Oligomerization of Uniquely Folded Mini-Protein Motifs: Development of a Homotrimeric $\beta\beta\alpha$ Peptide

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Received December 19, 2000

Abstract: The discovery of a discretely folded homotrimeric $\beta\beta\alpha$ motif (**BBAT1**) was recently reported (*J. Am. Chem. Soc.* **2001**, *123*, 1002−1003). Herein the design, synthesis, and analysis of a small library of peptides which led to the isolation of **BBAT1** is described. $\beta\beta\alpha$ peptides based on the monomeric sequence of **BBA5** (*Folding Des.* **1998**, *120*, 95−103) were synthesized to include the anthranilic acid/nitrotyrosine fluorescence quenching pair to rapidly detect interpeptide association. In the first generation of peptides synthesized, truncations in the loop region connecting the β-hairpin to the α-helix revealed that a two-residue deletion in the loop promoted an interpeptide association as detected by fluorescence quenching. An additional library of 22 loop-truncated $\beta\beta\alpha$ peptides was subsequently synthesized to include a variety of sequence mutations in an effort to enhance the observed peptide−peptide binding. From the fluorescence quenching screen, peptide **B2** was found to possess the strongest fluorescence-quenching response, indicative of a strong peptide−peptide association. Due the poor solubility of peptide **B2**, the *S*-methylated cysteine at position 9 in the loop was substituted with a glycine to generate peptide **BBAT1** which possessed greatly improved water solubility and formed discrete trimers. The successful design of this oligomeric $\beta\beta\alpha$ structure will likely aid the design of more complex $\alpha-\beta$ superstructures and further our understanding of the factors controlling protein−protein interactions at $\alpha-\beta$ protein interfaces.

Introduction

Protein−protein interactions are critical molecular recognition events in all living systems; however, there remains a great deal to be learned about the forces that mediate these key interactions.[1] A better understanding of these processes would greatly aid in the targeting and manipulation of important biological pathways. *De novo* protein design offers an excellent way to study the fundamental forces behind protein folding and protein−protein interactions because very complex naturally occurring interactions can be studied in more detail in smaller model systems.[2]

Great success has been achieved in designing oligomeric α-helical peptides with two, three, or four helices capable of forming well-defined quaternary structures.[3] As a result, the factors controlling interhelical associations are quite well-understood. The design of oligomeric peptides including β-forming structures in aqueous solution has been more difficult.[4] Recently, this group reported the design and characterization of a family of monomeric 23-residue $\beta\beta\alpha$ peptides based on the zinc-finger motif that are capable of forming well-defined tertiary structures in the absence of metal ions.[5] More recently, we described the biophysical characterization of a related peptide (**BBAT1**) which forms a discrete $\beta\beta\alpha$ homotrimer in water.[6] Herein we describe the process of design and discovery of the peptide **BBAT1** which involved the generation of a library of $\beta\beta\alpha$ peptides and screening of this library for fluorescence quenching.

Design

Our design efforts were based on the peptide **BBA5** which is a 23-residue monomeric $\beta\beta\alpha$ peptide consisting of three structural regions: (1) the hairpin region (residues 1−8) incorporating an unnatural D-Pro residue to nucleate a type-II′ turn,[7] (2) a helical region (residues 12−23) incorporating amino acids with a high propensity for α-helical structure, and (3) a loop region (residues 9−11) which serves to connect the hairpin and helix (Figure 1). The structure of **BBA5** is stabilized by a hydrophobic core formed between the helix and the hairpin.[5b]

(1) Uetz, P.; Giot, L.; Cagney, G.; Mansfield, T. A.; Judson, R. S.; Knight, J. R.; Lockshon, D.; Narayan, V.; Srinivasan, M.; Pochart, P.; Qureshi-Emili, A.; Li, Y.; Godwin, B.; Conover, D.; Kalbfleisch, T.; Vijayadamodar, G.; Yang, M. J.; Johnston, M.; Fields, S.; Rothberg, J. M. *Nature* **2000**, *403*, 623−627.

(2) (a) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779−819. (b) Bryson, J. W.; Betz, S. F.; Lu, H. S.; Suich, D. J.; Zhou, H. X. X.; O'Neil, K. T.; DeGrado, W. F. *Science* **1995**, *270*, 935−941.

(3) (a) Hill, R. B.; DeGrado, W. F. *J. Am. Chem. Soc.* **1998**, *120*, 1138−1145. (b) Walsh, S. T.; Cheng, H.; Bryson, J. W.; Roder, H.; DeGrado, W. F. *Proc. Nat Acad. Sci. U.S.A.* **1999,** *96*, 5486-5491. (c) Skalicky, J. J.; Gibney, B. R.; Rabanal, F.; Urbauer, R. J. B.; Dutton, P. L.; Wand, A. J. *J. Am. Chem. Soc.* **1999**, *121*, 4941−4951. (d) Micklatcher, C.; Chmielewski, J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 724−729.

