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De novo design and structural characterization of an α-helical hairpin peptide: A model system for the study of protein folding intermediates

(secondary structure/tertiary structure/diffusion collision/framework/molten globule)

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ABSTRACT **The *de novo* design and structural characterization of an α-helical hairpin peptide (α-helix/turn/α-helix, ατα) are reported. The peptide is intended to provide a model system for the study of the interactions of secondary structural elements during protein folding. Both the diffusion-collision and framework models of protein folding envision that the earliest intermediates in protein folding are transient secondary structures or microdomains which interact and become mutually stabilizing. Design principles for the ατα peptide were drawn from the large body of work on the structure of peptides in solution. Computer modeling was not used in the design process. Study of ατα by circular dichroism and two-dimensional nuclear magnetic resonance indicates that the designed peptide is monomeric, helical, and stable in aqueous solution at room temperature. Analysis of two-dimensional nuclear magnetic resonance experiments indicates that the two helices and the turn form in the intended positions and that the helices associate in the designed orientation. Development of ατα represents an advance in protein design in that both the secondary structural elements and designed tertiary interactions have been realized and can be detected in solution by nuclear magnetic resonance. The resulting model system resembles a protein folding intermediate and will allow the study of interacting helices in a context that approximates an early stage in protein folding.**

A principal goal in protein folding studies is to determine the mechanism of folding and the properties of folding intermediates. Protein folding is postulated to begin with the formation of individual secondary structures (microdomains) (1, 2). These transient structures are envisioned to be stabilized by packing against each other; later steps continuing in a hierarchical manner until the native structure is achieved (1, 3). Folding intermediates are difficult to study because they are minimally populated in equilibrium experiments and transiently populated in kinetic experiments.

An alternative approach is to use natural or designed peptides as model systems, the aim being to reduce the complexity of the folding system so as to isolate and characterize folding intermediates and study the kinetics of their formation. Peptides representing isolated structural elements of proteins such as turns (4) and helices (5) have been extensively studied. Linked elements of secondary structures have been less studied. A disulfide-linked helix/sheet model based on the structure of bovine pancreatic trypsin inhibitor has been developed (6). A peptide containing two helices connected by a turn region has been used as a stage in an incremental strategy to produce a four-helix bundle protein (7). Disulfide-linked coiled coils have been used to study the contribution of hydrophobic interactions to stability (8), and an amphiphilic peptide containing minimalist helical regions (Lys-Lys-Leu-Leu) and an Asn-Pro-Gly turn region has been used to study conformational changes induced by salt and pH (9). The peptides containing linked helices used minimalist sequences (sequences containing only a few different amino acids which are repeated) in the helical regions, which has the drawback of complicating the study of the solution structure by nuclear magnetic resonance (NMR) due to spectral overlap (10, 11). For this reason the structural attributes of these models were studied primarily with circular dichroism (CD) and the geometry of any helix association in solution is not known with certainty.

The goal of the present work was to develop by *de novo* design an α-helical hairpin peptide to be used as a model of an early protein folding intermediate. The peptide, ατα (α-helix/turn/α-helix), was designed to have a protein-like sequence (12) and be amenable to NMR structure determination. The initial structural characterization, reported here, of ατα by CD and two-dimensional NMR shows that the helices form and associate in the designed orientation, suggesting that the design goals have been achieved.

MATERIALS AND METHODS

Peptide Synthesis and Purification. ατα was initially synthesized by David Teplow at Harvard Medical School Brigham and Women's Hospital on an Applied Biosystems 430A peptide synthesizer using fluoren-9-ylmethoxycarbonyl chemistry and modified synthesis cycles. The peptide was subsequently synthesized at the 0.25-mM scale at the Rowland Institute with an Applied Biosystems 431A peptide synthesizer employing fluoren-9-ylmethoxycarbonyl chemistry and modified synthesis cycles developed at the institute. Modification of the side chain of Trp^2 by side-chain blocking groups from the three Arg(Pmc) and three Gln(Trt) residues was a problem during cleavage of the peptide from the resin (Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Trt, trityl). Cleavage of the peptide from the resin with a mixture of trifluoroacetic acid, water, ethanedithiol and thioanisole (85:5:5:5) for 3.5 hr minimized the modification of the Trp^2 side chain. The peptide was purified on a Vydac C_{18} reversed-phase column (22 mm × 25 cm) by using a 40-min linear gradient of 41–55% acetonitrile in 0.1% trifluoroacetic acid. Amino acid analysis of purified ατα gave the expected amino acid composition, and the molecular weight was confirmed by electrospray mass spectroscopy. Peptide concentrations were determined from triplicate amino acid analyses.

