The role of amino acid sequence in conformational switching observed in prions and proteins associated with amyloid diseases is not well understood. To study α to β conformational transitions, we designed a series of peptides with structural duality; namely, peptides with sequence features of both an α-helical leucine zipper and a β-hairpin. The parent peptide, Template-α, was designed to be a canonical leucine-zipper motif and was confirmed as such using circular dichroism spectroscopy and analytical ultracentrifugation. To introduce β-structure character into the peptide, glutamine residues at sites away from the leucine-zipper dimer interface were replaced by threonine to give Template-αT. Unlike the parent peptide, Template-αT underwent a heat-inducible switch to β-structure, which reversibly formed gels containing amyloid-like fibrils. In contrast to certain other natural proteins where destabilization of the native states facilitate transitions to amyloid, destabilization of the leucine-zipper form of Template-αT did not promote a transformation. Cross-linking the termini of the peptides compatible with the alternative β-hairpin design, however, did promote the change. Furthermore, despite screening various conditions, only the internally cross-linked form of the parent, Template-α, peptide formed amyloid-like fibrils. These findings demonstrate that, in addition to general properties of the polypeptide backbone, specific residue placements that favor β-structure promote amyloid formation.

Gross conformational transitions (or switches) in proteins are increasingly coming to light. Broadly speaking these may be classed into two types: those that have evolved to tailor or elicit specific normal protein functions, and those that lead to aggregated forms that render proteins defunct or even pathogenic. Examples of the first group include the large structural changes of certain viral-coat proteins that accompany virus-host membrane interactions (1). The other type of structural change is associated with the prions and proteins that form amyloid (2, 3).

It is accepted that misfolding of peptides and proteins cause the fibrillar aggregates known as amyloid, which characterize the diseases collectively known as amyloidoses (3–5). Therefore, the elucidation of the underlying molecular principles for the transformation of soluble proteins into amyloid has potential for understanding and tackling these diseases.

A diverse set of peptides and proteins form amyloid (6, 7). This set is not limited to peptides and proteins that form amyloid deposits in vivo and are associated with the various diseases (8, 9), and even non-natural, designed peptides and proteins can assume amyloid-like structures (10–12). In these cases, the structural changes vary from slow conversions of random-coil peptides to gross structural changes of larger, natively folded, globular structures brought about by destabilization of the native state. Furthermore, for the latter, there are no clear themes in the types of native state that undergo transitions to amyloid: all-α-helical proteins can be transformed into amyloid (13), as can all-β-structures (14) and structural types between these extremes (6, 7). Nonetheless, amyloid and amyloid-like structures have common cores based on β-structure (15, 16); fibrils are assembled from protofilaments in which β-strands are aligned perpendicular to the long fiber axis. Thus, an increasingly adopted view is that the ability to form amyloid is largely a general property of the polypeptide backbone (9, 17). However, the role, if any, of protein sequence in amyloid fibril formation is not clear.

A number of studies indicate that sequence, and therefore amino acid side chains, do influence the formation of amyloid (10, 18–22). However, the question is whether sequence changes simply affect the relative stabilities of the various folded, partly folded, and unfolded states of the subject proteins and hence their propensity to aggregate (18, 20, 21, 23); in this sense, the role of sequence in the formation of the amyloid structure itself may be regarded as passive. Alternatively, sequence may take a more active role in promoting β-structured elements in the unfolded states or within the amyloid-fibril structures themselves (10, 19, 22).

We are interested in addressing a specific issue in protein conformation switching, namely, how sequence brings about and influences the rather extreme α to β structural transitions in proteins. To do this, we set out to design a peptide with a structural conflict; namely, with sequence features compatible with both α-helical and β-hairpin structures. Such a system would allow us to assess the role of specific side chain placements in effecting the conversion between the two structures; in addition, if the β-structured form acted as precursor for amyloid formation, such a system may provide insight into the mechanism(s) of conversion to amyloid-like structures.

