Discovery and Characterization of a Discretely Folded Homotrimeric $\beta\beta\alpha$ Peptide

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The design of well-defined self-associating peptide motifs enriches our understanding of the fundamental forces involved in protein folding and protein−protein interactions.[1] Much success has been achieved in designing and understanding quaternary structure formation in α-helical structures;[2] however, the rational design of oligomeric pepides containing β-structure in aqueous solution has been far more challenging.[3] Our group recently reported the design and structure analysis of a family of 23-residue monomeric $\beta\beta\alpha$ (BBA) peptide motifs capable of forming discrete tertiary structures in the absence of metal ions or covalent cross links.[4] One member of this family, **BBA5**,[4c] served as the starting point for the development of a BBA motif with discrete oligomerization properties. The structure of **BBA5** includes a β-hairpin region containing a D-Pro amino acid to nucleate a type II′ turn (amino acids 1−8), a connecting loop region (amino acids 9−11) and a highly optimized helical region (amino acids 12−23) which packs against the hairpin to form a discrete hydrophobic core. We hypothesized that by removing residues in this loop region, the helix would be unable to fold back onto the hairpin. Therefore, the resulting solvent-exposed hydrophobic surface would be forced to promote peptide oligomerization (Figure 1).

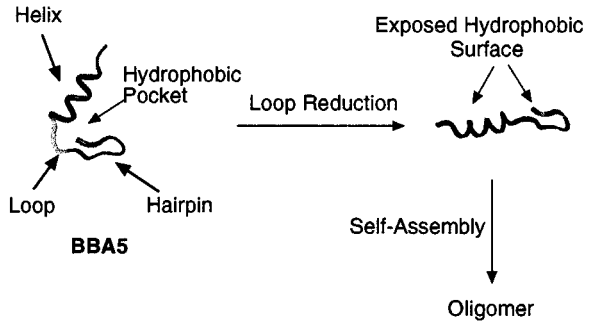
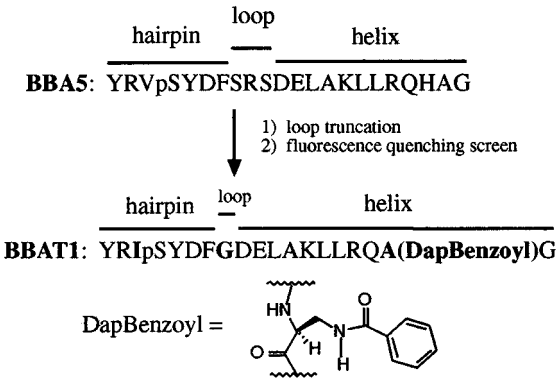


Figure 1. Schematic representation of the strategy used to induce oligomerization in monomeric **BBA5**.

Herein, we report the characterization of a homotrimeric BBA mini-protein motif (**BBAT1**). This oligomeric superstructure shows the hallmarks of a native protein including cooperative thermal denaturation and excellent dispersion in the ^{1}H NMR spectrum.

Scheme 1



In the discovery process, a library of 29 BBA peptide sequences was synthesized[5] which contained either a fluorophore (anthranilic acid)[6] or a quencher (nitrotyrosine). Fluorescence quenching of the mixture of peptides was therefore indicative of inter-peptide association. From this screen, a 21-residue peptide, **BBAT1**, was selected which is capable of forming discrete homo-trimers in aqueous solution (Scheme 1) at low concentrations (<10 μM). The sequence of **BBAT1** is similar to **BBA5**—the main feature being a two-residue deletion in the loop region (SerArgSer in **BBA5** replaced with Gly in **BBAT1**). Other modifications include an Ile3→Val, His21→Ala, and an unnatural diaminopropionic acid group derivatized with a benzoyl functionality in place of Ala22 in **BBA5**. This amino acid is a remnant of the fluorescence-quenching screen, which required an anthranilic acid amide.[5]