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Abbreviations: NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy.

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CD Measurements. CD spectra were acquired on a model 62D Aviv spectropolarimeter in the laboratory of Don Wiley, Harvard University. Typical spectra were collected with 50 μM $\alpha t\alpha$ peptide in 20 mM NaCl/1 mM phosphate buffer, pH 3.6, in a 1-mm-path-length rectangular cell. Temperature was controlled with a Hewlett–Packard Peltier device interfaced to an IBM PC computer. Ellipticity is reported as per residue molar ellipticity, θ (deg·cm^2·dmol^{-1}).

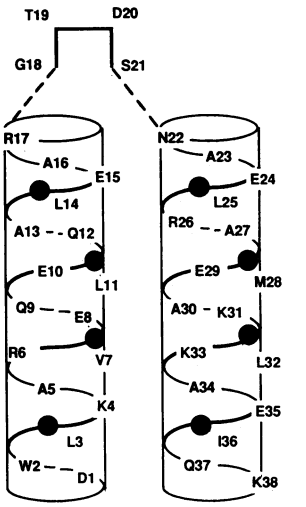
Two-Dimensional NMR. Two-dimensional NMR spectra were collected at 25°C on a JEOL GX-400 NMR spectrometer. Samples consisted of 3 mM $\alpha t\alpha$ in 20 mM NaCl, pH 3.6, in 10% ^2H_2O/90% H_2O. Data were collected in the phase-sensitive mode according to States *et al.* (13). Thirty-two scans of 2048 complex points were accumulated for each of 256 $t1$ values. The spectral width was 4201.7 Hz in both dimensions. The spectra were apodized with 45° phase-shifted sine bells in both dimensions and were zero-filled to yield 2048 × 2048 real matrices. Spectra were referenced to internal sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4. Spectra were processed on a Stardent Titan computer with software developed at the Rowland Institute (14). Spectra were assigned using purged correlated spectroscopy (P.COSY) (15), total correlated spectroscopy (TOCSY) (16), and nuclear Overhauser effect spectroscopy (NOESY) (17) and standard sequential assignment techniques (18). The sequential assignment process was aided by the use of the EASY software (19) running on a Sun 3/60 computer. Assignments will be published elsewhere.

RESULTS

Design of $\alpha t\alpha$. $\alpha t\alpha$ is the second attempt from this laboratory to synthesize connected helices. The first attempt involved a minimalist design (20) in which just six amino acids were used: Ala, Leu, Glu, Lys, Trp, and Gly. The helices were made up of Ala, Leu, Glu, and Lys and were connected by a simple "tether" consisting of four Gly residues. A single Trp residue was included in one helix as a fluorescence probe. The peptide was not completely free of aggregation, and preliminary two-dimensional NMR experiments indicated that it would be very difficult, if not impossible, to assign the peptide, much less determine anything about the secondary or tertiary structure. The minimalist approach was abandoned at this point in favor of a design which would contain a variety of amino acids and lead to a peptide open to NMR structure determination. $\alpha t\alpha$ is our first attempt at such a design.

$\alpha t\alpha$ was designed to contain two helices of 17 amino acids each [within the range of helix lengths observed in four-helix bundle proteins (21)] and a turn region consisting of 4 amino acids (Fig. 1). The side chains of the hydrophobic amino acids in the interface were arranged to interact in a manner similar to that observed in coiled-coil peptides, although the strict heptad repeat of coiled coils was not used in the design. Three of the amino acids in the hydrophobic interface—Leu, Ile, and Val—were chosen for their ability to support stable hydrophobic interfaces in artificial coiled-coil peptides (8). Met was also used, since it is commonly found in naturally occurring coiled-coil peptides (22). Most coiled coils have interactions between similar amino acids (a result of their being dimers associating in a parallel fashion), but the adoption of such an arrangement in the designed peptide would lead to difficulties in resolving NOEs within the hydrophobic interface. For this reason only one Leu-Leu pair was used, the rest of the interactions being between one Leu and one other amino acid: Ile, Val, or Met.