(4) (a) Mayo, K, H.; Ilyina, E. *Protein Sci.* **1998**, *7*, 358−368. (b) Illyina, E.; Roongta, V.; Mayo, K. H. *Biochemistry* **1997**, *36*, 5245−5250. (c) Quinn, T. P.; Tweedy, N. B.; Williams, R. W.; Richardson, J. S.; Richardson, D. C. *Proc. Natl Acad. Sci. U.S.A.* **1994**, *91*, 8747−8751. (d) Yan, Y.; Erickson, B. W. *Protein Sci.* **1994**, *3*, 1069−1073. (e) For a review of recent successes in the design of monomeric β-sheet peptides, see: Lacroix, E.; Kortemme, T.; Lopez de la Paz, M.; Serrano, L. *Curr. Opin. Struct. Biol.* **1999**, *9*, 487−493.

(5) (a) Struthers, M. D.; Cheng, R. P.; Imperiali, B. *Science* **1996**, *271*, 342−345. (b) Struthers, M. D.; Cheng, R. P.; Imperiali, B. *J. Am. Chem. Soc.* **1996**, *118*, 3073−3081. (c) Struthers, M. D.; Ottesen, J. J.; Imperiali, B. *Folding Des.* **1998**, *3*, 95−103.

(6) Mezo, A. R.; Ottesen, J. J.; Imperiali, B. *J. Am. Chem. Soc.* **2001**, *123*, 1002−1003.

(7) Stanger, H. E.; Gellman, S, H. *J. Am. Chem. Soc.* **1998**, *120*, 4236−4237.

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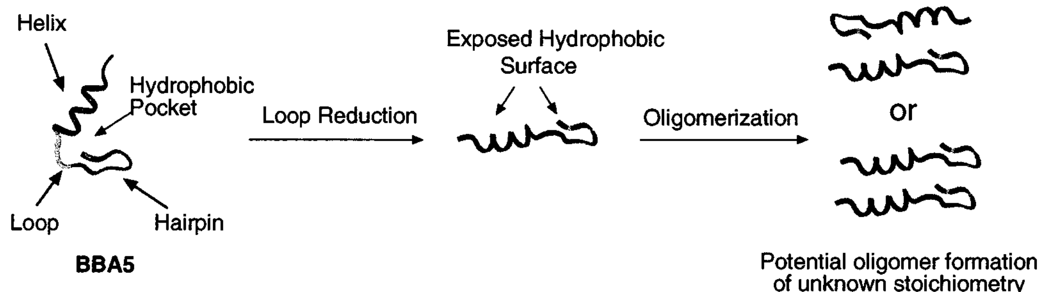
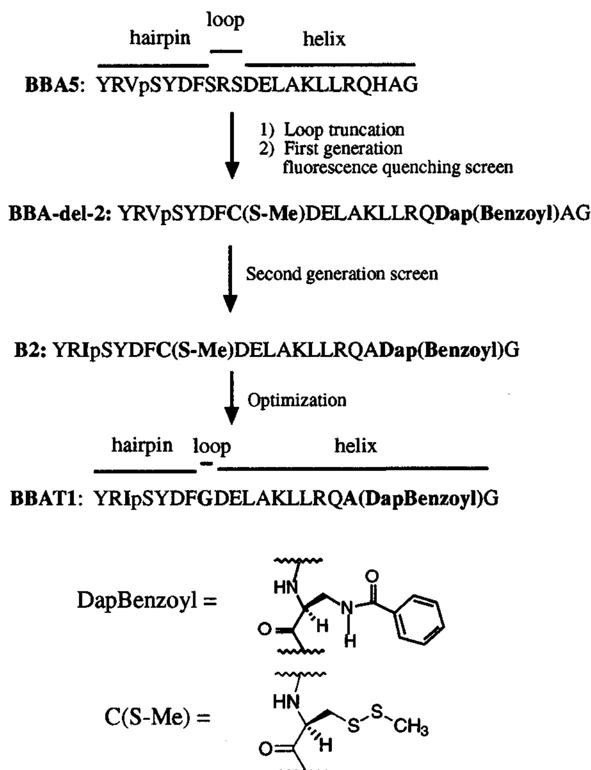
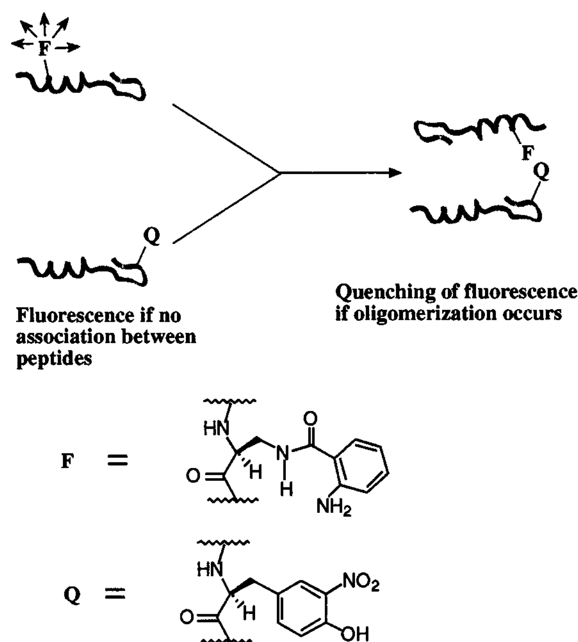


Figure 1. Schematic representation of the strategy used to induce oligomerization in the $\beta\beta\alpha$ motifs.

