Energetics of Weak Interactions in a β-hairpin Peptide: Electrostatic and Hydrophobic Contributions to Stability from Lysine Salt Bridges

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Abstract: Analysis of the contribution of ion pairing interactions to the stability of a β-hairpin in aqueous solution has been studied quantitatively by NMR. A thermodynamic cycle has been constructed involving a combination of a single mutation (Lys→Gly) and a "pH switch" (CO_2^-→CO_2H) to remove stepwise the contributions to stability from the interaction between the C-terminal carboxylate group of Ile16 and the side chains of Lys1 and Lys2. Turning these interactions "on" and "off" is shown to affect the chemical shifts of *all* residues, including those in the turn, such as to suggest that folding of the hairpin approximates to a two-state process. Two independent NMR methods have been used to analyze the thermodynamics of folding and are found to be in good agreement. Differences in hairpin stability have been analyzed in terms of an electrostatic interaction between charged groups on the terminal residues and the hydrophobic component of the Lys1 side chain: we estimate the primary electrostatic interaction to contribute 1.0−1.2 kJ mol^{-1} to stability, consistent with previous estimates for salt bridges in solvent-exposed sites in proteins and α-helical peptides, while the hydrophobic component is smaller but still significant (0.3−0.8 kJ mol^{-1}). The hairpin stability is extremely sensitive to small structural perturbations (single residue mutations) or environmental changes (such as pH) providing a novel vehicle for quantitative studies of weak interactions.

The rationalization of chemical and biological molecular recognition phenomena frequently relies on our understanding of weak noncovalent interactions, their magnitude, and their cooperative interplay. Quantitative analysis of individual binding contributions is problematic because individual interactions are seldom viewed in isolation but frequently as an incremental component of a stronger interaction.[1] The energetic importance of ion pairing contributions in regulating protein structure, in stabilizing α-helical peptides and in molecular associations in aqueous solution, is well established.[2−5] However, their energetic contribution is frequently highly dependent on context and degree of solvent exposure, with water molecules competing very effectively for recognition sites.[6] Synergistic effects that link one interaction with other neighboring interactions further complicate the analysis.[2] The application of thermodynamic cycles, in combination with structural mutations, has proved successful in deconvoluting these various factors and in measuring interaction energies between residue side chains in protein hosts[7,8] and in the context of supramolecular host−guest recognition.[9,10]

Refolding of mainly α-helical proteins into largely β-sheet structures has been widely implicated in protein folding-related disease states, including Alzheimer's and BSE,[11] suggesting that model peptide β-sheets may provide some insight into the underlying molecular basis for β-sheet stabilization, self-association, and prion-like structural transformations.[12] β-hairpin and three-stranded β-sheet peptide motifs, derived from native protein sequences or through rational design, have already been shown to fold autonomously in aqueous solution.[13] The suitability of β-hairpin peptides as vehicles for quantitative analysis of weak noncovalent interactions has not been examined in detail,[14] nor has the cooperative nature of the linear array of interactions that stabilize them.[15,16]

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We address these issues in the context of the thermodynamic contribution of weak ion pairing interactions in a model β-hairpin peptide which we analyze through a thermodynamic cycle involving a combination of a single mutation (Lys→Gly) and a "pH switch" (CO_2^-→CO_2H) to remove stepwise these contributions to β-hairpin stability. We show that the folding transition approximates reasonably well to a two-state process with the folded population proving extremely sensitive to changes in sequence and to the ionization state of specific functional groups.

Materials and Methods

Materials and NMR Methodology. The preparation and purification of peptides has been described in detail previously together with the NMR methodology used.[15,16]

Analysis of Peptide Aggregation. Dilution experiments were carried out to examine the concentration dependence of $\delta_{H\alpha}$ values primarily at 298 K, since this is close to the temperature at which the peptides shows maximum stability, and at which it is likely to be most sensitive to temperature-dependent aggregation. Deviations from random coil chemical shifts were measured in the range 30 μM to 2 mM, and no significant differences in $\delta_{H\alpha}$ values or line widths were detected. The concentration range was extended by examining CD spectra down as low as 7.5 μM. Estimates of the folded population from analysis of the ellipticity at 216 nm were in good agreement with the NMR analysis despite the large difference in concentration required for these two methods (a dilution of close to 500-fold). We conclude that we are observing folding of the monomeric peptides under these conditions.[15,16]

