Use of Aromatic Amino Acid Residues To Restrict the Dynamics in the Hydrophobic Core of a Designed Helix−Loop−Helix Dimer

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The design of polypeptides and proteins with well defined tertiary structures remains a considerable challenge,[1,2] and to date, only a single designed four-helix bundle has been reported[3] that fulfills criteria used to discriminate proteins from molten globule and "gemisch"[4] states. The reason may be the predominant use of isoleucine, leucine, and valine residues in the hydrophobic core which is a common design feature of the overwhelming majority of designed four-helix bundles.[5,6] The side chains of these residues have low barriers to rearrangement and may be quite mobile in the hydrophobic core of designed proteins. In this paper, we wish to report that for the first time aromatic amino acid residues have been successfully used as the main design principle to restrict the internal dynamics of a four-helix bundle.

GTD-43, a polypeptide with 43 amino acids (Figure 1) was designed to fold into a helix−loop−helix motif. The key design feature was the use of the aromatic residues Phe-10 and Trp-13 in helix I and Phe-34 in helix II to stabilize the hydrophobic core. GTD-43 has a well-dispersed NMR spectrum and is in slow exchange on the NMR time scale (Figure 2). It also has a narrow temperature interval for thermal denaturation.[7] It appears therefore that the use of the conformationally restricted side chains in the hydrophobic core provides a significant improvement in protein design. The results suggest that this principle may be of general use in the design of hydrophobic interfaces between amphiphilic helices in the engineering of proteins with well-defined tertiary structures. The incorporation of the restraining residues within the hydrophobic core also represents a significant improvement over existing four-helix bundle designs in the development of functionalized proteins, since the exposed face of the helix−loop−helix motif can be kept free from reactive residues.

The ^{1}H NMR spectrum of GTD-43 (Figure 2) shows narrow lines and good shift dispersion in aqueous solution at pH 3.0 and 288 K. At 298 K the spectrum shows decreased resolution and increased linewidth and at 288 K GTD-43 therefore approaches slow exchange on the NMR time scale. Molten globules show the opposite behaviour with decreased line widths with higher temperature indicative of fast exchange between a large number of conformers.[6] The assignments of the amino acid residues, the sequential assignments, and some stereospe-

S-L-Aad-A-Nva-L-Q-E-A-F-R-A-W-L-Q-Y-H-A-A-K
1 20

G-T-G-P
21 24

A-Q-D-Q-E-A-L-R-A-F-A-Aad-Q-L-Nva-A-K-I-N
25 43

GTD-43

Figure 1. The amino acid sequence of GTD-43. The one-letter code is used for the natural amino acid residues where A is Ala, D is Asp, E is Glu, F is Phe, G is Gly, H is His, I is Ile, K is Lys, L is Leu, N is Asn, P is Pro, Q is Gln, R is Arg, S is Ser, T is Thr, W is Trp, and Y is Tyr. Aad is α-aminoadipic acid; Nval is norvaline.

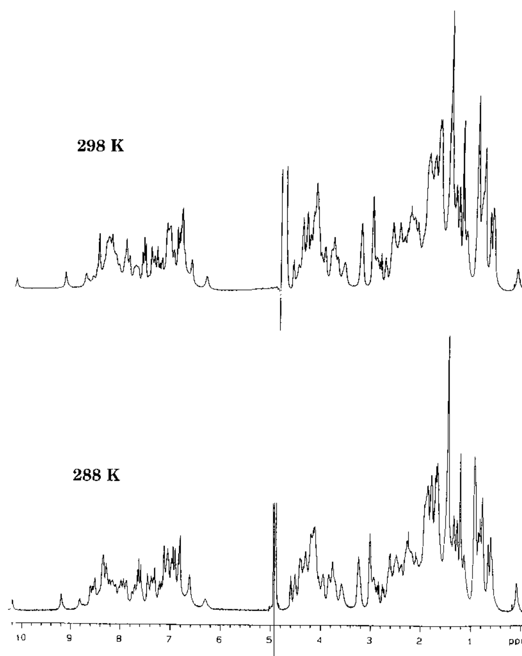

Figure 2. The 500 MHz ^{1}H NMR spectrum of GTD-43 recorded at pH 3.0 in 90% H_2O/10% D_2O at 288 and 298 K. The spectrum is well resolved, and it has been assigned at 288 K. With increased temperature, the line width is increased and the shift dispersion is partially lost showing that GTD-43 is in slow exchange on the NMR time scale. See for example the amide and aromatic regions from 6.6 to 8.6 ppm and the methyl region from 0.1 to 1.0 ppm.

