General Strategy for Covalently Stabilizing Helical Bundles: A Novel Five-Helix Bundle Protein

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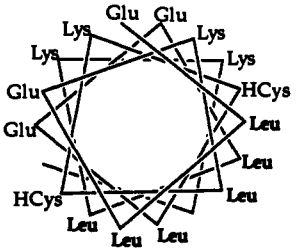
The self-assembly of peptide and protein structures is an essential feature of the *de novo* design of proteins with biological activity.[1] The propensity of amphiphilic helical peptides to assemble in aqueous solution has been used in the design of coiled coils[2] and four- and six-helix bundles.[3] Due to the instability of peptide assemblies at low concentration and high temperature, peptidic loops have been successfully used to link together the individual helices of four-helix bundles.[4] Templates have also been used to promote the formation of a particular aggregation state of peptides.[5] Ideally, it would be desirable to stabilize the helical bundle formed by a small peptide by directly cross-linking the assembly. Here we describe a novel method using disulfide bonds to trap the assembly formed by a 16 amino acid amphiphilic peptide (**1**).

Peptide **1** was designed to have an amphiphilic helical conformation (Figure 1) with the potential to self-assemble into a four-helix bundle. Using the design criteria of DeGrado and co-workers, Glu and Lys were arranged to promote intramolecular salt bridges, the amino terminus was acetylated, and the carboxy terminus was converted into a primary amide to reduce helix destabilization.[3] Homocysteine (HCys) was used at the helical interfaces to cross-link the peptides; computer modeling suggested that HCys would have the appropriate length for disulfide bond formation in the bundle.[6]

Peptide **1a** was synthesized by standard solid-phase methods using an Fmoc-based strategy[7] and purified to homogeneity by HPLC.[8] Circular dichroism (CD) confirmed that **1a** was highly helical (95% helical at 70 μM based on the mean residue ellipticity at 222 nm[9]), and size exclusion chromatography experiments



Ac-Glu-Leu-Leu-Glu-Lys-Leu-HCys(SR)-Lys-HCys(SR)-Leu-Glu-Glu-Leu-Leu-Lys-Lys-NH_2
(**1a**) R = Acm
(**1b**) R = SCH_3
(**1c**) R = H

Figure 1. Helical wheel representation of peptide **1**.

confirmed an aggregation state of approximately 4 in aqueous solution.[10] Peptide **1a** was cross-linked either by removing the acetamidomethyl (Acm) protecting group with dimethyl(methylthio)sulfonium tetrafluoroborate (DMST) to give **1b**, followed by reduction of the mixed disulfide with DTT and oxidation of **1c** with $K_3Fe(CN)_6$,[11] or by treatment of **1a** with cyanogen iodide to directly remove the Acm protecting group and form disulfide bonds.[12,13]

Both reactions provided the same major compound as determined by HPLC, but with a much shorter retention time on a reverse-phase C_8 column as compared to **1a–c**.[8] No free sulfhydryls existed within the assembly as determined by Ellman's test, confirming that all sulfhydryls were converted to disulfide bonds.[14]

If a four-helix bundle was covalently stabilized, we would have expected a molecular weight by mass spectrometry of 7998. Contrary to our expectations, the molecular weight of the purified compound (5HB) was determined by electrospray mass spectrometry to be 9997.5. This corresponds well with the molecular weight of a molecule which, remarkably, contains *five* covalently linked peptides (9997.7). To confirm that 5HB is a covalently linked pentamer, SDS-PAGE was also used to determine an approximate molecular weight. Under nonreducing, denaturing conditions, a band corresponding to molecular weight 9239 was obtained for 5HB, confirming the pentameric, covalent structure.

Circular dichroism experiments confirmed that 5HB was highly helical (87% at 70 μM), although slightly less helical than **1a** (95%) at a comparable concentration. As the concentration of **1a** was lowered, there was a concomitant decrease in the helical content as measured by CD (97 to 61% from 170 to 1 μM), whereas 5HB showed no concentration-dependent decrease in helicity (Figure 2a). The helical content of peptide **1a** also showed temperature-dependent behavior: as the temperature was increased from 4 to 80 °C, the helical content decreased from 86 to 47% (Figure 2b). The temperature dependence of the helicity of 5HB, however, was markedly less, with helical content ranging from 86 to 71% over the same temperature range.

A number of potential structures were investigated for this disulfide-linked pentamer, but only the five-helix bundle structure was consistent with the formation of five disulfides in a helical material capable of burying the hydrophobic surfaces from solvent.

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(8) HPLC conditions: all reactions were monitored and purified by HPLC using Vydac-C_8 reverse-phase columns (analytical, 1 × 25 cm; preparative, 2.2 × 25 cm) with mobile phase A (100% CH_3CN/0.1% TFA) and mobile phase B (100% H_2O/0.1% TFA) with linear gradients (40–90% A for peptide **1a**, 10–40% A for 5HB) of 30 (analytical) or 60 min (preparative).

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(10) Size exclusion studies were performed at 4 °C using a 1.6-cm by 90-cm column of Sephadex G50–80. Compounds were applied at a concentration of 0.2 mM and eluted with 0.5 M NaCl, 0.05 M phosphate buffer, pH 7.0.

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(13) Method A: a solution of **1a** (1 mM) in 1:1 H_2O/methanol was treated with DMST (10 equiv) for 3 days to yield **1b**, which was treated with DTT (50 equiv) to yield **1c**. A solution of **1c** (250 μM) in H_2O was treated with $K_3Fe(CN)_6$ until a yellow color persisted to yield 5HB. Method B: a solution of **1a** (200 μM) was treated with ICN (50 equiv) for 3 days to yield 5HB.