The turn residues, Gly^{18}, Thr^{19}, Asp^{20}, and Ser^{21}, were chosen as consensus residues from β-turn regions involving four consecutive amino acids in protein structures (23). Gly at position 1 of the turn serves the dual function of participating in the turn and being a strong helix-breaking amino acid (24). Pro was not included in the turn to avoid potential problems with cis/trans isomerization of the Xaa-Pro bond, which could give rise to multiple resonances in NMR spectra (4, 25). Thr, which has a high frequency of occurrence at position 2 in β-turns (23), was used instead. Asp is found more frequently in β-turns at the third position than in other positions. Ser, which has been identified as a good N-capping residue (26, 27), was used at the last position of the turn.



Sequence:

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     1                   5                  10
Succinyl-Asp-Trp-Leu-Lys-Ala-Arg-Val-Glu-Gln-Glu-Leu-Gln-Ala-
         15                  20                  25
Leu-Glu-Ala-Arg-Gly-Thr-Asp-Ser-Asn-Ala-Glu-Leu-Arg-
             30                  35
Ala-Met-Glu-Ala-Lys-Leu-Lys-Ala-Glu-Ile-Gln-Lys-NH2
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FIG. 1. Design and amino acid sequence of $\alpha t\alpha$. The hydrophobic amino acids which make up the helix–helix interface are indicated by filled circles. The helices have been twisted outward so that their hydrophobic surfaces are facing the viewer.

To disfavor dimerization or nonspecific aggregation, charged amino acids in the helices were arranged to flank the hydrophobic interface. Charged residues were also arranged so as to give possible salt bridges between the two helices. The hydrophobic parts of the long side chains of the charged amino acids could also interact with the hydrophobic core. The remainder of the positions in the hydrophilic region were filled by amino acids with good intrinsic helix-forming propensities (28). Seven Ala residues were included, three in the first helix and four in the second helix. Three residues of Gln (neutral hydrophilic amino acid with good helix-forming potential) were also employed in the helical regions.

A major design criterion for $\alpha t\alpha$ was that it be amenable to NMR experimentation. The peptide had to be monomeric at the concentrations used for NMR experiments and it had to be assignable. The problems of dimerization or association were addressed by employing a narrow hydrophobic interface surrounded by charged residues as described above. To facilitate the sequential NMR assignment of $\alpha t\alpha$, a variety of different amino acids were used (15 of the 20 naturally occurring amino acids) and care was also taken to ensure that each sequence of three amino acids was unique (12, 29).

Trp was included near the amino terminus to provide a fluorescent probe. The side chain of Lys^{38} was arranged so as to be in proximity to that of Trp^2 when the helices associate. A fluorescence acceptor group can be attached to the side-chain amino group of Lys^{38} and will allow helix association to be measured by energy transfer. This also provides a means of measuring the kinetics of helix association. A complete description of the design process will be published separately.