cific assignments of β protons have been obtained from the TOCSY, DQFCOSY, and NOESY spectra. Some spectral parameters, such as the chemical shift dispersion, may be compared to those of native proteins. The chemical shift range of for example the NH protons of GTD-43 covers 2.2 ppm[7] which may be compared with the 2.7 ppm of the NH region of the native protein IL-4.[8] So far, 176 NOEs have been identified with 18 long-range, 60 medium-range, and 98 sequential ones. The observed NOEs show that GTD-43 folds into two helical regions connected by a short loop, since the fingerprint NOEs typical of helix formation (i.e., the medium range NOEs) have been observed in the helical segments.[7] Preliminary distance−geometry calculations followed by simulated annealing converge into a group of structures show the designed hairpin helix-loop-helix motif of the NH region. A full account of the NMR structure determination is in preparation.

The melting point[7] of GTD-43 shows irreversible denaturation as the polypeptide precipitates in solution at 338 K, and lowering the temperature does not bring GTD-43 back into solution. The sharp decrease of the negative value of the mean residue

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ellipticity (θ) observed above 323 K indicates a relatively narrow temperature range for denaturation. Since GTD-43 precipitates, the mean residue ellipticity of the thermally denatured peptide cannot be obtained, but the melt starts at 50−55 °C when θ_{222} for GTD-43 is −15 000 deg cm^2 dmol^{-1} and precipitation occurs at 65 °C when θ is −6000 deg cm^2 dmol^{-1}. To our knowledge the reported values of θ_{222} of denatured helix−loop−helix dimers are all negative, and the transition for GTD-43 is therefore probably more than halfway at the point of precipitation, suggesting a temperature interval for complete denaturation of only 20−30 °C. The measured mean residue ellipticity at 222 nm was −20 000 deg cm^2 dmol^{-1}, which is similar to what has been measured for other designed four-helix bundles.[5,6] The concentration of GTD-43 was determined from the extinction coefficients of Trp and Tyr at 280 nm (5559 and 1197 M^{-1} cm^{-1}, respectively) in the presence of 6 M guanidine hydrochloride at pH 7. The measured helical content was thus in agreement with that expected from a helix−loop−helix motif.

The CD spectrum was independent of concentration in the range from 4 to 195 μM at 298 K. Equilibrium sedimentation ultracentrifugation[7] at 37 μM concentration of peptide showed that the apparent molecular weight was 8050 ± 200. After correction for nonlinearity in the detection system by multiplying with a calibrated factor of 1/0.92, the experimentally determined apparent molecular weight was 8970 ± 200. Electrostatic repulsion in the salt-free solution is known to reduce the apparent molecular weight by 5−10%, and the experimental value corresponds well to twice the molecular weight of GTD-43, which is 9544. GTD-43 is therefore a dimer that has to be symmetric since only one set of resonances is observed in the ^{1}H NMR spectrum. No concentration dependence of the apparent molecular weight was found. Under the assumption that a concentration independent CD spectrum between 4 and 195 μM concentration can be interpreted to mean that there is less than 5% of monomer in solution at 4 μM concentration of peptide, the upper limit of the dissociation constant can be estimated to be 2×10^{-8} M.

Weak ANS (8-anilino-1-naphthalenesulfonic acid) binding was observed for GTD-43, although an equilibrium constant could not be obtained since the polypeptide precipitated at high concentrations of ANS. Thus, although the temperature dependence of the ^{1}H NMR spectrum shows that GTD-43 is better defined structurally than a molten globule, some region of the hydrophobic core may still be imperfectly packed.