Thermodynamic Analysis. Hairpin folding has been analyzed by assuming a two-state model in which the peptide is either folded or unfolded; the basis for this assumption is justified below and as described previously.[15,16] The equilibrium constant for folding is given by the expression: $K = \nu/(1-\nu)$, where ν is the fraction of folded peptide assessed using two methods to measure the folded population, either the root-mean-square (RMS) value (taken over all residues) for the deviation in Hα chemical shifts from random coil values (RMS$\Delta\delta_{H\alpha}$), or $\Delta\delta^{Gly}$, the difference between the Hα chemical shifts of Gly9 in the β-turn. $\Delta G°$ for folding was estimated from $\Delta G° = -RT \ln K$. The temperature-dependence of RMS$\Delta\delta_{H\alpha}$ and $\Delta\delta^{Gly}$ were fitted to the following expression, where A is the experimental parameter (RMS$\Delta\delta_{H\alpha}$ or $\Delta\delta^{Gly}$), and A_{limit} the limiting value for the fully folded state:

$$A = A_{limit}\,[\exp(x/RT)]/[1 + \exp(x/RT)] \quad (1)$$

where

$$x = [T(\Delta S°_{298} + \Delta C_p° \ln(T/298)) - (\Delta H°_{298} + \Delta C_p° (T - 298))] \quad (2)$$

Initially, eq 1 was used iteratively to determine $\Delta H°_{298}$, $\Delta S°_{298}$, and $\Delta C_p°$ as RMS$\Delta\delta_{H\alpha}$ and $\Delta\delta^{Gly}$ varied with T. The value for A_{limit} in each case was determined from data for peptide **1** in 50% aqueous methanol at 278K where the peptide was essentially fully folded, as judged by CD.[15] The same limiting values were assumed for the two peptides at both pH 2.2 and 5.5. A_{limit} was also determined by iteration and found to be in good agreement with the 50% aqueous methanol data. The limiting values for RMS$\Delta\delta_{H\alpha}$ and $\Delta\delta^{Gly}$ in the fully unfolded state were taken as zero. Analysis of chemical shifts in an eight-residue peptide corresponding to the C-terminal β-strand GKKITVSI showed very small deviations from random coil values in both water and aqueous methanol, as previously reported.[15] Similar results have been obtained for other model peptides of unrelated sequence (up to 20 residues), suggesting that reported random coil values are reasonably accurate and that solvation effects from methanol are small compared with secondary structure-induced changes in chemical shifts.[15] Several mutated (or truncated) hairpin analogues have also been investigated in which hydrophobic interactions between strands have been deleted.[16] These hairpins show only a very low propensity to fold and have RMS$\Delta\delta_{H\alpha}$ and $\Delta\delta^{Gly}$ values close to zero, justifying our assumption of limiting values in the fully unfolded state. Since RMS$\Delta\delta_{H\alpha}$ and $\Delta\delta^{Gly}$ are measured accurately from chemical shift data, errors in $\Delta G°$ are small (±0.2 kJ mol^{-1}). We note that there are small systematic differences in the folded population estimated using the two methods; $\Delta\delta^{Gly}$ Hα values give slightly higher values in all cases. However, systematic errors in the differences in free energies ($\Delta\Delta G°$ values) taken from the thermodynamic cycle in Figure 4 are likely to be significantly reduced because the same limiting values are used in the analysis of each data set. Errors derived from the fitting procedure for $\Delta H°$ and $\Delta S°$ are indicated in Table 1, but more realistic estimates of errors have been derived on the basis of the range of values determined here, and previously,[15] using the two independent methods described. In all cases $\Delta H°$ is estimated to be small: for peptide **1** we observe a range of values between +7.2 and +0.8 kJ mol^{-1} over the pH range 2.2 to 5.5, i.e., a mean value for $\Delta H°$ of 4.0 ± 3.2 kJ mol^{-1}. For peptide **2** over the same pH range values lie between +0.9 and −1.6 kJ mol^{-1}, mean value −0.3 ± 1.3 kJ mol^{-1}. For $\Delta S°$ values: peptide **1**, range −3.4 to +23 J K^{-1} mol^{-1}, mean value +10 ± 13 J K^{-1} mol^{-1}, and peptide **2**, range −6.1 to −12.1 J K^{-1} mol^{-1}, mean value −9 ± 3 J K^{-1} mol^{-1}. $\Delta C_p°$ also shows some variation; although the effects of changes in pH and residue mutation (Lys1→Gly) are expected to have some impact on $\Delta C_p°$, these structural changes result in the deletion of a relatively small proportion of the total number of interactions present in each hairpin. Neglecting the effects of the mutation, the range of values observed for $\Delta C_p°$ is −520 to −1330 J K^{-1} mol^{-1}, suggesting that errors may be as large as ±45%, that is, $\Delta C_p° = -920 \pm 400$ J K^{-1} mol^{-1}.