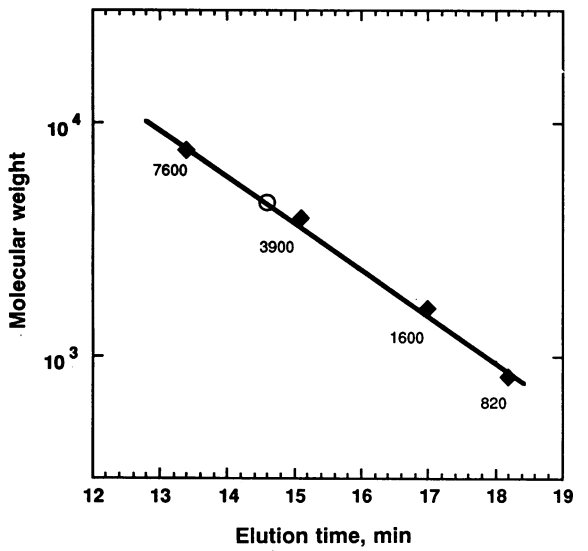

FIG. 2. Size-exclusion chromatography of $\alpha t\alpha$. Peptide standards (◆) and $\alpha t\alpha$ (○) were subjected to size-exclusion HPLC on a Waters Protein-Pak 60 column (7.8 mm × 30 cm) in 0.2 M KCl/0.05 M phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min. Absorbance was monitored at 210 nm. Under these conditions the peptide standards (◆) and $\alpha t\alpha$ (○) exhibited ideal size-exclusion behavior. Peptide standards with molecular weights of 820, 1600, and 3900 were synthetic peptides developed for use in size-exclusion chromatography (30) and were purchased from SynChrom, Lafayette, IN. The largest standard peptide is a synthetic coiled-coil peptide, Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_5$-NH_2 with Leu^{23} changed to Cys, that is related to previously described peptide models (8). This peptide dimerizes (dimer molecular weight, 7600) under the conditions used and was a gift from Colin Mant, University of Alberta, Edmonton, Canada. The line is the result of a regression analysis of the data. Although the hydrodynamic properties of the coiled-coil high molecular weight standard might be expected to differ from those of the smaller peptides, which are believed to have little structure in solution, the data are well fit by the regression line.

$\alpha t\alpha$ Is Monomeric in Solution. In order to determine the structural attributes of $\alpha t\alpha$ in solution, the peptide must be monomeric over the concentration range studied. The helical content of $\alpha t\alpha$ would likely vary if the peptide were to dimerize or to aggregate. No significant differences in θ_{222} were observed over a concentration range of 5–200 μM, nor were there variations in the chemical shifts of the amide protons from 200 μM and 5 mM (data not shown). The peptide was also subjected to molecular-sieve HPLC (Fig. 2). The results indicate that the peptide is monomeric. These experiments indicate that $\alpha t\alpha$ is monomeric over a concentration range of 5 μM to 5 mM.

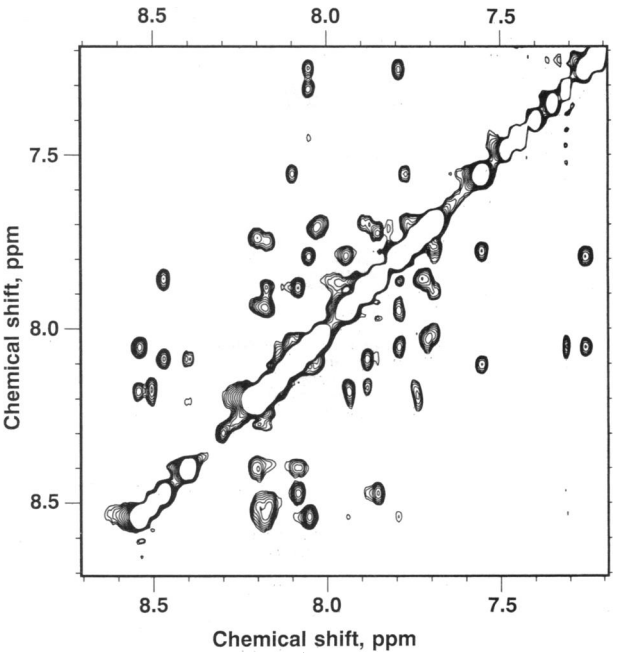

FIG. 4. Contour plot of the amide–amide region of the NOESY spectrum (mixing time, 200 msec) of $\alpha t\alpha$ at 3 mM in 10% ^2H_2O/90% H_2O in 20 mM NaCl at pH 3.6 and 25°C. Most of the off-diagonal peaks in this region are $d_{NN}(i, i+1)$ NOEs which are consistent with helix formation.