Scheme 1



Scheme 2



We proposed that by eliminating portions of the loop region, the helix and hairpin would be unable to pack against one another, thus exposing the hydrophobic residues to water. In this case, oligomerization would likely occur to bury these exposed hydrophobic surfaces (Figure 1). We were particularly interested (1) to learn whether an oligomer of discrete stoichiometry would be formed and (2) to find out whether the helix domain would preferentially associate with another hairpin domain as in the monomer, or, whether common helix−helix interactions would prevail. If the helical and hairpin domains were truly complementary, as observed in the monomer, then such helix−hairpin interactions should remain intact upon oligomerization. The overall iterative design process is shown in Scheme 1.

We chose to detect potential peptide−peptide associations using a fluorescence quenching-based assay.[8] This technique provides a very rapid and sensitive assessment of intermolecular interactions provided that the fluorophore and quencher are on different peptides: Noninteracting peptides would result in a strong fluorescence signal, while an interpeptide association would lead to a decrease in the fluorescence signal (Scheme 2). The anthranilic acid/nitrotyrosine pair[9a] was used such that their small size would have a minimal effect on the resulting peptide structure (Scheme 2). The fluorophore was incorporated near the end of the helix at position 21 of **BBA5**, while the nitrotyrosine quencher amino acid was used in place of Tyr6 on the hairpin. In this arrangement, packing of the helix against a hairpin would bring the two reporter groups in close proximity, resulting in fluorescence quenching.[10]

To rapidly synthesize and access many different full-length peptide sequences for study, we developed a strategy whereby two smaller peptide libraries of helices and hairpins would be first synthesized. Ligation of the various helices and hairpins would more rapidly provide a larger variety of full-length peptides than by simply synthesizing each intact peptide separately. For example, a library of 10 hairpins and 10 helices would provide access to 100 peptides via ligation chemistry. Note, however, that for each hairpin or helix in our fluorescence study, we were required to synthesize two peptides to accommodate the fluorescence-quenching strategy. Hence, in the example above, 40 total peptides need to be synthesized to access 100 peptides via ligation chemistry.

(8) Wu, P. G.; Brand, L. *Anal. Biochem.* **1994**, *218*, 1−13.

(9) (a) Meldal, M.; Breddam, K. *Anal. Biochem.* **1991**, *195*, 141−147. (b) The use of pairs of larger fluorophores has been shown to greatly perturb peptide association properties, see: Daugherty, D. L.; Gellman, S. H. *J. Am. Chem. Soc.* **1999**, *121*, 4325−4333.

(10) This fluorophore/quencher pair is quite efficient with complete quenching across 20 Å and 82% quenching across 50 Å in a reported example. See ref 9a.

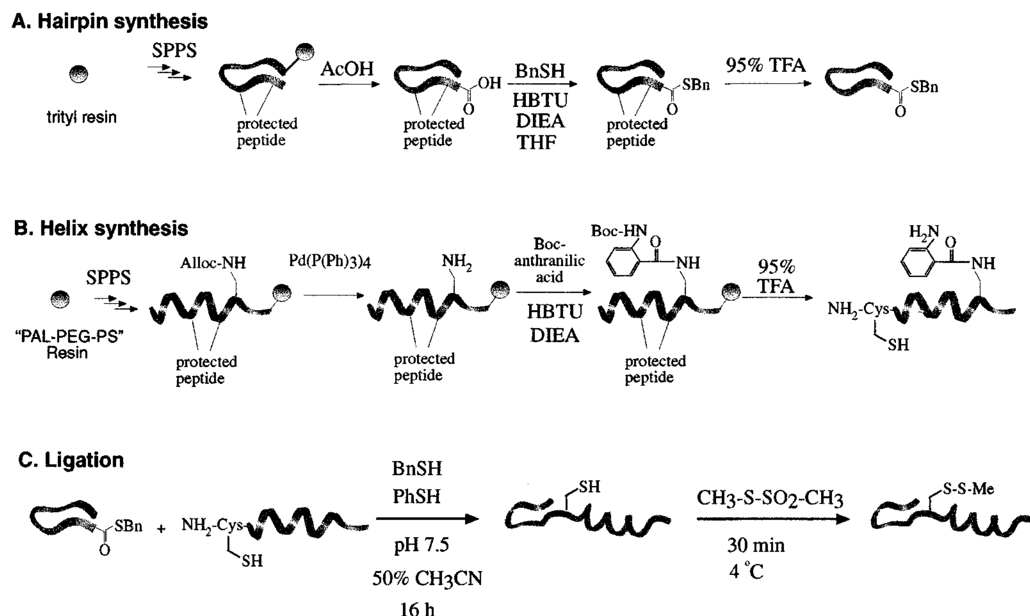


Figure 2. Synthetic strategy for (a) the thioester hairpin, (b) the fluorophore-labeled helix, and (c) ligation of the two peptide segments.