Table 1: Free Energy, Enthalpy, Entropy and Change in Heat Capacity for the Folding of β-hairpin Peptides in Aqueous Solution at 298 K

	$\Delta G°$ [a] (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J K^{-1} mol^{-1})	$\Delta C_p°$ (J K^{-1} mol^{-1})
1 pH 5.5	+1.0 (0.2)[b]	+3.7 (0.4)[b]	+9.1 (1.4)[b]	−1130 (80)[b]
	+0.7 (0.2)[c]	+3.7 (0.7)[c]	+12.4 (2.4)[c]	−1330 (120)[c]
1 pH 2.2	+2.4 (0.2)[b]	+1.4 (0.2)[b]	−3.5 (0.6)[b]	−760 (30)[b]
	+2.3 (0.2)[c]	+0.8 (0.4)[c]	−3.4 (1.5)[c]	−1000 (80)[c]
2 pH 5.5	+2.8 (0.2)[b]	−0.6 (0.2)[b]	−11.0 (0.7)[b]	−520 (40)[b]
	+2.2 (0.2)[c]	−1.5 (0.4)[c]	−12.1 (1.2)[c]	−800 (60)[c]
2 pH 2.2	+3.2 (0.2)[b]	+0.9 (0.2)[b]	−7.8 (0.6)[b]	−550 (30)[b]
	+2.6 (0.2)[c]	−0.2 (0.5)[c]	−6.1 (1.5)[c]	−650 (80)[c]

[a] $\Delta G°$ calculated directly from estimated population of folded state at 298 K with maximum error calculated from uncertainty in RMS$\Delta\delta_{H\alpha}$ and $\Delta\delta^{Gly}$ Hα values; $\Delta H°$ and $\Delta S°$ derived from analysis of the temperature-dependence of RMS$\Delta\delta_{H\alpha}$ values and $\Delta\delta^{Gly}$ Hα splitting. Errors are those derived from the nonlinear least-squares fitting. A more detailed description of errors is presented in methods; the range of values measured for $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ for each peptide probably better reflects error limits. [b] RMS$\Delta\delta_{H\alpha}$ values. [c] $\Delta\delta^{Gly}$ Hα splitting.

Results and Discussion

A Model β-Hairpin Peptide. We have reported a 16-residue β-hairpin peptide that folds in water without the need for incorporation of nonnatural amino acids or disulfide bonds (Figure 1a; peptide **1**, X = Lys).[15] The β-hairpin has marginal stability ($\Delta G° \approx 0$ at 303 K) providing a sensitive model system for quantitating weak interactions through sequence mutation or changes in environmental conditions. In Figure 2a we show deviations of Hα chemical shifts from random coil values ($\Delta\delta_{H\alpha}$) for peptide **1** at pH 5.5 and 298K. The pattern of $\Delta\delta_{H\alpha}$ values is consistent with a β-hairpin structure with two extended β-strand regions (+$\Delta\delta$ values) separated by a β-turn (−$\Delta\delta$

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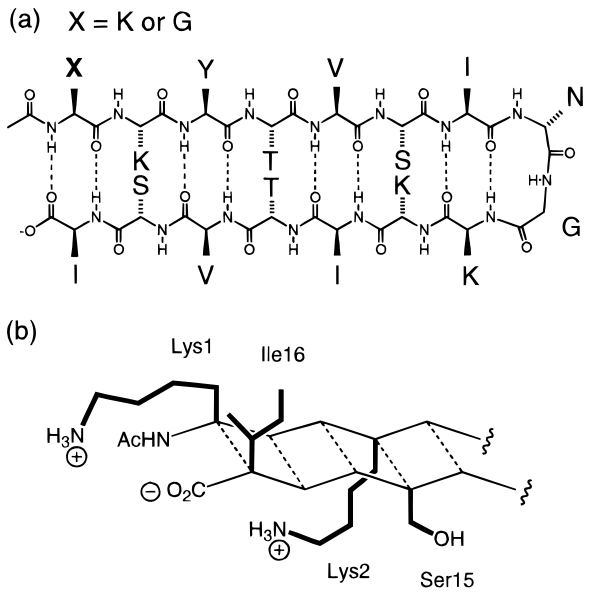