The oligomeric state of **BBAT1** was assessed using sedimentation equilibrium analytical ultracentrifugation. The data reveal that the peptide acts as a single ideal species with a molecular weight consistent with that of a trimeric peptide in solution from 50 to 935 μM at 5 °C.[7]

The CD spectrum of **BBAT1** shows minima at 222 and 208 nm which are characteristic of α-helical structures (Figure 2A). Interestingly, the intensity of the signal is significantly enhanced when compared to **BBA5**[4] which suggests that the helical portion of the peptide has been greatly stabilized upon oligomerization. As a comparison, the CD spectrum of the 12-residue helix portion alone (peptide **Helix1**) shows a minimum at 205 nm, which is indicative of a random coil structure. This suggests that the hairpin portion of **BBAT1** is an important contributor to the observed stability of the helix and its oligomerization (vide infra). Helicity ($[\theta]_{222}$) was also monitored as a function of concentration for both **BBAT1** and **Helix1** separately (Figure 2A inset). The CD spectrum of **BBAT1** shows a strong dependence on concentration and can be fit well to a monomer−trimer model with an estimated dissociation constant of 5.1×10^{-11} M^2 for a two-state monomer−trimer equilibrium.[7] In contrast, the helicity of **Helix1** is only slightly dependent on concentration over the same concentration range.[8]

The stability of **BBAT1** was studied by monitoring the CD spectrum as a function of temperature. At 43 μM, **BBAT1** displays a reversible cooperative thermal transition not observed in monomeric **BBA1**[9] or **BBA5** (Figure 2B). Such cooperative thermal unfolding transitions are typical of proteins with unique

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(8) In a related experiment, titration of a 150 μM sample of **Helix1** with up to 3 equiv of peptide hairpin (residues 1−8 of **BBAT1**) showed no enhancement in helicity ($[\theta]_{222}$) and therefore suggests that there is negligible binding between these two peptides over these concentrations (data not shown).
(9) **BBA1** is a closely related peptide to **BBA5** and exhibits many of the same biophysical characteristics. See ref 4.

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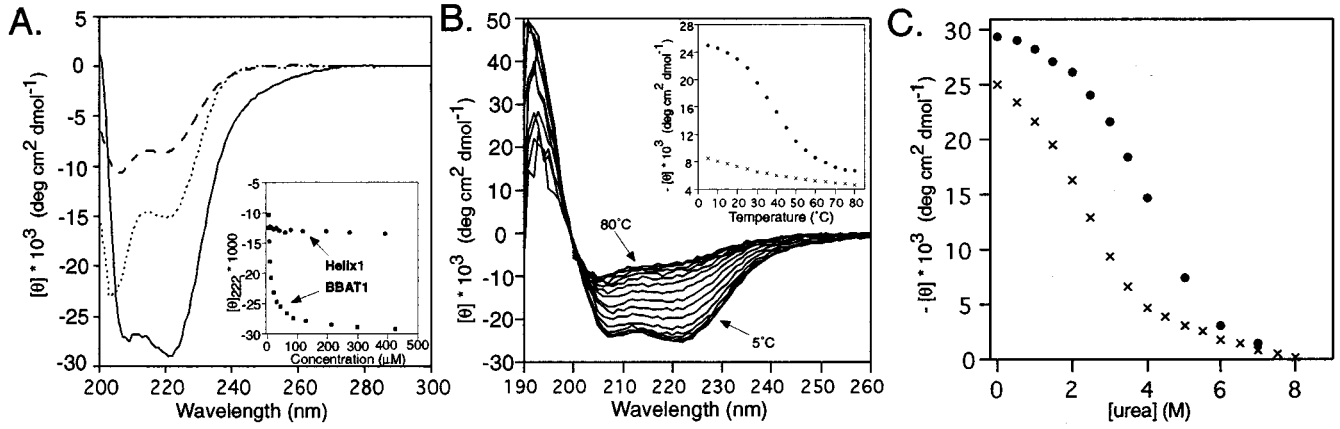