Results

Synthesis of Library Hairpin and Helix. We used the native ligation technique[11] for assembling the helix and hairpin domains. A necessity for this reaction is the incorporation of an N-terminal cysteine and a C-terminal thioester. We thus developed a methodology for generating the C-terminal thioester which is similar to a procedure which has since been published.[12,13] The hairpin portion of the peptide was synthesized on a trityl resin and incorporated the quenching functionality of 2-nitrotyrosine in place of Tyr6 in **BBA5**. The peptide was then cleaved from the resin using mildly acidic conditions to afford the fully protected peptide hairpin segment with a free carboxyl terminus. This C-terminal acid was then reacted with benzyl mercaptan, HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), and DIEA in THF to give the protected thioester. We chose conditions under which the epimerization of the C-terminal amino acid was kept to a minimum.[14] Purification using flash column chromatography, followed by cleavage of the protecting groups with TFA afforded the C-terminal thioester in high yield (Figure 2A).[15]

The helix was synthesized using a standard TFA-labile resin and included a N-terminal cysteine for ligations *and* an unnatural diaminopropionic (Dap) acid group with an allyloxycarbonyl-(Alloc)-protected amino side chain (Figure 2B). After solid-phase peptide synthesis, the Alloc group was selectively deprotected to expose its free amine side chain. Boc-anthranilic acid was then coupled to the amine to introduce the fluorophore.[16] Cleavage with TFA afforded the crude helix segment peptides which were used in the ligation chemistry without purification.

Ligation of Library Fragments. The ligations of various C-terminal thioester hairpins and N-terminal cysteine-containing helices were performed over 16 h in 50 mM phosphate buffer (pH 7.5):acetonitrile (1:1) in the presence of 2% each of benzyl mercaptan and thiophenol. Following the ligation, the crude reaction mixture was treated with methyl methanethiolsulfonate (MMTS) to cap the free cysteine thiols and eliminate the possibility of intermolecular disulfide formation (Figure 2C). Purification of each full-length ββα peptide was performed using standard reversed-phase HPLC.

First Generation Peptides. To first explore the effect of a loop truncation and to model the synthetic methodology described above, eight first-generation peptides were synthesized as shown in Table 1. One hairpin sequence was synthesized with either tyrosine or nitrotyrosine at position 6. In addition, six helical peptides were synthesized, representing three sequences of different loop lengths which contained either Dap-anthranilic acid or Dap-acetyl at position 19. **BBA5** contains the amino acids Ser9-Arg10-Ser11 in its loop sequence. For this loop-truncation study, Ser9 was replaced with the Cys as required for the ligation chemistry, and Arg10 was replaced with Ser to remove the net positive charge on the ligated peptides which may inhibit peptide self-association via electrostatic effects.

Suitably matched helical and hairpin peptides were ligated together as described above such that each full-length ββα

(11) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994,** *266*, 776–779. (b) Dawson, P. E.; Churchill, M. J.; Ghadhiri, M. R.; Kent, S. B. H. *J. Am. Chem. Soc.* **1997**, *119*, 4325–4329. (c) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923–960.

(12) The following paper used a similar strategy using a trityl resin but obtained substantially lower yields than our modified method: Futaki, S.; Sogawa, K.; Maruyama, J.; Asahara, T.; Niwa, M.; Hojo, H. *Tetrahedron Lett.* **1997**, *38*, 6237–6240.

(13) In addition, two closely related methods have been recently published which employ a safety-catch linker to generate the C-terminal thioester: (a) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369-11374. (b) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689.

(14) The use of THF likely inhibited possible epimerization of the C-terminal amino acid. See: Quibell, M.; Packman, L. C.; Johnson, T. *J. Chem. Soc., Perkin Trans. 1*, **1996**, 1219–1225. However, significant (30%) epimerization of the C-terminal position was observed when the following two sequences were used in the thioesterification reaction: YDIPSYRF and YDVPSYRF. These two sequences were not included in the present study.

(15) To verify that the observed single peak by HPLC was not the result of overlapping peaks of epimerized product, a thioesterification reaction was conducted under the known epimerization conditions of: HATU/HOAt/DIEA/DMF (ref 15). Two resolved peaks were observed by reversed phase HPLC, confirming that epimerization was not occurring under our conditions for the peptides reported.

(16) In half of the helical peptides synthesized, an acetyl group was coupled to the amine to generate a peptide suitable for ligation with a quencher-bearing hairpin segment.

Table 1. List of Peptide Hairpins and Helices Synthesized in the First Generation

peptide name	amino acid length	sequence[a,b]
Hairpin-1	8	Ac-NH-[YRVpSYDF]-CO-SBn
Hairpin-1-q	8	Ac-NH-[YRVpSY_{NO2}DF]-CO-SBn
Helix-f	15	NH_2-[**CSS**DELAKLLRQDap(Anthr)AG]-CO-NH_2
Helix-q	15	NH_2-[**CSS**DELAKLLRQDap(Ac)AG]-CO-NH_2
Helix-del1-f	14	NH_2-[**CS**DELAKLLRQDap(Anthr)AG]-CO-NH_2
Helix-del1-q	14	NH_2-[**CS**DELAKLLRQDap(Ac)AG]-CO-NH_2
Helix-del2-f	13	NH_2-[**C**DELAKLLRQDap(Anthr)AG]-CO-NH_2
Helix-del2-q	13	NH_2-[**C**DELAKLLRQDap(Ac)AG]-CO-NH_2

[a] (Anthr) represents an anthranilic acid amide cap on Dap, (Ac) represents an acetyl cap on Dap. [b] Y_{NO2} represents 2-nitrotyrosine; p represents D-proline; Dap represents diaminopropionic acid.