Figure 1. (a) Peptide backbone alignment of the β-hairpin peptide; side chains are indicated by the one-letter amino acid code with **X** representing the position of the mutation Lys1→Gly (peptide **1**, X = Lys; peptide **2**, X = Gly). (b) Schematic representation of the β-pleated sheet structure of the hairpin and the location of the side chains of Lys1 and Lys2 with respect to the C-terminal carboxylate group.

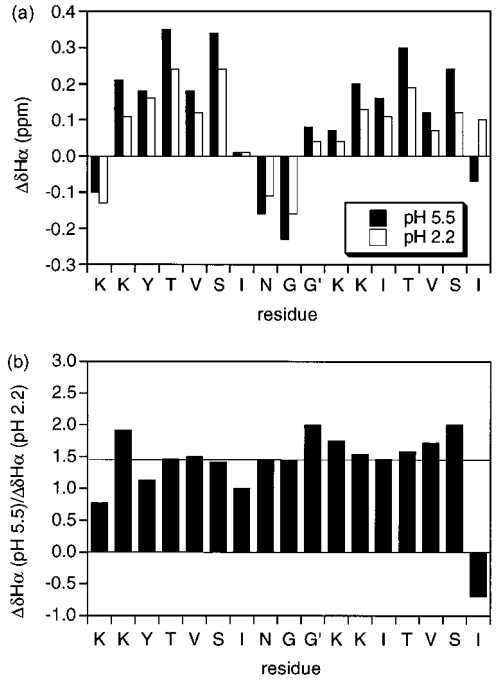

Figure 2. (a) Histogram of the deviation of Hα chemical shifts from random coil values ($\Delta\delta_{H\alpha}$) for peptide **1** at pH 2.2 and 5.5. (b) Ratio of $\Delta\delta_{H\alpha}$ values at pH 5.5 and pH 2.2; the horizontal line at 1.46 represents the ratio determined from the RMS value for $\Delta\delta_{H\alpha}$ taken over all residues at the two pHs.

values).[17] The C-terminal carboxylate group of Ile16 has a pK_a of 3.40, determined by an NMR pH titration of the δ_{NH} of Ile16. Changing the pH from 5.5 to 2.2 permits the carboxylate of Ile16 to be switched selectively to its free-acid form. The $\Delta\delta_{H\alpha}$ values for **1** determined at pH 2.2 are also shown in Figure 2a. Deviations from random coil chemical shifts are less pronounced, indicating that the hairpin is less folded at low pH. An important observation is that there is a uniform reduction in the magnitude of $\Delta\delta_{H\alpha}$ values for *all* residues including those furthest away in the Asn-Gly β-turn. This is more clearly illustrated by taking the ratio of $\Delta\delta_{H\alpha}$ values at the two pHs (Figure 2b) which reflects the ratio of populations of the folded conformation at the individual residue level. It is evident that the majority of residues show a very similar ratio for the change in shift, which is close to the ratio determined from the RMS value averaged over *all* residues at each pH. The data strongly suggest that the pH switch results in a cooperative destabilization of the β-hairpin conformation rather than just a localized unfolding close to the ionisable group, which would produce only localized effects on Hα chemical shifts. Temperature-dependent changes in Hα chemical shifts also show a similar cooperative pattern of changes as the hairpin thermally unfolds, with all residues again reflecting a similar change in folded population.[15,16] Taken together, the data suggest, to a reasonable approximation, that the hairpin folds and unfolds via a two-state process.

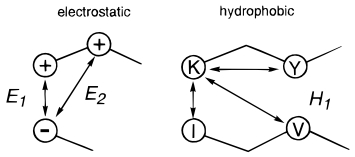

Figure 3. Contributions to hairpin stability: (left) electrostatic interactions are broken down into E_1 and E_2 contributions from the salt bridges between Lys1 (+) and Lys2 (+), and the C-terminal carboxylate group of Ile16 (−); (right) hydrophobic interactions (H_1) between the aliphatic portion of the side chain of Lys1 and neighboring hydrophobic residues on the same face of the hairpin.