GTD-43 was designed to fold into a helix−loop−helix motif. Currently accepted design principles for amphiphilic helices and helix−loop−helices have been summarized by Bryson et al.[1] Both helices were designed to form mutually complementary hydrophobic interfaces in the folded state.

It has previously been proposed that complementary packing alone of the hydrophobic regions in four-helix bundles is not enough to induce protein-like properties in polypeptides, but that it is also necessary to increase the free energy of the closely similar conformers.[9] In order to restrict the conformational degrees of freedom in the GTD-43 dimer, the aromatic residues Phe-10 and Trp-13 were incorporated in helix I and Phe-34 in helix II, Figure 3. In a hairpin helix−loop−helix conformation in the folded state, Phe-34 may interact with both Phe-10 and Trp-13. The side chains of Phe and Trp are more conformationally restricted than those of leucines, and the reorganization of helix−helix interfaces may therefore give rise to larger energy barriers. Aromatic stacking interactions may also contribute.

A leading design principle of GTD-43 was the extensive variation of the amino acid composition (Figure 1) in order to facilitate the assignment of the ^{1}H NMR spectrum and the determination of the solution structure.

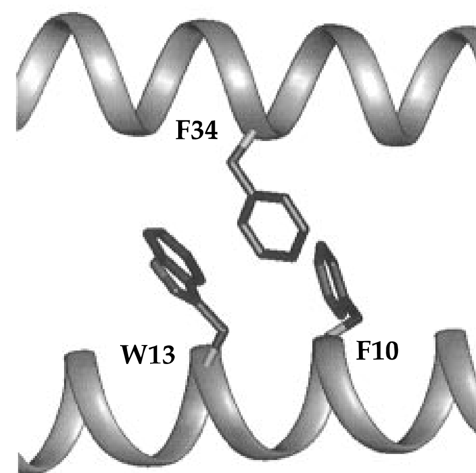

Figure 3. Modeled representation of the spatial organization of Phe-10, Phe-34, and Trp-13 in the hydrophobic interface of GTD-43. The aromatic ring of Phe-34 fits into the cavity formed between Phe-10 and Trp-13.

On a more subtle level of design the helical propensities of the helical regions were kept relatively low to allow a more cooperative folding process.

GTD-43 was designed in a rational way where the main emphasis was placed on mutual complementarity of the hydrophobic interfaces, the reduction of internal mobility by Phe and Trp and the overall helical propensity of the helical segments. α_2D was designed according to similar principles[3] but with a different strategy to reduce internal mobility. Two charged residues on the periphery of the hydrophobic region were designed to interfere with the hydrophobic residues in case of helix−helix rearrangements. α_2D, too, has two Phe and one Trp residues but not in positions to interact the way they can in GTD-43, since they are too far apart, and the authors do not mention the aromatic interactions as being a design principle. A different approach was taken recently when a library of four-helix bundles was engineered where the hydrophobic amino acid residues were varied in a random way.[10] Phenylalanines appear in some of the mutants to be able to form similar aromatic interactions as Phe-10, Phe-34, and Trp-13 in GTD-43, and it will be interesting, when more structural information is available, to see whether these appear in native-like proteins that may well be found in the large number of engineered proteins. So far, however, the positions used to incorporate hydrophobic residues have been strictly limited to fixed positions in each helix, not including those that have been used in the design of GTD-43.

The synthesis of GTD-43 was carried out on an automated peptide synthesizer using *t*-BOC-protected amino acids. It was cleaved using anhydrous HF on a Teflon vacuum line and purified by size exclusion and reversed phase HPLC. It was identified by electrospray MS (measured 4774.6, theoretical 4772.4). The NMR spectroscopic measurements were carried out at 500 MHz (Varian Unity 500) in aqueous solution at pH 3.0. The CD spectrum of GTD-43 was recorded on a JASCO-720 circular dichroism spectrometer in aqueous solution at 298 K.

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Supporting Information Available: Experimental and spectroscopic details (8 pages). See any current masthead page for ordering and Internet access instructions.

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