shift in the fully folded state (derived from **Uβ4**) and δ_U is the random coil Hα shift for the unfolded state.[15] In Figure 3b, ϕ_F values are indicated for each residue, showing good agreement between sites, consistent with a two-state model. A mean value for ϕ_F of 0.78 and a standard error of ± 0.02, equates to a free energy of folding $\Delta G_F = -RT\ln[\phi_F/(1-\phi_F)] = -3.1(\pm 0.3)$ kJ mol^{-1}. A similar estimate of the folded population from NH chemical shift data (Figure 3c), though generally considered less reliable in population estimates, seems to be in good agreement, it gives a mean ϕ_F of 0.72 (± 0.08). Thus, the designed hairpin **β4** appears to be significantly folded in aqueous solution, and attains a large proportion of the maximum stability estimated from **Uβ4**. This result may, in part, reflect the effects of entropy–enthalpy compensations where the entropic advantages of the conformational flexibility of peptide **β4** offset any reduction in enthalpic benefits from poorer side-chain packing or weaker hydrogen bonds.

The approach described provides an alternative to disulfide or backbone cyclization of hairpin peptides to estimate reference chemical shift data for the fully folded state. The alternative approach to derive δ_F values has been the cosolvent induction method to enhance β hairpin stability (typically using methanol (MeOH) or 2,2,2-trifluroethanol (TFE)). However, cosolvent addition has been reported in a number of cases to lead to a plateau at around 40–50% (v/v) cosolvent, after which further additions result in no further increase in apparent stability.[16] Thus, δ_{obs} values derived at this plateau point, and subsequently used as an estimate of reference shifts, may fall short of those for the 100% folded population. To examine this possibility, we have compared the δ_F values derived from **Uβ4** in aqueous solution with those from the **β4** peptide in 50% (v/v) methanol at 298 K, pH 5.5. Cosolvent induces a uniform increase in magnitude of $\Delta\delta$Hα values reflecting a solvent-induced increase in hairpin stability. The effects are modest with increases of typically 25% reflecting the fact that **β4** is already highly folded. We have demonstrated that Hα shifts are independent of peptide concentration in the range 0.01–1 mM eliminating the possibility that aggregation effects may be influencing peptide chemical shifts.[7] The two data sets show that in many cases the shifts are quite similar. Within the **bN$_8$–bG$_9$** β-turn, shifts are larger in the cosolvent stabilized hairpin, while at other sites the shifts of **Uβ4** are slightly larger. Calculating the folded populations (ϕ_F) for **bV$_5$→bI$_{12}$**, as described above, we derive a mean value and standard error of $\phi_F = 0.94(\pm 0.08)$ for **β4** in 50% (v/v) MeOH. Within the errors of the calculation, **β4** is close to fully folded under these conditions. Using the shifts for **β4** in 50% (v/v) MeOH at 298 K as the limiting values for the fully folded state enables us to calculate the stability of **β4** in aqueous solution as $\Delta G_F = -3.9$ (± 0.5) kJ mol^{-1}. Again, within the limits of this approach, the two different reference states produce comparable stabilities.

In conclusion, we have demonstrated that the 16-residue hairpin peptide **β4** is highly folded in aqueous solution ($\phi_F = 0.78$). The effects of cosolvent are sufficiently stabilizing to push the folded/unfolded equilibrium for an already highly folded hairpin close to the fully folded limit ($\phi_F \rightarrow 1$). There-

fore, the use of cosolvents to derive reference values, in this case, seems justified. However, in cases where the peptide sequence is very weakly folded ($\Delta G_F > 0$; $\phi_F \rightarrow 0$), the effects of cosolvents on hairpin stability reach a plateau, which, as others have suggested,[6b,15] may result in chemical shift values that do not represent the fully folded state and overestimate β hairpin populations. We have shown that introducing a β-finger motif into native ubiquitin, by extending the N-terminal β hairpin, has enabled us to estimate the contribution to protein stability of an independent structural motif, providing both an upper limit on stability for autonomously folding β hairpins of approximately 16-residues, and a spectroscopic reference state for estimating the stability of related autonomously folding peptides in solution.

Received: September 25, 2003 [Z52955]

Keywords: NMR spectroscopy · protein folding · protein modifications · proteins · structure elucidation

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[7] Spectroscopic studies of the 16-residue β hairpin peptide **β1** (KKYTVXINGKKITVXI, X = S) (see refs. [5a,b]) have shown that it is highly folded in aqueous solution. Subsequent rounds of sequence refinement to maximize the stability of the folded state showed that the **β4** analog with two cross-strand Lys–Glu salt bridges (X = E) was further stabilized by about 3.5 kJ mol^{-1} (B. Ciani, M. Jourdan, M. S. Searle, *J. Am. Chem. Soc.* **2003**, *125*, 9038 – 9047). The **β4** sequence, which is highly soluble, was used in the extension of the ubiquitin N-terminal hairpin.

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[9] A pKK223-3 plasmid construct containing the yeast ubiquitin gene was used to express the wild-type and mutant proteins in *Escherichia coli* strain BL21(DE3) under the control of the IPTG-inducible *tac* promoter. Mutant genes were cloned using overlap polymerase chain reaction (PCR) methodology and mutations confirmed by DNA sequencing. Proteins were isolated and purified using published methods (G. W. Platt, S. A. Simpson, R. Layfield, M. S. Searle, *Biochemistry* **2003**, *42*, 13762 – 13771).

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[11] NMR spectroscopic data were collected at 600 MHz on a Bruker Avance 600 spectrometer on approximately 1.0 mM samples of **WT*** and **Uβ4** in H_2O solution at pH 5.5. Near complete backbone (Hα and NH) assignments were obtained from a combination of homonuclear TOCSY and NOESY experiments as previously described, see ref. [9]. The structure of **Uβ4** was modeled using SYBIL in which the **β4** hairpin extension was grafted on to wild-type ubiquitin using the X-ray coordinates of ubiquitin (1UBQ: S. Vijay-Kumar, C. E. Bugg, K. D. Wilkinson, R. D. Vierstra, P. M. Hatfield, W. J. Cook, *J. Biol. Chem.* **1987**, *262*, 6396 – 6399) and our NMR structural model of hairpin **β4** (ref. [7]). The mutations P19S, E24D, and A28S were introduced to generate yeast ubiquitin from the human sequence. Unfavorable side chain interactions and steric clashes were minimized by a rotomer scan for each mutated residue. The model of **Uβ4** was subjected to several rounds of steepest-decent and conjugate-gradient minimization. The coordinates were exported as a pdb file and reformatted using the *xleap* module within the AMBER6 suite of programs (//http:amber.scripps.edu/doc6/install.html). The structure was equilibrated with counterions and TIP3 water molecules to a distance of 8 Å and unrestrained molecular dynamics run for 100 ps. Subsequently, 244 NOE restraints were introduced gradually over 30 ps at 100 K, while at the same time increasing the temperature to 300 K. Restrained molecular dynamics were run for a further 50 ps at 300 K. The low-resolution structure showed the hairpin extension to be stable and well-formed with NOE restraints showing no violations > 0.5 Å. The mean energy-minimized model of **Uβ4** is shown in Figure 2b. The entire structure of **Uβ4** is currently undergoing detailed refinement using NOE restraints; coordinates and NMR restraints will be published elsewhere

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