Others have engineered peptides and proteins with structural conflicts. Minor and Kim (24) show that an 11-residue peptide sequence can be accommodated at structurally distinct
sages within the same protein fold; in this case, overall tertiary context overrides local sequence and secondary structure preferences. At the level of a whole protein, Dalal and Regan (25) have met the Paracelsus Challenge and succeeded in transmuting a mixed α/β protein fold into an all α-helical fold by altering only ~50% of the sequence. In addition, several groups have succeeded either serendipitously, or with reasoned designs to construct peptide sequences that do switch conformational states (11, 12, 26–29).

To elucidate specific sequence features of natural proteins that drive α to β secondary structure switching, we designed and characterized a series of peptides in which positive design features for a dimeric, α-helical coiled coil and a β-hairpin were superimposed in a short sequence. The parent peptide, Template-α, was confirmed as a stable, cooperatively folded, dimeric, helical structure consistent with the designed leucine zipper. As expected, mutation of three exterior Gln residues to Thr reduced the stability of this folded state. Surprisingly, however, thermal unfolding of the mutant was accompanied by conversion to β-structure on a time scale of tens of minutes. In this state the samples gelled and were shown to contain fibrils with titinorial properties and the morphology of amyloid. Small sequence changes were made to probe the basis of the conversion to amyloid. We conclude that straight destabilization of the coiled-coil structure does not necessarily foster the conversion to amyloid. We designed and characterized a series of peptides in which positive design features for a dimeric, parallel coiled coil, Fig. 1A and B, schematic representations of a heptad sequence repeat (abcdefg) configured onto an α-helical wheel with 3.5-residues per turn, and a β-hairpin, respectively. In the latter the dashed lines indicate the intended inter-strand hydrogen-bonded sites; that is, positions where the backbones of the residues paired across the β-hairpin should hydrogen bond. C, designed peptide sequences. Key: standard one-letter codes are used for the amino acids; Ac, acetyl (CH3.C.O.-); Am, amidated C terminus (-NH2). Color is used to highlight residues designed to interact in both structures: green, hydrophobic residues; dark blue, positively charged lysines; red, negatively charged glutamates. The f positions where the Gln to Thr substitutions were made are colored light blue.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—Peptides were made on a Pioneer Peptide Synthesis System (Perseptive Biosystems) using standard Fmoc chemistry. Peptides were purified by reverse-phase high performance liquid chromatography and their identities confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Purified peptides were stored at pH 2, –20 °C in 7 M DTT.1 Oxidized peptides were prepared as follows: peptides (at ≤100 μM) were agitated at room temperature overnight in 100 mM Tris (pH 8.5), 50% Me2SO, 2 M guanidine hydrochloride. Oxidized peptides were stored at pH 2, –20 °C.

**Circular Dichroism Spectroscopy**—Circular dichroism measurements were made on a JASCO J-715 spectropolarimeter fitted with a Peltier temperature controller. Unless otherwise stated, the sample stocks were diluted into a standard buffer of 25 mM potassium phosphate (pH 7) containing 1 mM DTT (DTT was omitted for the oxidized peptides). All data were collected in 1-mm quartz cuvettes. Data points for CD spectra were recorded at 1-nm intervals using a 1-nm bandwidth and 4–16-s response times. After baseline correction, ellipticities in mdeg were converted to molar ellipticities (deg cm2 dmol res–1) by normalizing for the concentration of peptide bonds. Data points for the thermal unfolding curves were recorded through 1 °C min–1 ramps using a 2-nm bandwidth, averaging the signal for 16 s at 1 °C intervals.