Table 2. List of Ligated $\beta\beta\alpha$ Peptides Synthesized in the First Generation

peptide name	amino acid length	sequence[a,b]
BBA-f	23	Ac-NH-[YRVDpSY DF **C(S-Me)SS** DELAKLLRQDap(Anthr)AG]-CO-NH_2
BBA-q	23	Ac-NH-[YRVDpSY_{NO2}DF **C(S-Me)SS** DELAKLLRQDap(Ac)AG]-CO-NH_2
BBA-del1-f	22	Ac-NH-[YRVDpSY DF **C(S-Me)S** DELAKLLRQDap(Anthr)AG]-CO-NH_2
BBA-del1-q	22	Ac-NH-[YRVDpSY_{NO2}DF **C(S-Me)S** DELAKLLRQDap(Ac)AG]-CO-NH_2
BBA-del2-f	21	Ac-NH-[YRVDpSY DF **C(S-Me)** DELAKLLRQDap(Anthr)AG]-CO-NH_2
BBA-del2-q	21	Ac-NH-[YRVDpSY_{NO2}DF **C(S-Me)** DELAKLLRQDap(Ac)AG]-CO-NH_2

[a] (Anthr) represents an anthranilic acid amide cap (anthrolyl) on Dap, (Ac) represents an acetyl cap on Dap. [b] Y_{NO2} represents 2-nitrotyrosine; p represents D-proline, Dap represents diaminopropionic acid.

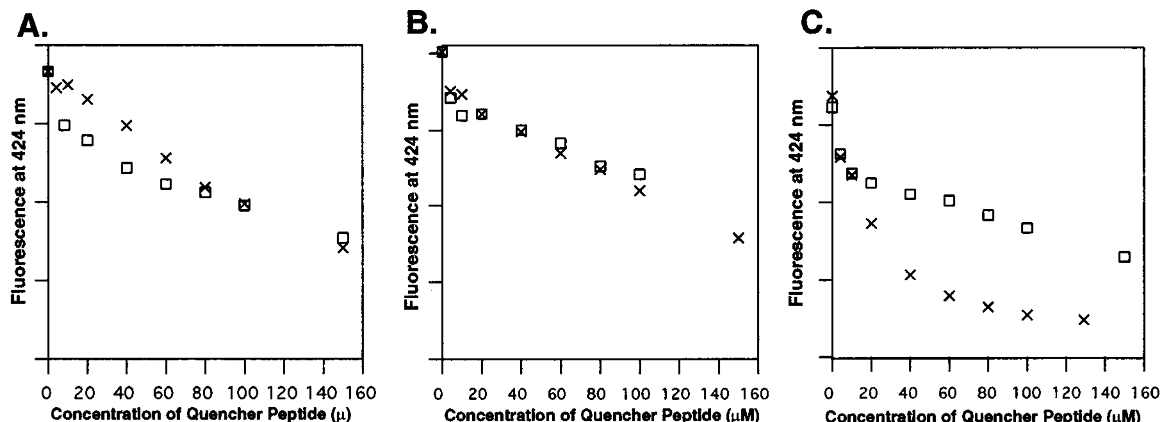


Figure 3. Fluorescence quenching titration experiments for the first generation of peptides. In each figure, a 4 μM solution of fluorophore-bearing peptide is titrated with incremental amounts of quencher-bearing $\beta\beta\alpha$ peptide (X). Separate titration experiments were performed for each fluorophore-labeled peptide with control quencher peptide (□) **CON-q** (Ac-NH-[KSY_{NO2}D]-$CONH_2$). In each case, excitation was at 315 nm, and emission was monitored at 424 nm. (A) Fluorescence quenching of **BBA-f**. (B) Fluorescence quenching of **BBA-del1-f**. (C) Fluorescence quenching of **BBA-del2-f**.

peptide contains either a fluorophore or a quencher but not both. The full length sequences are shown in Table 2.

The three sequence pairs were screened for fluorescence quenching by titrating in successive amounts of the quencher-bearing peptide to a 4 μM solution of fluorophore-bearing peptide at pH 7.5. The magnitude of the fluorescence-quenching response was compared to that of a control quencher-bearing peptide **CON-q** (Ac-Lys-Ser-Tyr_{NO2}-Asp-NH_2). From the results shown in Figure 3, it is clear that the sequence possessing the two-residue loop deletion had a much stronger fluorescence-quenching response than either of the two other sequences or the control peptide. Thus, it is likely that this two-residue deletion promoted *inter*molecular peptide association (see also Scheme 1).