CD Spectra. CD spectra of $\alpha t\alpha$ recorded at various temperatures indicate a high proportion of helix in the peptide (Fig. 3). At 25°C, pH 3.6 $\alpha t\alpha$ was estimated to be ≈60% helical (31) and the helicity increased slightly at lower temperatures (Fig. 3*A*). In the designed structure 34 of 38 amino acids are in the helices (89% helix). This discrepancy could be due to end fraying or helix–coil equilibria. The unfolding and refolding of $\alpha t\alpha$ was examined by monitoring the CD

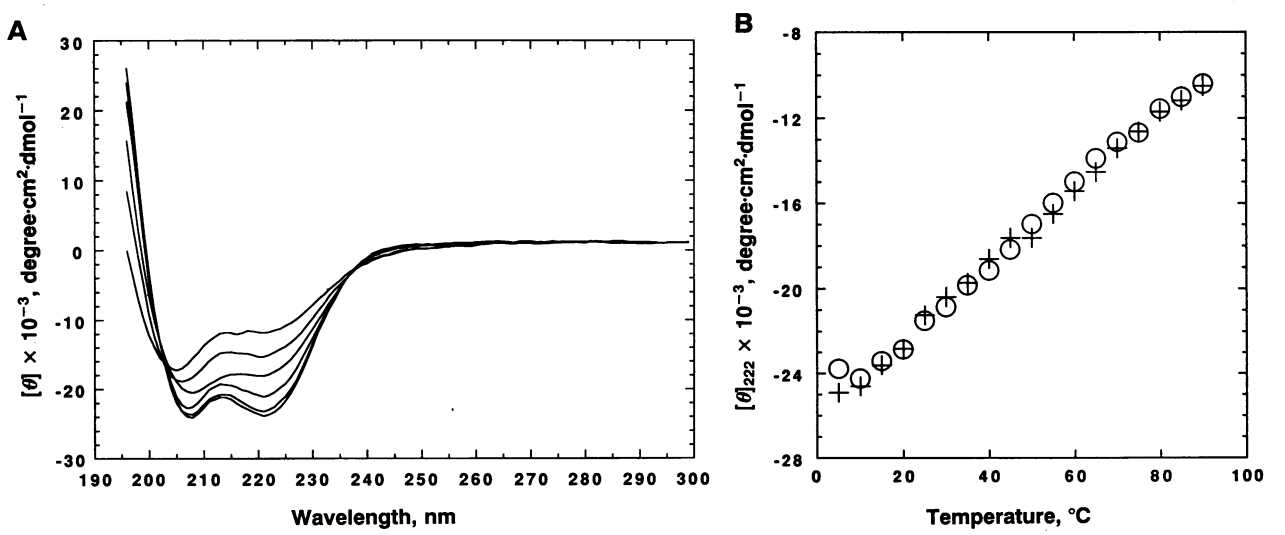

FIG. 3. CD experiments. (*A*) CD spectra of $\alpha t\alpha$ as a function of temperature. Experiments were performed with 50 μM peptide in 20 mM NaCl/1 mM phosphate, pH 3.6. Temperatures are 5, 10, 25, 40, 60, and 80°C from bottom to top. Each spectrum represents the results of a single scan and the data have been subjected to three-point smoothing. (*B*) The molar ellipticity at 222 nm of $\alpha t\alpha$ as a function of temperature under the conditions described in *A*. The symbols represent rising (○) and falling (+) temperature.