Origin of pH-Dependent Changes in Hairpin Stability. The C-terminal carboxylate group is the only ionisable group in the peptide that titrates in the pH range described. We conclude that electrostatic interactions involving the carboxylate group are largely responsible for the pH-dependent changes in stability that are observed. We have previously determined the structure of peptide **1** using NOE restraints[15] and have modeled the possible electrostatic interactions on the basis of this structure. Shown schematically in Figure 1b are ion pairing interactions involving the flexible protonated side chains of Lys1 and Lys2 which are able to come into close contact with the ionized C-terminal carboxylate group of Ile16. To deconvolute these contributions to stability we have mutated the N-terminal residue Lys1→Gly, to give peptide **2** (Figure 1a; **2**, X = Gly). Consistent with our model, peptide **2** has lower stability, in accord with the deletion of an important electrostatic interaction.

To confirm that peptide **2** folds in essentially the same manner, we have carried out a detailed structural analysis at pH 2.2 and 5.5. Inter-strand Hα−Hα NOEs confirm the main chain alignment of the two β-strands as shown in Figure 1a. Many side chain-side chain NOEs (Figure 3a) point to the same hydrophobic packing arrangements previously identified for **1**.[15] Thus, a mutation of the N-terminal residue (Lys1→Gly) does not result in gross changes in the manner in which the hairpin folds. With regard to the disposition of the C- and N-terminal residues of peptide **1**, we detect an NH→NH NOE between Lys1 and Ile16, which demonstrates that the terminal residues spend a significant fraction of their time in close proximity.[15] We also detect unambiguous NOEs from the δCH_2 of the Lys1 side chain to Ile16 Hα and NH, supporting the proposed salt bridge, and consistent with at least partial burial of the Lys1 side chain in order to facilitate the formation of the ionic interaction.

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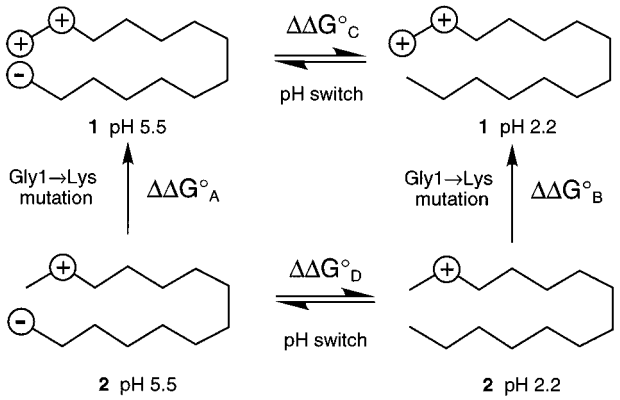

Figure 4. Thermodynamic cycle equating differences in free energy $\Delta\Delta G°$ (A→D) with structural changes as a consequence of residue mutation (Lys1→Gly) or pH switch (CO_2^-→CO_2H). Positively charged Lys side chains (+), and negatively charged carboxylate group (−).

On the basis of this structural analysis, we conclude that the Lys1→Gly mutation is likely to affect hairpin stability in two ways: first, the potential electrostatic interaction (E_1) between the side chain of Lys1 and Ile16 CO_2^- is removed; second, the hydrophobic surface area of the Lys1 side chain, which is at least partially buried against Ile16, is also deleted, removing the combined contribution from the hydrophobic effect (H_1) with neighboring residues (Figure 3). We have constructed the thermodynamic cycle shown in Figure 4 to analyze these interactions using a combination of the Lys1→Gly mutation (**1**→**2**) coupled with the pH switch to turn-off the two electrostatic contributions (E_1 and E_2) from the Lys1 and Lys2 side chains.

Thus, stability measurements for the two hairpins at different pHs enable the contributing electrostatic interactions E_1 and E_2, and the hydrophobic component H_1 to be equated with the differences in stability $\Delta\Delta G°_A$, $\Delta\Delta G°_B$, $\Delta\Delta G°_C$, and $\Delta\Delta G°_D$ shown in Figure 4, leading to the following approximate relationships:

$$\Delta\Delta G°_A \approx E_1 + H_1 \quad (3)$$

$$\Delta\Delta G°_B \approx H_1 \quad (4)$$

$$\Delta\Delta G°_C \approx E_1 + E_2 \quad (5)$$

$$\Delta\Delta G°_D \approx E_2 \quad (6)$$

Several assumptions are implicit in the above analysis that are worthy of further comment. We have assumed that the hydrophobic contribution to the stability of the hairpin from the Lys1 side chain is similar when the charge interaction E_1 is switched-off. Since the population weighting for the different Lys1 side chain conformations will change when the electrostatic interaction is removed, this may also result in changes in the nature of the hydrophobic interactions that take place, which we are not readily able to detect by NMR. We suggest that such differences may result in only small effects on the hydrophobic contribution of Lys1 and that to a reasonable approximation eqs 5 and 6 represent a physically realistic breakdown of the contributions to hairpin stability. From these expressions, $\Delta\Delta G°_B$ and $\Delta\Delta G°_D$ lead directly to values for H_1 and E_2, while substitution of these values into the other expressions enables E_1 to be determined.

Determination of *β*-Hairpin Stability. The temperature-dependence of $\Delta\delta_{H\alpha}$ values for both peptides **1** and **2** shows the same cooperative behavior already highlighted by the pH switch. That is, *all* residues simultaneously show changes in the proportion of the folded conformer present suggesting a cooperative folding transition. On this basis, we have calculated a single paramater to reflect the stability of the hairpin at a particular temperature. Since all $\Delta\delta_{H\alpha}$ values show a similar temperature-dependent behavior, we have calculated a root-mean-square value for $\Delta\delta_{H\alpha}$ (RMS$\Delta\delta_{H\alpha}$) taken over all residues.[15] The temperature-dependence of this parameter is shown in Figure 5a for peptides **1** and **2** at both pH 2.2 and 5.5. The *β*-hairpin shows the unusual characteristic of having a maximum stability close to 303 K but unfolds at both higher and lower temperature, showing a pronounced curvature in its temperature-dependent stability profile. Such a behavior is characteristic of globular proteins which fold with a large change in heat capacity, and is usually attributed to the hydrophobic contribution to folding.[18] Fitting the data in Figure 5a, assuming temperature-dependent enthalpy and entropy terms (see methods), confirms this thermodynamic signature; folding is found to be entropy-driven at room temperature and is associated with a negative change in heat capacity (Table 1). The temperature-dependence of $\Delta\delta_{H\alpha}$ values for peptides **1** and **2** at pH 2.2 and 5.5 reveal small differences in overall stability but the characteristic curvature in the stability profiles is clearly similar (Table 1).

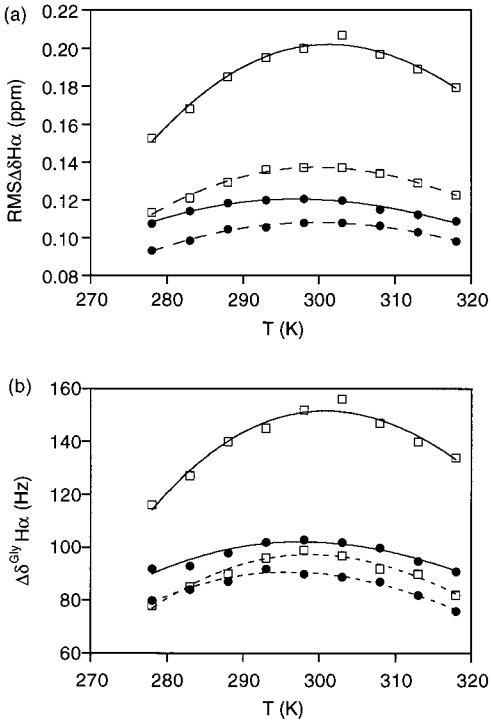

Figure 5. (a) Plot of temperature-dependence of RMS$\Delta\delta_{H\alpha}$ (ppm) values for peptides **1** and **2** at pH 2.2 and 5.5; (b) temperature-dependence of $\Delta\delta^{Gly}$ (Hz) values for peptides **1** and **2** at pH 2.2 and 5.5; (□) peptide **1**, (●) peptide **2**, (- - -) pH 2.2, (—) pH 5.5; lines represent the best fit to the experimental data (see Table 1).