Second Generation Peptides. We expanded on this initial discovery and designed a second set of peptides to try to optimize the interactions between the peptide surfaces (Table 3). We have found that many of the hairpin residues are critical for the type-II′ turn, and thus we synthesized two additional hairpin sequences possessing fairly conservative substitutions. Hairpin peptide **2** incorporated an isoleucine in place of valine in position 3. Hairpin **3** swapped the positions of Tyr1 and Phe8.

More drastic changes were made in the helix since we believed the helix to be more robust to sequence variations. The sequence **Helix-del2-q** was resynthesized as helix **A** to incorporate a benzoyl cap in place of the acetyl cap in its "quencher" form. We expected that the benzoyl cap would more accurately approximate the size of the anthranilic acid amide, and the benzoyl cap was used in the remaining helices for this reason. The Ala and Dap(X) residues were swapped in helix **B**; CPK modeling suggested that the fluorophore would be better aligned with the leucines to form a hydrophobic face. An isoleucine was substituted for a leucine in helix **C** to probe the effect of a β-branched amino acid in the core of the helix.[17] The hydrophobic Dap(X) was placed closer to the hydrophobic core in helix **D**—this increased steric bulk was compensated

(17) (a) Brive, L.; Dolphin, G. T.; Baltzer, L. *J. Am. Chem. Soc.* **1997**, *119*, 8598−8607. (b) Jiang, X.; Bishop, E. J.; Farid, R. S. *J. Am. Chem. Soc.* **1997**, *119*, 838−839. (c) Gibney, B. R.; Rabanal, F.; Skalicky, J. J.; Wand, A. J.; Dutton, P. L. *J. Am. Chem. Soc.* **1997**, *119*, 2323−2324.

Table 3. List of Purified Ligated ββα Peptides in the Second Generation

name[a,b]	no. residues	sequence[c,d,e]
A1	21	YRVpSY*DFCDELAKLLRQDap(X)AG
B1	21	YRVpSY*DFCDELAKLLRQ**ADap(X)**G
C1	21	YRVpSY*DFCDELAKL**I**RQDap(X)AG
D1	21	YRVpSY*DFCDELAKL**Dap(X)**RQ**A**AG
E1	21	YRVpSY*DFCDELAKLLRQDap(X)**L**G
F1	21	YRVpSY*DFCDELAKL**A**RQDap(X)**L**G
G1	20	YRVpSY*DFC**DLE**KLLRQDap(X)AG
I1	21	YRVpSY*DFCDELA**K**LLRKDap(X)AG
A2	21	YR**I**pSY*DFCDELAKLLRQDap(X)AG
B2	21	YR**I**pSY*DFCDELAKLLRQ**ADap(X)**G
C2	21	YR**I**pSY*DFCDELAKL**I**RQDap(X)AG
D2	21	YR**I**pSY*DFCDELAKL**Dap(X)**RQ**A**AG
E2	21	YR**I**pSY*DFCDELAKLLRQDap(X)**L**G
F2	21	YR**I**pSY*DFCDELAKL**A**RQDap(X)**L**G
G2	20	YR**I**pSY*DFC**DLE**KLLRQDap(X)AG
I2	21	YR**I**pSY*DFCDELA**K**LLRKDap(X)AG
A3	21	**F**RVpSY*D**Y**CDELAKLLRQDap(X)AG
C3	21	**F**RVpSY*D**Y**CDELAKL**I**RQDap(X)AG
D3	21	**F**RVpSY*D**Y**CDELAKL**Dap(X)**RQ**A**AG
F3	21	**F**RVpSY*D**Y**CDELAKL**A**RQDap(X)**L**G
G3	20	**F**RVpSY*D**YCDLE**KLLRQDap(X)AG
I3	21	**F**RVpSY*D**Y**CDELA**K**LLRKDap(X)AG

[a] Each name is derived from a hairpin name (number) and helix name (letter). [b] Each peptide name represents a pair of peptides: one peptide possessing a natural tyrosine residue at Y* and an anthrolyl Dap(X), the other peptide possessing nitrotyrosine at residue Y* and a benzoyl cap at Dap(X). [c] Dap(X) refers to either an anthranilic acid amide (anthrolyl) on the side-chain of Dap or benzoic acid cap (benzoyl) on its side-chain. The identity of X depends of the identity of Y* (see footnote b). [d] All cysteines are capped with an *S-Me* group to avoid interpeptide disulfides; All peptides possess a N-terminal acetyl group and a C-terminal amide. [e] Residues in **bold** denote a change from sequence **A1**.

for by placing an alanine near the end of the helix. **BBA5** possesses a "clam-shell" type structure where the end of the helix diverges well away from the hairpin.[5c] In an attempt to form a more well-packed interface, a leucine was placed near the end of the helix in helix **E**. Helix **F** also included this leucine substitution but compensated for this increased size by inserting an alanine into the core of the hydrophobic surface. We explored further loop-residue deletions in helices **G** and **H.** In helix **G**, residues near the loop were redesigned to delete one residue but retain the helix N-cap[18] Asp and an overall neutral charge. Helix **H** simply deleted the Asp residue and substituted a Gln for the Lys to, again, retain a neutral charge. Finally, helix **I** was synthesized with a Lys in place of the Gln in an attempt to form a cation−π interaction[19] with an aromatic residue on the hairpin.

