Quantitative Evaluation of Stabilizing Interactions in a Prenucleated α-Helix by Hydrogen Exchange

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The α-helix[1] is a major feature in the native structure of proteins,[2] as well as in early intermediates in protein folding.[3] Inspection of the high-resolution crystal structures of proteins suggests numerous interactions that affect the stability of α-helices, including H-bonds,[4] capping interactions,[4,5] salt bridges,[6] hydrophobic clusters,[7] and helix dipole effects.[8] A clear understanding of protein folding will require quantitative information on the contribution of each of these interactions to the free energy of the folded state of a protein. Mutational analysis of proteins[9] and comparisons of the helix content in sequence variants of peptide models[10] have been used to approach this problem. Results obtained with proteins are limited by the difficulty of engineering specific, perturbation-free changes in the native structure and by the problem of residual structure in the unfolded state.[11] Analysis of data from peptide models depends on assumptions concerning helix nucleation and propagation[3,b,10] and quantitative interpretation of circular dichroism spectra for small peptides with fractional helicity.

The relationship between the slowing of peptide hydrogen exchange (HX) rate and equilibrium thermodynamic stability offers, in principle, a direct route to evaluation of individual helix stabilizing interactions.[10c,12,13] HX slowing can be expressed in terms of a protection factor, $P = k_{rc}/k_{obs}$, where k_{obs} is the observed rate of exchange and k_{rc} is the reference free peptide rate.[12b] The reference rate, k_{rc}, has now been accurately determined for all the naturally occurring amino acids as a function of sequence, pH, temperature, and hydrogen isotope.[14] When H-exchange exhibits EX2 behavior,[13] the parameter P relates to the equilibrium constant for the conformational H-bond breaking reaction. Therefore, analysis of the P factors determined for individual peptide NHs in a model helix can, in principle, reveal the free energy of stabilizing interactions to a resolution of individual amino acids.

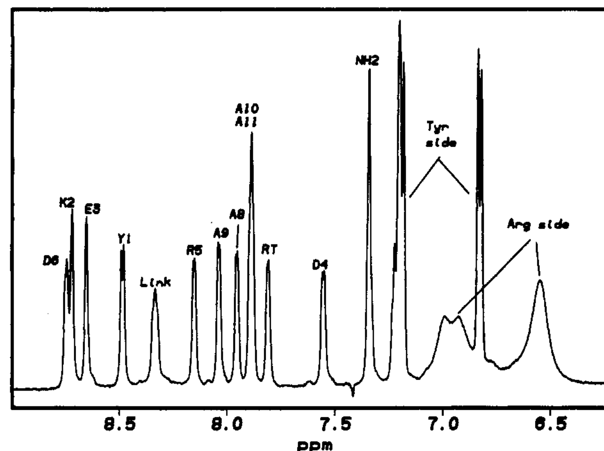

Figure 1. 1D ^{1}H-NMR spectrum illustrating the resolution of the exchangeable amides in the XA4 oligopeptide. Resonance assignment used 2D TOCSY experiments to identify spin systems and NOESY experiments to reveal short interproton distances.

The measurement of P factors for individual sites in a model helix proves difficult in practice. In 1D NMR spectra, amide resonances tend to be poorly dispersed.[15] Two-dimensional NMR spectroscopy is time consuming and usually inadequate to monitor fast exchanging amides. To overcome these limitations, we have designed and synthesized an 11-residue peptide that forms a stable helix, called XA4, with sequence as follows:

Ac-Tyr Lys Glu Asp Arg Asp Arg Ala Ala Ala Ala-NH_2

The bar indicates a covalent amide linkage connecting the side chains of Lys2 and Asp6. Covalent cross-bridges have been demonstrated to promote helical structure in oligopeptides.[16] Other helix stabilizing interactions in XA4 include a salt bridge connecting Glu3 and Asp4 to Arg7, capping of the helix N-terminus at the amide NHs of Glu3 and Asp4 by the acetyl CO, and capping by the C-terminal carboxamide.

Figure 1 shows that this strategy successfully resolves almost all the amide resonances in the 1D ^{1}H-NMR spectrum. Except for the Ala10–Ala11 overlap, all the backbone NHs, the cross-bridged NH, and the carboxyl-terminal NH_2 protons can be distinguished. The circular dichroism spectrum shows typical helical character in the far ultraviolet region, but quantitative interpretation in terms of helix content is complicated by the peptide's short length, the presence of the terminal tyrosine side chain, and the cross-bridging amide group. The presence of a complete H-bonded structure is affirmed by the hydrogen exchange results described below. Systematic NOE cross peaks, including NH(i) to NH(i + 1) and CαH(i) to NH(i + 3) connectivities, document helical conformation that extends through the length of the peptide (possibly excepting Tyr1). That

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this represents α-helix and not 3–10-helix is shown by CαH(i) to NH(i + 4) NOEs and the absence of CαH(i) to NH(i + 2) NOEs (except for Tyr1 to Glu3).