**Analytical Ultracentrifugation**—Sedimentation equilibrium experiments were conducted at 5 °C in a Beckman Optima XL-1 analytical ultracentrifuge fitted with an An-60 Ti rotor. A ~100-μl sample of Template-α (100 μM) in standard buffer containing 100 mM sodium chloride was used. The sample was equilibrated for ~48 h at rotor speeds of 40,000, 50,000, and 60,000 rpm. Sedimentation curves were measured by absorbance at 240 nm (the ε222 for Template-α was calculated as 2220 M–1 cm–1). The resulting data sets were fitted simultaneously using routines in the Beckman Optima XL-A/1 computer analysis software (version 4.0). Two fitting models were used: the first assumed a single ideal species; the second assumed a monomer-dimer equilibrium and fixed monomer molecular weight. The molecular weight and partial specific volume of the peptide were calculated from the amino acid sequence as 3118 and 0.755, respectively. The viscosity of the buffer at 5 °C was taken to be 1.008 mg ml–1.

**Thioflavin T Binding**—Emmission fluorescence spectra of thioflavin T (10 μM) with ~10 μM peptide added were recorded between 480 and 600 nm with an excitation wavelength of 345 nm using a Varian Eclipse spectrofluorimeter. 1-cm quartz cuvettes. A scan rate of 600 nm min–1 and data interval of 1 nm was used throughout.

**Electron Microscopy**—Droplets of peptide solution were applied to carbon-coated copper specimen grids and dried with filter paper before negative staining with 2% phosphotungstic acid at pH 7. Grids were examined in a Hitachi 7100 TEM at 100 kV and digital images were acquired with a (800 × 1200 pixel) charge-coupled device camera.

**RESULTS AND DISCUSSION**

**Design Principles and Characterization of Template-α**—The starting point for our study was a designed canonical α-helical leucine-zipper sequence (Template-α). Using established rules for coiled-coil assembly (30–33), Template-α was designed to form a leucine zipper (i.e., a dimeric, parallel coiled coil, Fig. 1A): the combination of Val at α and Leu at α sites of the heptad repeat was used to direct dimer formation; Lys at one α position and the juxtaposition of Lys and Glu at α and β respectively, were placed to ensure parallel dimers; the β and γ sites were filled with helix-promoting Ala residues and the outer f sites were made polar Gln. The heptad repeat was flanked by Cys residues, which could be oxidized to an intramolecular disulfide bond to favor the alternative β-hairpin conformation (Fig. 1B) as required. An N-terminal Tyr-Gly tag was added to allow peptide concentrations to be determined (34). The N and C

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1 The abbreviation used is: DTT, dithiothreitol.
The CD spectrum of Template-A had minima at 208 and 222 nm, characteristic of a helical structure. To confirm this, we prepared samples of this state for the gelled peptide and its derivatives, giving a dissociation constant of 66 μM (95% confidence limits of 49 and 91 μM). These data are consistent with the CD measurements, which indicated near, but not complete folding at 5°C and 100 μM peptide concentrations. In summary, the CD and equilibrium sedimentation data for Template-A are comparable with those collected for the designed and natural leucine zippers under similar conditions (35) and are, thus, consistent with the peptide forming a dimeric helical structure as designed.

Promoting a Conformational Switch in the Template-A System—As a first step to introduce β-structure character into the designed system, we replaced the Gln residues at the 3 sites of the heptad repeat of Template-A with Thr to give Template-αT (Fig. 1C). The f sites lie away from and, so, should not compromise the helical-zipper interface (Fig. 1A). As Thr has a lower α-helix propensity and a higher β-sheets propensity than Gln (36, 37), Template-αT was expected to form a leucine zipper, but with a reduced stability compared with Template-A. This was observed (compare Figs. 2, A and B, and 3, A and B). However, the thermal unfolding of Template-αT was less reversible; that is, it was recorded after returning to the starting temperature showed a lower helicity than was measured at the start of the experiment (Fig. 3A). When thermal unfolding was repeated at the higher peptide concentration of 300 μM, Template-A again unfolded with a normal, reversible, sigmoidal transition (Fig. 2, A and B). The unfolding of Template-αT showed an inflection above 60°C, which suggested some refolding (Fig. 3B). Spectra recorded after cooling this sample were wholly different from anything recorded previously; the minima at 208 and 222 nm were absent and replaced by a single minimum at 218 nm, indicative of β-structure (Fig. 3A). It was possible to follow the transition of Template-αT from a near-unfolded conformation to a β-conformation directly on the time scale of minutes by maintaining a freshly prepared 300 μM sample of the peptide at 7°C (Fig. 3C). All of the component fibrils were twisted with similar periodicity and, on average, had a diameter of ~10 nm. These features are all consistent with amyloid formation.