As before, each suitably labeled thioester hairpin was ligated with the appropriate helix such that each ligated peptide either contained a fluorophore or a quencher. This generated 27 peptide pairs (3 hairpins × 9 helices); 5 of which were inseparable from major impurities by reversed-phase HPLC. The remaining 22 ligated ββα peptide pairs are shown in Table 3.

Each second-generation ββα peptide pair was screened for fluorescence quenching in a rapid fashion using a 96-well microwell plate reader connected to a fluorometer. Fluorescence measurements were taken for each peptide pair at a constant fluorophore concentration (5 μM) with six different quencher concentrations (0, 5, 10, 25, 50, and 75 μM). Additional experiments were performed with the control quencher peptide **CON-q** for comparison with sequences containing hairpin **1**. The results for each peptide pair are summarized in Table 4.

(18) Aurora, R.; Rose, G. D. *Protein Sci.* **1998**, *7*, 21−38.
(19) Ma, J. C.; Dougherty, D. A. *Chem. Rev.* **1997**, *97*, 1303−1324.

Table 4. Summary of the Fluorescence Screening Results of the Second Generation[a]

helix/hairpin combination	1	2	3	**1 + CON-q**
A	5	5	20	>75
B	<5	<5	b	>75
C	5	5	20	>75
D	25	15	65	>75
E	<5	<5	*b*	>75
F	40	insoluble	insoluble	>75
G	30	30	35	>75
H	*b*	*b*	*b*	*b*
I	5	5	25	>75

[a] Each entry represents the approximate concentration (μM) of quencher-containing peptide required to diminish the observed fluorescence of 5 μM of the fluorophore-containing member by 50%. Each data point was interpolated from a curve generated by six data points from six different concentrations of quencher-bearing peptide (0, 5, 10, 25, 50, 75 μM). Column 4 shows the data from fluorophore-bearing peptides containing hairpin 1 and control quencher peptide CON-q. Excitation was at 315 nm, and emission was monitored at 424 nm. [b] These peptides could not be purified by reversed-phase HPLC.

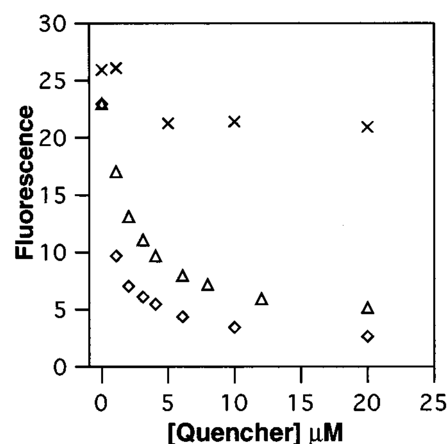


Figure 4. Three separate fluorescence quenching titration experiments of 1 μM fluorophore-labeled peptide and incremental amounts of a quencher-bearing peptide: Δ = titration of **B1-f** with **B1-q**; ◇ = titration of **B2-f** with **B2-q**; X = titration of **B1-f** with **CON-q**. Excitation was at 315 nm, and emission was monitored at 424 nm.

In search of a tight binding peptide interaction, we observed that peptides **B1** and **B2** had two of the strongest fluorescence-quenching responses from this screen where a tight binding interaction appeared to be forming at concentrations below 5 μM. Peptides **E1** and **E2** were not as soluble as peptides **B1** and **B2** and thus were not pursued further. The quenching experiment was subsequently repeated in a cuvette titration experiment to obtain a more accurate comparison of peptides **B1** and **B2** (Figure 4). It was found that peptide **B2** had the greatest fluorescence quenching response and thus appeared to be forming the tightest interpeptide association of all the peptides synthesized.

B2 was resynthesized with a natural tyrosine residue at position 6 and the Dap(Benzoyl) functionality at position 20. It was found that this peptide had very limited solubility in aqueous solution; it appeared that the solubility limit had been reached in performing the quenching experiments (∼100 μM). Having shown that the amino acids linking the hairpin and the helix were critical for inducing peptide association, we suspected that substitution of the Cys-S-Me "hinge" residue may have an influence on the structure and hence the solubility of the peptide. Replacement of Cys-S-Me (residue 9) with Gly resulted in a peptide with substantially increased solubility (>1 mM) (Scheme

1). This peptide was subsequently renamed **BBAT1** and has been further characterized using a complement of biophysical methods which revealed it to be a discrete well-structured symmetrical homotrimer in water.[6] Briefly, some of the biophysical characteristics of **BBAT1** include: (1) a greatly enhanced CD signal in comparison to monomeric **BBA5** consistent with a more structured helical portion of the peptide, (2) significant peptide−peptide binding at peptide concentrations of <10 μM, (3) a cooperative thermal denaturation which is typical of native protein structures and was not observed in the monomer, (4) sharp and disperse NMR signals which are typical of native protein structures, (5) significant protection of the amide protons from H/D exchange, and (6) several helix-sheet NOE contacts which suggest that the homotrimer consists of some form of domain-swapped[20] topology.[21]