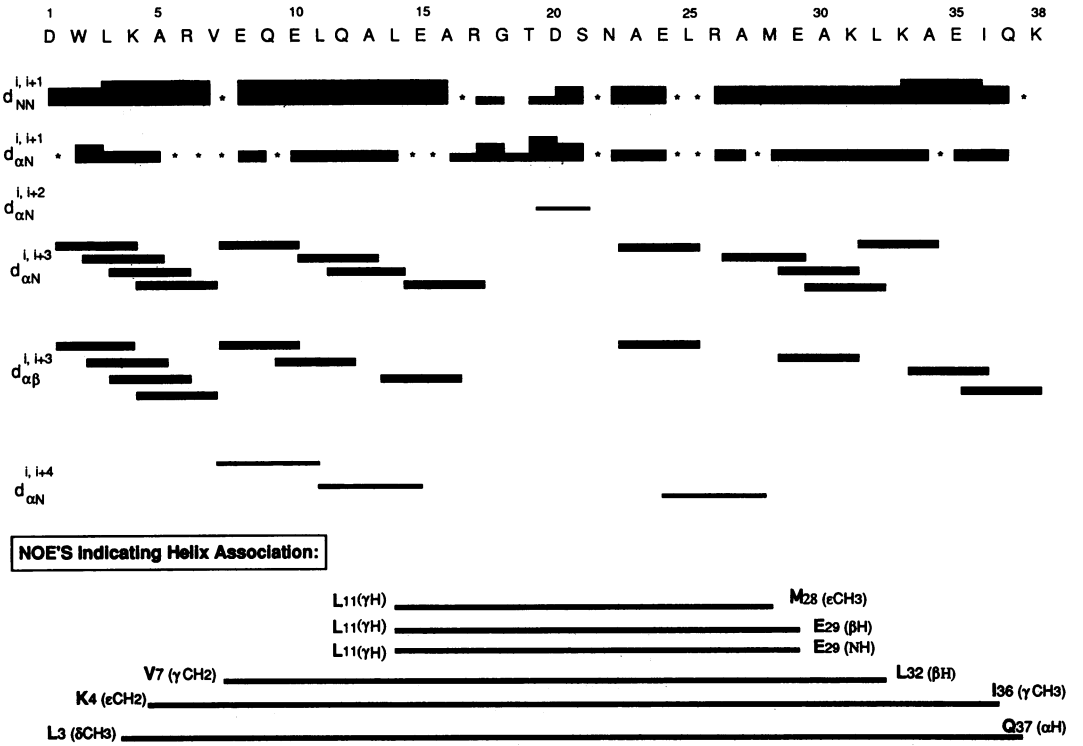

FIG. 5. Diagram of NOE connectivities in $\alpha t\alpha$. NOESY experiments were performed under the conditions described in Fig. 4. NOEs which could not be observed because of spectral overlap are indicated by stars. The relative strength of NOEs are indicated by the thickness of the lines. Amino acid residues are shown by one-letter symbols.

signal at 222 nm (Fig. 3*B*). The resulting curve shows residual helical structure in the peptide at temperatures as high as 90°C. The lack of a sharp temperature unfolding transition in $\alpha t\alpha$ is similar to the behavior of shorter monomeric polyalanine-based peptides (32), certain designed coiled-coil peptides (33), and the molten globule states of certain proteins (34, 35).

NMR Experiments. A large number of $d_{NN}(i,i+1)$ NOEs are observed in the amide–amide region of a NOESY spectrum of $\alpha t\alpha$ in water at 25°C and pH 3.6 (Fig. 4). These and other NOEs are summarized in Fig. 5. $d_{NN}(i, i+1)$ NOEs are often observed in helical regions of proteins and peptides (18, 36), although they have also been found in nascent helices as well (37). A large number of medium-range $d_{\alpha\beta}(i, i+3)$ and $d_{\alpha N}(i, i+3)$ NOEs are also detected. These medium-range NOEs require that successive turns of helix be formed, so the presence of these NOEs is more indicative of helix formation than the short-range $d_{NN}(i, i+1)$ NOEs alone (36, 37). Taken together, the $d_{NN}(i, i+1)$ NOEs and the series of overlapping medium-range $d_{\alpha\beta}(i, i+3)$ and $d_{\alpha N}(i, i+3)$ NOEs define the regions of the peptide which are helical. These NOEs indicate that the helices in the peptide extend from Asp^1 to Arg^{17} and from Asn^{22} to Lys^{38}.

NOEs are observed which are indicative of a turn at positions 18–21 (4, 18) (Fig. 5). The primary diagnostic NOE for a turn in this region is a $d_{\alpha N}(i, i+2)$ NOE which is observed between Thr^{19} and Ser^{21}. This NOE is consistent with a tight turn with Thr^{19} in position 2 of the turn and Ser^{21} in position 4. A strong $d_{\alpha N}(i, i+1)$ NOE between Thr^{19} and Asp^{20} with a weaker $d_{NN}(i, i+1)$ NOE is consistent with a significant population of a type II turn (4, 18).

A number of long-range NOEs are observed which indicate helix association. These NOEs represent close associations near three of the four interhelical pairs of hydrophobic amino acids. The Leu^{11} γ–Met^{28} ε, Leu^{11} γ–Glu^{29} β, and Leu^{11} γ–Glu^{29} N NOEs are near the designed Leu^{11}–Met^{28} hydrophobic pair. A Val^7 γ–Leu^{32} β NOE is observed between the Val^7–Leu^{32} pair, and the Lys^4 ε–Ile^{36} γ and Leu^3 δ–Gln^{37} α NOEs are near the Leu^3–Ile^{36} pair. No NOEs have been identified which indicate the association of Leu^{14} and Leu^{25}, the pair closest to the turn region, but not all of the NOEs in the NOESY spectrum have been assigned. The long-range NOEs all indicate that the helices are associating in the designed orientation. Further spectroscopic and modeling studies are necessary to obtain a detailed three-dimensional structure of $\alpha t\alpha$. However, the present data are consistent with a solution structure that closely resembles the designed structure.