Template-αT Formed Amyloid-like Fibrils—Switches to β-structure accompanied by gel formation are indicative of amyloid-like fibrils. Therefore, we tested the gelled peptide samples for tinctorial properties characteristic of amyloid. The fluorescence of thioflavine T (38) was compared in the presence of Template-αT in its α-helical and β-structured states. The fluorescence intensity at 480 nm for the latter was ~2.5 times greater (Fig. 4A). This was evidence for the presence of amyloid-like fibrils in the heat-treated, 300 μM Template-αT sample. To confirm this, we prepared samples of this state for electron microscopy. The resulting images revealed filamentous, 25–50-nm bundles of extended, non-branching fibrils (Fig. 4, B and C). The component fibrils were twisted with a similar periodicity and, on average, had a diameter of ~10 nm. These features are all consistent with amyloid formation.

Manipulating the α to β Transition in the Template-A System—Having established a system that underwent an α to β transition with the added curiosity that the β-structured state formed amyloid, we set out to examine how small, specific and...
rational sequence changes influenced the transition. For instance, could the transition be eased and made to occur at lower temperatures by destabilizing the native coiled-coil conformation of Template-αT? Capping the ends of free-standing α-helical peptides is known to stabilize helical structures (39), and this effect is enhanced in leucine-zipper and other coiled-coil peptides (40). Thus, Template-αT was re-synthesized with free N and C termini to give Template-αTu (Fig. 1C). This had the desired effect of destabilizing the leucine-zipper state; indeed, the peptide was random coil and did not form appreciable helical structure over a wide range of concentrations. It is not clear how uncapping the peptide might affect amyloid formation itself. Nevertheless, Template-αTu could be converted to a β-structured state. However, this still required elevated temperatures and, in fact, the transition was more difficult to effect than with Template-αT: under the conditions used to convert Template-αT (300 μM, 70 °C), Template-αTu did not transform over a 1-h time scale, but a transition was observed within 1 h at 80 °C. Fibrils were observed for these samples by electron microscopy.

With Template-αTu the formation of amyloid was assisted by salt; 0.5 M KF lowered the temperature required for the conversion described above to 70 °C, i.e. to that required for the capped peptide in the absence of salt. Curiously, however, salt also stabilized the low-temperature α-helical state of the peptide increasing the α-helical content from ~33 to 50% at 300 μM peptide. Thus, again for our system, there was no correlation between destabilization of the native state and ease of fibrillogenesis. Salt also accelerated the conversion of Template-αT at 70 °C to the β-structured state; the CD-monitored transitions in 0, 0.5, and 1 M KF were complete in ~60, 30, and 10 min, respectively.

Promoting a β-Hairpin Structured Intermediate?—Summarizing the above, simply destabilizing the leucine-zipper conformation of Template-αT did not facilitate the α to β transition. Others have used β-hairpins to build a high-resolution structure for an β amyloid fibril (15), and implicated their involvement in amyloid-like fibril formation by a peptide from OspA (41). Therefore, our next step was to determine if the transition was affected by increasing the β-hairpin propensity of Template-α (Fig. 1B). Although by no means an ideal β-hairpin sequence, Template-α was designed to be compatible with this conformation. The key features were the Cys residues flanking the central heptad-based sequence. In our design logic, oxidation of these to an intramolecular disulfide link would bring the termini of the peptide together. This should simultaneously promote the β-hairpin and destabilize the leucine zipper. The design principles for the β-hairpin follow from an understanding of amino acid pairings in anti-parallel β-sheets (42, 43). On this basis, the Cys-Cys pair should occupy