Hydrogen–deuterium exchange results were obtained at each amide NH position by mixing the peptide into D_2O and recording 1D spectra in time. The HX rate measured for the overlapped Ala10–Ala11 resonance was assigned to Ala10. The rapidly exchanging C-terminal amide NH_2 protons were measured by saturation–recovery; one proton exchanges at the free amide rate while the other, slowed by ~2-fold, effectively contributes one residue unit to the C-terminal stabilization. The prenucleated character of the helix allows the data to be treated using a Zimm–Bragg[18] nucleation constant, σ, near unity so that the fully helical chain is assigned a statistical weight of $\sigma\prod(S_i)$, where the S_i denotes helix propagation constants for each residue on the peptide.[18] The opening probability at each site is then computed as described previously[10a] to fit S values for each of the alanine residues in the C-terminal fray.

Figure 2 shows values of $-RT \ln (P - 1)$, the free energy associated with the HX slowing factors P, at all but one of the positions in the helix.[12] At the N-terminus, the Tyr1 NH exchanges at close to the free peptide rate. Lys2 was too fast to be measured accurately, as expected if it is not involved in an H-bond.[14] The dramatic onset of a large and nearly identical HX slowing at Glu3 and Asp4 indicates that the N-acetyl carbonyl cooperatively caps the NHs of these two residues. The ~20-fold HX protection indicates a free energy for this interaction of ~1.6 kcal/mol on an absolute scale. HX slowing reaches a maximum at Asp6, which has both its donor NH and its acceptor carbonyl within the covalent cross-bridge. Residues in the C-terminal half of the helix exhibit classical fraying behavior, reflecting sequential opening of individual H-bonds from the C-terminal end of the helix. The fraying behavior of the C-terminyl alanines points to a Zimm–Bragg S value for alanine of 1.7 ± 0.2.

The S values found in these studies are significantly greater than those obtained from some model systems[19] but are close to other values in the literature. Doig et al.[20] measured the effect of an N-terminal acetyl group on the helical ellipticity signal of a partly helical, 18-residue, alanine-based polypeptide (0 °C, pH 9.5) and computed a value of 1.2 kcal/mol relative to an alanine "capping" reference. Rohl et al. found an S factor of 1.5 for the alanine H-bonding interaction in a hydrogen–deuterium exchange study of unresolved peptide NHs in a family of alanine copolypeptides with serially increasing chain lengths,[10c] consistent with recent results using ^{15}N-edited NMR to resolve the exchange of individual residues.[21]

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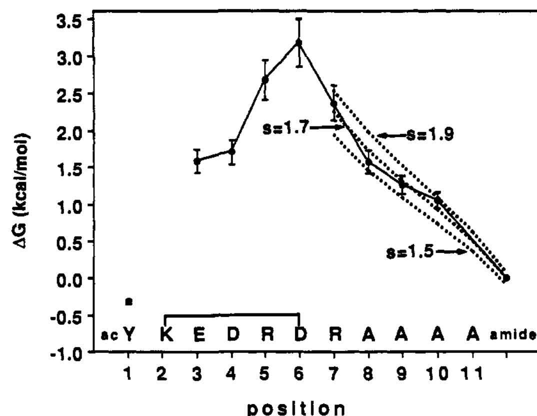

Figure 2. Free energy associated with protection factors of the NH proton at each position in XA4 except for the NHs of Lys2 and Ala11 (5 °C). The dotted lines illustrate fits to the C-terminal fray with the S values shown. Spectra were recorded on a Bruker AM500 spectrometer. ^{1}H–^{2}H exchange was initiated by passing a sample of the peptide in H_2O buffer through a small column of Sephadex G10 (Pharmacia) equilibrated with D_2O buffer in a benchtop centrifuge at 5 °C.[22] NMR data were processed on a Silicon Graphics workstation using the program Felix (Hare Research). HX rates were obtained from the time-dependent single exponential decay in NH amplitude. HX protection factors ($P = k_{rc}/k_{obs}$) were obtained by comparing rates observed to rates expected in an unstructured peptide with the same sequence (k_{rc}).[14a] For this calculation, the side-chain effect of Asp6, which is involved in the covalent link, was approximated by using the side-chain correction appropriate for asparagine. The k_{rc} values for the carboxamide protons were obtained from the peptide Ac-Ala-Ala-Ala-NH_2.

The results described here show that HX measurements in stable α-helices short enough to afford resolution of individual amides by 1D NMR spectra open a general avenue for determining the free energies of specific helix stabilizing interactions. Accuracy of the determination depends on measurements of HX rate constant and the accuracy of reference values used to calculate P. An overall error of 10% in P leads to an error of 0.06 kcal/mol in ΔG. Application of the method to the determination of other helix stabilizing interactions will be detailed elsewhere.

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