We have sought an alternative handle on the temperature-dependent stability of the hairpins to assess the possible errors in the above analysis. While the RMS$\Delta\delta_{H\alpha}$ values described reflect largely perturbations to the chemical shifts of residues in the *β*-strand sequences (since these residues are in the majority), we also note that the chemical shift difference between the Hαs of Gly9 in the turn is also sensitive to the

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Table 2: Differences in Hairpin Stability at 298 K for Changes in pH (2.2→5.5) and for Mutation Lys1→Gly

	free energies[a] (kJ mol^{-1}) from RMS$\Delta\delta_{H\alpha}$	free energies[a] (kJ mol^{-1}) from $\Delta\delta^{Gly}$ Hα
$\Delta\Delta G°_A$	−1.8 (0.2)	−1.5 (0.2)
$\Delta\Delta G°_B$	−0.8 (0.2)	−0.3 (0.2)
$\Delta\Delta G°_C$	−1.4 (0.2)	−1.6 (0.2)
$\Delta\Delta G°_D$	−0.4 (0.2)	−0.4 (0.2)

[a] $\Delta\Delta G°$ values calculated directly from estimated populations of folded state at 298 K; maximum error calculated from uncertainty in RMS$\Delta\delta_{H\alpha}$ and $\Delta\delta^{Gly}$ Hα values.

Table 3: Energetics of Ion Pairing Interactions (E_1 and E_2) and Hydrophobic Interaction (H_1) in Aqueous Solution

	$\Delta G°$ (kJ mol^{-1}) (RMS$\Delta\delta_{H\alpha}$)	$\Delta G°$ (kJ mol^{-1}) ($\Delta\delta^{Gly}$ Hα)
E_1	−1.0 (0.2)	−1.2 (0.2)
E_2	−0.4 (0.2)	−0.4 (0.2)
H_1	−0.8 (0.2)	−0.3 (0.2)

folded population of the hairpin. These two protons are magnetically nonequivalent in the folded state but the chemical shift difference between them ($\Delta\delta^{Gly}$) decreases as the proportion of random coil conformation increases. The two signals for peptide **1** at pH 5.5 and 298 K are well resolved ($\Delta\delta^{Gly} \approx$ 155 Hz at 500 MHz), but begin to coalesce at higher and lower temperatures. The temperature-dependent stability profile, reflected in the magnitude of $\Delta\delta^{Gly}$, is very similar to that observed using the RMS$\Delta\delta_{H\alpha}$ approach, with both showing the same stability maximum at ∼303 K. The temperature-dependent stability profiles for both peptides at pH 2.2 and 5.5 are shown in Figure 5b; the data have similarly been fitted using a two-state approximation, and all thermodynamic parameters for folding derived using the two methods are shown in Table 1.

***E*$_1$, *E*$_2$, and *H*$_1$ Contributions to Hairpin Stability.** Estimates for the differences in hairpin stability at the different pHs, as sketched out in Figure 4, are shown in Table 2; calculated values for the energetic contributions of E_1, E_2, and H_1, derived from eqs 3−6, are presented in Table 3. The two data sets are remarkably consistent. The analysis shows that the energetic contributions from the three terms are small (<1.5 kJ mol^{-1}), but all contribute favorably to hairpin stability. The electrostatic interaction between Lys1 and the C-terminal carboxylate group appears to make the primary contribution to stability, which we estimate to be 1.0−1.2 kJ mol^{-1}. In contrast, the side chain of Lys2, which is conformationally more restricted in its interaction with the carboxylate group, contributes less energy, 0.4 kJ mol^{-1}. This is clearly evident from the relatively small effect of pH on the stability of peptide **2**. The hydrophobic contribution of the Lys1 side chain is determined in the range 0.3−0.8 kJ mol^{-1} and is comparable with the electrostatic contribution of E_2. The hydrophobic contribution from the burial of the aliphatic portion of the Lys1 side chain is clearly an important part of the analysis. Despite the terminal positions of the residues between which the interactions take place, which intrinsically lead to a greater degree of conformational flexibilty, the sum of these interactions contributes significantly to the population of the folded conformation. Ionic interactions, even in solvent-exposed sites where there is competition with water molecules, have a significant stabilizing influence.

The destabilization of β-hairpins through the loss of an electrostatic interaction has been examined for a number of model peptides, and similar, though qualitative, conclusions have been reached.[19] How do our estimates of apparent interaction energies compare with quantitative estimates from other systems? Detailed analysis using protein engineering methods to dissect out pairwise interaction energies suggests that the contribution of salt bridges is quite variable and context-dependent. Surface-exposed ion pairs have been shown to contribute relatively little, 0−2 kJ mol^{-1},[20] but in other cases interaction energies are more significant, 0.9−5.3 kJ mol^{-1}.[2] Partially buried ion-pairs in T4 lysozyme have been shown to make large contributions to protein stability, 12−21 kJ mol^{-1}.[21] Glu−Lys interactions on the surface of α-helical peptides have also been analyzed quantitatively and reveal interaction energies of 0−2 kJ mol^{-1}.[22,23] Our estimate of 1.0−1.2 kJ mol^{-1} for the interaction between the two terminal β-hairpin residues is consistent with values determined for analogous solvent-exposed sites in both proteins and α-helical peptides.