\[ ^2 \text{M. R. Hicks and D. N. Woolfson, unpublished results.} \]
a so-called non-hydrogen-bonded site in the hairpin (Fig. 1B). In turn, this would lead to the alignment of complementary, inter-strand hydrophobic interactions (between the a and d positions of the heptad repeat) and electrostatic pairs (between e and g) (Fig. 1B). Furthermore, in this conformation, two of the Thr residues introduced in Template-αT should also align across the structure at a favored non-hydrogen-bonded site (43). With these potential interactions, and if a β-structured precursor could seed/promote amyloid-like fibril formation, one might expect that forcing the intra-molecular Cys-Cys bridge would facilitate the α to β transitions in the Template-α peptides and promote fibrillogenesis.

To probe the role of the intramolecular cross-link, oxidized variants for Template-α, Template-αT and Template-αTu, which we distinguish with the suffix “-ox,” were prepared. For each peptide, mass spectrometry confirmed the intramolecular disulfide bonds, and either sedimentation equilibrium experiments or analytical size-exclusion chromatography was used to show that the low-temperature states were monomers. At 100 μM and 5 °C Template-αT-ox was α-helical and thermally stabilized (Fig. 5, A and B). Thermal unfolding converted the sample to a β-structured state (Fig. 5A). Compared with Template-αT, this conversion of Template-αT-ox to β-structured aggregates was facilitated; a 100 μM sample could be transformed in ~20 min at 70 °C. The gels and fibrils from these samples had all the previously described characteristics consistent with amyloid. For Template-αTu-ox the disulfide link did not induce additional structure at low temperature, but the temperature required to observe the conversion to β-structured in the presence of salt was lowered to 50 °C (compared with 70 °C for the reduced peptide). Although we screened a number of conditions, only the oxidized form of Template-α showed any tendency to form amyloid-like fibrils (Fig. 5C). Thus, tethering the termini of the peptides using a disulfide bond increased their ability to form amyloid consistent with the possible involvement of a β-hairpin in the fibrillogenesis process.

**Conclusions—**We have shown that peptides designed and characterized as a canonical, dimeric leucine-zipper can be induced to switch to β-structured states that form amyloid-like fibrils. The switches were heat-induced, indeed they occurred from the heat-denatured states of the peptides, and they were facilitated by modifications that raise the β-propensity of the sequence. Straight destabilization of the native leucine-zipper state did not promote the switch per se; elevated temperatures were still required to induce β-structure in a mutant that did not form a leucine-zipper dimer at ambient temperatures. This contrasts with reports for certain globular proteins in which amyloid formation correlates with the destabilization of the native state (18, 20).

What drives the formation of the β-structured states and amyloid-like fibrils in our designed system? The following are possible contributors. The fact that the transitions in Template-α and Template-αT were induced by heat is consistent with other studies that show the requirement for the polypeptides to be partly or fully unfolded to transform to amyloid (9, 20). However, in our system (as judged by far-UV CD spectra), Template-αTu was largely unfolded under all conditions, but still required heating to switch state. This requirement for heat also suggests the involvement of the hydrophobic effect. This is consistent with our observation that the formation of the β-structured state was accelerated by salt, and foregoing work on other peptides that undergo structural switches (12, 26), and most recently on insulin variants that form fibrils (22). The hydrophobic effect cannot be the only factor at play, however. This is for several reasons. First, the mutation from Gln to Thr, which first resulted in the structural switch, is a swap of one...
polar amino acid for another. Second, low pH abrogates the α to β switch: none of the peptides converted to the β-form at pH 2 (data not shown); and the soluble, helical form of Template-αT could be recovered by re-suspending the fibril-containing gels in pH 2 buffer. This implicates electrostatic interactions in the formation and assembly of the β-structured state; either the designed cross-strand salt bridges (Fig. 1) are being broken at low pH, or the overall positive charge on the peptide destabilizes the assemblies. Third, the parent peptide only formed fibrils when its termini were tethered together. This cross-link could favor the alternative fibrils when its termini were tethered together. This cross-linking study is that

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