DISCUSSION

The problem of developing a helical hairpin peptide system could be approached in one of two ways: by building a peptide corresponding to a hairpin structure in a protein or by *de novo* design. The decision to design a model peptide was made in order to avoid problems with aggregation (38) and marginal stability (39) that occur with peptides derived from protein sequences. A successful design could also provide a starting point for the design of larger structures such as a four-helix bundle protein.

Although $\alpha t\alpha$ is still being subjected to structural analysis, the NMR data presented here indicate that certain elements of the design have been realized. The peptide contains two helical regions which are well defined by the observation of short- and medium-range NOEs. NOEs between side-chain hydrogens on opposite helices indicate that the helices associate and that the alignment is approximately as designed. The observed NOEs are also consistent with the occurrence of a turn in the designed position.

Although the helices in $\alpha t\alpha$ are rather stable, the unfolding of the peptide by temperature is not very cooperative (Fig. 3*B*). The cooperativity of $\alpha t\alpha$ resembles that of short alanine-based peptides which have lengths similar to the lengths of the individual helices of $\alpha t\alpha$ (32), the designed coiled-coil peptides of Hodges and coworkers (33, 40), and the molten globule state of certain proteins (34, 35). The cooperativity is

less than that of longer alanine peptides whose lengths are equal to or greater than the entire αtα peptide (31). Comparison of the temperature unfolding curves of αtα and several coiled-coil peptides indicates that the stability of αtα compares favorably with the much larger synthetic coiled-coil peptides described by Hodges and coworkers (40) but is less than the versions of these peptides containing disulfide crosslinks (8). These results suggest that the hydrophobic interface contributes significantly to the stability of the helices in αtα but not to the cooperativity of unfolding. Cooperativity could arise because of extensive networks of hydrogen bonding, from coiling, or from specific interdigitation of amino acid side chains. The lack of cooperativity in the unfolding of αtα could be explained by the length of the helices (which precludes either extensive hydrogen-bonding networks or extensive coiling) and the lack of specifically interdigitated side chains in the hydrophobic interface.

A question of particular interest in protein folding is the role of hydrophobic interactions in the early stages (3). A compact molten globule-like intermediate with at least partially formed secondary structure appears to be formed in a variety of proteins on the millisecond time scale (35, 41, 42). Since these intermediates are formed rapidly, typically in the dead time of stopped-flow instruments, the relation between hydrophobic collapse and secondary-structure formation has been difficult to investigate and remains an open question. It may be possible to address the kinetic aspects of this problem with αtα. Related questions involve the role of hydrophobic residues in localizing secondary structure and whether specific or nonspecific interactions are involved in the early stages of folding (43). These questions can also be addressed with αtα.

In addition αtα can serve as a model to study helix–helix interactions as an aid to protein design, independent of folding considerations. Thanks to a large body of experimental work on monomeric model peptides (reviewed in ref. 31), the factors which stabilize isolated α-helices are beginning to be understood. In addition, it is clear from work with synthetic peptides that tertiary hydrophobic interactions play a critical role in the stabilization of helices in peptide aggregates (8, 28, 32, 40). So it seems that the design of relatively simple assemblages of helices and turns is within our grasp. Greater challenges lie in the design of close-packed hydrophobic interiors, which would lead to *de novo* designed proteins with cooperative temperature unfolding transitions and protein-like hydrogen-exchange behavior.

The helical hairpin peptide reported here, αtα, represents a successful *de novo* peptide design and provides a model system with which to investigate the balance of forces localizing and stabilizing secondary structures and the kinetics of helix association. The relative contributions to the stability of the system by intrinsic helix-forming capacities, hydrophobic interactions, specific side-chain interactions, and the nature of the turn are open to investigation with this system.

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