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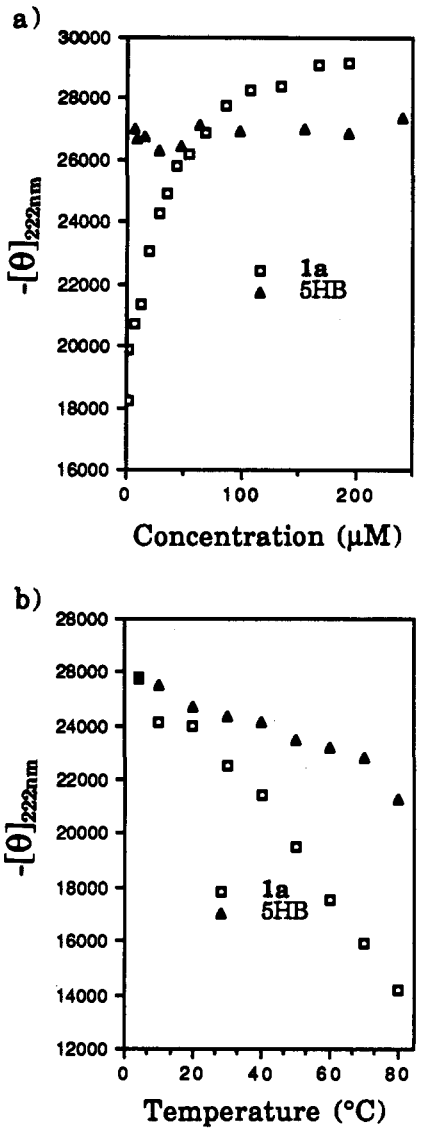

Figure 2. Mean residue ellipticity at 222 nm of **1a** and 5HB vs (a) concentration in 10 mM phosphate, 100 mM NaCl, pH 7.0 buffer, at 20 °C, and (b) temperature at a concentration of 40 μM in 10 mM phosphate, 100 mM NaCl, pH 7.0 buffer. Concentrations were determined from quantitative amino acid analysis and are based on monomer.

If 5HB forms a five-helix bundle, it is predicted that ligands might bind into the hydrophobic interior. To test this we measured the change in fluorescence intensity of two probes, acridine orange (AO) and acridine orange–10-dodecyl bromide (AODB), with increasing concentration of 5HB (Figure 3). Increasing the concentration of 5HB had no effect on the fluorescence intensity of AO, whereas AODB showed a 10-fold increase in fluorescence intensity, presumably due to binding of the dodecyl chain into the hydrophobic interior of 5HB which shielded the acridine moiety from solvent.[15]

It is interesting that, although **1a** was designed to self-assemble into a four-helix bundle, a covalently stabilized pentamer was obtained. It is possible that the placement of the hydrophobic Hcys residues at the interfaces of the amphiphilic helices was not

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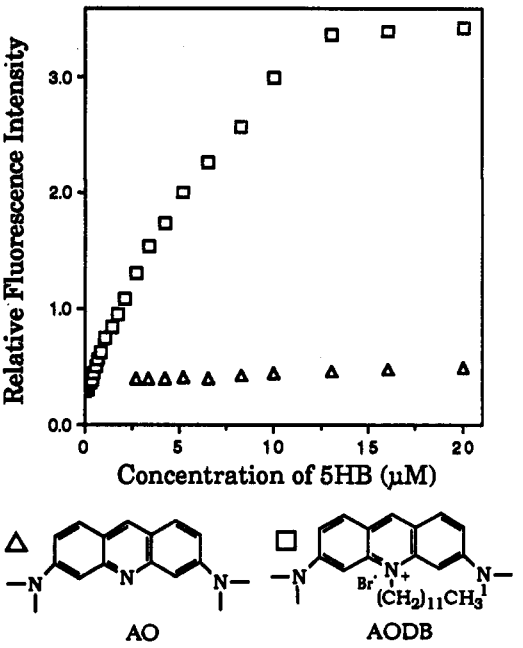

Figure 3. Fluorescence intensity of AO and AODB as a function of the concentration of 5HB. Probe concentrations were 1.0 μM in 10 mM phosphate, pH 7.0 buffer, with 0.1% ethanol.

optimal for covalently linking four peptides due to restrictions in the torsional angle of the resultant disulfide bonds. Any amount of five-helix bundle in solution, therefore, would be kinetically trapped if there was a less strained transition state for disulfide bond formation.[16]

In conclusion, we have developed a general strategy for covalently stabilizing helical bundles via disulfide linkages. With the peptides studied (**1a** and **1c**), a pentameric assembly was obtained which was held together via five disulfide bonds. The protein, 5HB, was very helical and highly stable to increases in temperature and decreases in concentration as compared to the non-cross-linked helical bundle of **1a**. 5HB was also shown to specifically bind an acridine moiety containing a dodecyl chain, which is consistent with a five-helix bundle structure. This method provides a powerful means for converting a 16 amino acid peptide into an 80 amino acid protein *in one step*. Work is underway to modify the design strategy in an effort to stabilize other unique peptide assemblies.

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Supplementary Material Available: Reaction schemes and characterization for the compounds reported (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(16) Since 5HB contains an odd number of peptide chains, it is interesting to speculate if the helices within the bundle adopt a parallel or a mixed parallel/antiparallel orientation. Molecular modeling of 5HB was used to investigate these possibilities. A model of 5HB with the helices in a mixed parallel/antiparallel orientation placed either all Glu or all Lys residues at the interface between the antiparallel helices, whereas a model of 5HB with the helices in a parallel orientation showed charge complementarity at the interfaces of the helices which may have a stabilizing effect.