Enthalpic and Entropic Contributions to Folding: Origin of β-Hairpin Stability. The β-hairpin peptides described in this study represent a unique family that has proved amenable to thermodynamic analysis. These hairpins show the unusual characteristic of having a stability maximum with subsequent unfolding occurring both above and below room temperature. The pronounced curvature in plots of $\Delta G°$ versus T is characteristic of a significant change in heat capacity between folded and unfolded states that is usually interpreted in terms of the hydrophobic interaction contributing significantly to folding in aqueous solution.[18] A more detailed analysis of the changes in $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ that accompany folding are consistent with this model as is evident from the data in Table 1, and as previously discussed.[15,16]

While the stability of the folded conformations of peptides **1** and **2** has been shown to be sensitive to pH, the thermodynamic signature for folding remains very similar with a significant $\Delta C_p°$ for folding evident in all cases, with small enthalpy and entropy terms. This is in marked contrast to the stability profile of peptide **1** in 50% aqueous methanol, where folding is strongly enthalpy-driven with a compensating large negative entropy term which we have associated with the much larger conformational restriction of the peptide backbone associated with stronger electrostatic (hydrogen bonding) interactions between the β-strands.[15] The marked curvature in the $\Delta G°$ versus T plots for peptides **1** and **2** in water at the different pHs, each giving a characteristic negative $\Delta C_p°$ for folding, emphasizes the important contribution of the hydrophobic effect to hairpin stability in all cases. While we are confident in interpreting small changes in folded populations ($\Delta\Delta G°$ values) from these data to evaluate contributions from E_1, E_2, and H_1, the corresponding enthalpic and entropic contribution to each of these parameters are subject to larger uncertainty such that errors are likely to be at least of the same magnitude as any differences we wish to measure. We have presented some discussion of error analysis in the Materials and Methods Section.

Two-State Folding of β-Hairpin Peptides. An important assumption in the above analysis is that folding approximates reasonably well to a two-state process. We have attempted to justify this approximation in this and previous work.[15,16] To support our conclusions, recent studies of the folding kinetics

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of a β-hairpin peptide, monitored by temperature-jump tryptophan fluorescence experiments, have identified a single-exponential relaxation process indicating a unique effective kinetic barrier separating the folded and unfolded states.[24] These authors suggest that such a bimodal population distribution (two-state behavior) can be explained by "growing" the hairpin from a nucleating β-turn. A simple statistical mechanical model was presented that offers a structural framework for this two-state behavior.

In support of the nucleating effects of the turn sequence in hairpin folding, we have shown from NMR studies of a truncated 11-residue peptide analogue of **1** (residues 6−16), in which the N-terminal β-strand has been deleted,[16,25] that the SINGKK sequence significantly populates a type I′ turn in water. Thus, in the absence of significant interstrand hydrogen bonding or hydrophobic interactions the turn appears to be predisposed for β-hairpin formation.

In this study, we have shown that the β-hairpin peptide folds and unfolds in response to a single mutation or a change in pH, which we interpret in terms of a two-state process. The system described is sufficiently sensitive that changes in folded population are readily detected in response to the small structural perturbations. We have estimated the magnitude of the electrostatic and hydrophobic contributions by their effects in perturbing the overall stability of the hairpin. The cooperative folding model requires that the interactions between residues must be "linked", that is, any change to the system at one site will affect the energetics of other interactions along the hairpin.[26] For this reason the parameters that we measure must be interpreted as *apparent* binding contributions to hairpin stability rather than *intrinsic* interaction energies between isolated functional groups. By examining interactions between the ends of the hairpin we have endeavored to leave the linear array of interactions at the core of the structure relatively unperturbed by the effects of sequence mutation and changes in ionization state of individual functional groups. Despite the fact that the interactions considered in this analysis are between the terminal residues, they have a significant and quantifiable effect on hairpin stability. We have shown that a model β-hairpin can provide a useful vehicle to derive numerical estimates of apparent binding contributions in a weakly interacting system.

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