Figure 2. (A) CD spectrum of **BBAT1** at 300 μM (solid line), **Helix1** at 390 μM (dotted line) and **BBA5** at 50 μM (dashed line). (A: inset) Concentration dependence of $[\theta]_{222}$ for **BBAT1** and **Helix1**. (B) Variable temperature CD of **BBAT1** at 43 μM. (B: inset) Plot of $[\theta]_{222}$ vs temperature for **BBAT1** (circles) and **BBA1** ($\times$). (C) Stability of **BBAT1** at 43 μM ($\times$) and at 212 μM (circles) to urea. Experiments performed in pH 7.5 50 mM phosphate buffer, 5 °C.

nativelike structure.[10] Interestingly, the residual CD signal of **BBAT1** at 80 °C closely matches that of **BBA1** at 80 °C[4a,b]—this result is consistent with the hypothesis that the β-hairpin is still folded at this temperature.[11] We also investigated the stability of **BBAT1** at two different concentrations using the chemical denaturant urea (Figure 2C). At 43 μM, there appears to be a three-state process occurring whereby the majority of the CD signal is lost in a partially cooperative process up until ~4 M urea. This is then followed by a more linear denaturation step up until 8 M urea. It is possible that this represents the unfolding of the helix and dissociation of the trimer up to 4 M urea, followed by unfolding of the hairpin in the monomers. This result again supports the hypothesis that the hairpin is stable without stabilizing helical interactions.[4b,11] Note that the resistance of **BBAT1** to unfolding by each denaturation method is greatly enhanced at higher concentrations[7] which is consistent with enhanced stabilization in the oligomeric state.

The presence of nativelike structure[10] was investigated using ^{1}H NMR.[12] The 1-D ^{1}H NMR spectrum of **BBAT1** shows sharp and well-dispersed peaks which are typical of proteins/peptides with well-defined structures.[7] Remarkably, a peak near -0.4 ppm is visible which corresponds to the protons in the isoleucine side chain and suggests tight packing of the methyl groups near the face of an aromatic side chain. Furthermore, H/D exchange experiments[13] performed at pD 4.9 and 5 °C indicate that many amide protons are substantially protected from solvent. Several amide protons in the core of the helix were not fully exchanged after 4 days, while one proton in particular was visible for more than 8 days.[7] This is in contrast to the amides found in **BBA1** which were fully exchanged after ~3 h under the same conditions.[4a]

We investigated the topology of **BBAT1** using 2-D ^{1}H NMR TOCSY and NOESY experiments. Although a full structural determination was not possible at this stage due to the degeneracy of the trimeric structure, many residues were readily identified. Several distinctive helix-to-hairpin NOEs[7] were observed which preclude a parallel topology for the trimer, whereby only helix-to-helix and hairpin-to-hairpin contacts would be present. Rather, these results suggest some form of a *symmetrical domain-swapped* topology. Domain swapping[14] is a common mode of oligomerization in nature since the interdomain contacts observed in the monomer are preserved in the oligomer. Indeed, several examples of domain-swapped proteins are known to be the result of truncations in their domain-connecting loop regions.[15]

In summary, we have reported a new loop-truncated form of $\beta\beta\alpha$ peptide **BBA5** that forms a well-defined homotrimer in water. Key to switching the motif to a discrete oligomeric state was the removal of two amino acids in the loop region of the monomer. **BBAT1** possesses many of the characteristics found in native protein structures. We are currently studying mutants of **BBAT1** to better understand the effect of sequence variations on the structure and oligomeric state.

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Supporting Information Available: Sedimentation equilibrium data, ANS binding plot, fit of the concentration dependent CD data, temperature denaturation plot at higher concentration and ^{1}H NMR data including 1-D and 2-D spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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