Trends from Screening. Many interesting trends emerge upon analysis of the fluorescence-quenching data. First and most importantly, it appears that a two-residue deletion between the hairpin and helix portions of **BBA5** is optimal for inducing interpeptide association. Neither **BBA-del1** (removal of one residue) nor peptides **G1-G3** (removal of three residues) provide comparable fluorescence quenching responses. From the strategy outlined in Figure 1, it is likely that such a two-residue deletion in the loop prevents an intramolecular folding event between the two domains, causing the burial of the exposed hydrophobic surfaces only through interpeptide associations. Interestingly, the three-residue deletion is ineffective at promoting effective associations. This may be due to the removal of critical structure-inducing residues in the helix.

Hairpin **3** possesses Tyr1→Phe and Phe8→Tyr substitutions effectively swapping residues at positions 1 and 8. This change has a dramatic effect on the ability of the peptide undergo self-association (Table 4). All of the peptides with hairpin **3** are only slightly better binders than the control peptide **CON-q**. That a single hydroxyl group can dramatically alter the folding of this peptide suggests that Phe8 likely plays a critical role in the hydrophobic core of these oligomeric structures.

An additional trend observed with the hairpins is that peptides containing hairpin **2** appear to be associated with mildly stronger-binding peptides than those with hairpin **1**. The addition of a single methyl group in the side-chain of Ile versus Val suggests that this side-chain contributes significantly to the stability of the hydrophobic core of the oligomer.

The first trend observed with the helix portion of **BBAT1** involves the change from Dap(Acetyl) to Dap(Benzoyl) between the first and second generation of peptides. The presence of a benzoyl group in place of an acetyl group enhanced the fluorescence-quenching response as observed in **BBA-del2** versus **A1**: the concentration of quencher-bearing peptide required to diminish 50% of the observed fluorescence in **BBA-del2** was ~20 μM, while it was ~5 μM for **A1**. This suggests that the benzoyl group (and likely the fluorophore itself) contributes to the observed binding and is likely buried within a hydrophobic core. Studies are currently in progress to evaluate these considerations.

Helix **B** was associated with the strongest binding in both hairpins **1** and **2**. This sequence likely aligns the more hydrophobic Dap(Benzoyl) residue with the hydrophobic face formed by the three leucines already in the helix—perhaps aiding its burial with complementary hydrophobic groups.

Attempts to enhance the binding of the end of the helix were made by incorporating Leu residues in place of Ala near the helix terminus. In the case of helix **E**, binding was enhanced in comparison to helix **A** but its limited aqueous solubility precluded further study. Nevertheless, it demonstrates that this helix-end position is important for enhancing the binding of these $\beta\beta\alpha$ peptides. Helix **F** also incorporated an Ala in the hydrophobic core of the helix in an attempt to compensate for the steric bulk associated with the additional Leu residue. Peptides with this helix had limited solubility *and* more limited fluorescence quenching. Disruption of the all-leucine core likely removed necessary hydrophobic residues required for stronger binding.

Discussion

A small library of fluorophore- and quencher-containing peptides was screened for fluorescence quenching in search of an oligomeric form of the monomeric mini-protein motif **BBA5**. By eliminating two residues from the loop of **BBA5** and making some minor adjustments to the sequence, peptide **B2** was identified and found to possess the strongest fluorescence-quenching response. Replacement of the central Cys-S-Me residue with a glycine residue gave a peptide (**BBAT1**) that was studied further and found to form well-structured and discrete homo-trimers in aqueous solution.[6]

Replacement of the Cys-S-Me group was critical to the discovery of the discrete homotrimer. In particular, the fact that the addition or deletion of additional residues from peptide **B2** did not enhance fluorescence quenching led us to believe that the Cys-S-Me group may play a critical role itself. By choosing the most flexible amino acid available for the loop, the α and β domains were allowed to fold with the least amount of influence from the loop. Indeed, it is quite possible that the structure formed by peptide **BBAT1** does not accurately reflect the structure of parent peptide **B2** due to the differences in their loop regions.[22] In fact, the increased solubility of peptide **BBAT1** is likely the result of its better defined fold. Further analysis of this loop region will help us understand the role of the loop in the **BBAT1** fold, and such studies are ongoing.

Fluorescence quenching played a key role in this discovery process. The technique allowed for a rapid and sensitive assay for intermolecular associations using peptide concentrations in the low micromolar range. In addition, the method has the potential to determine the stoichiometry of the interaction.[23] It should be noted, however, that there are limitations to such a screening method. First, the manner in which the screen was interpreted may favor higher-order oligomers. For example, it is possible that we isolated a trimeric peptide over the dimer as a consequence of the screen: A trimeric association in the screen would possess two nitrotyrosine moieties which may better quench the anthrolyl fluorescence. Furthermore, in the interpretation of the screening results, we assume that each peptide combination is capable of quenching the fluorescence equally well. This may not be the case as different peptide−peptide orientations may have dramatically different quenching profiles and may have skewed our results toward peptide **B2**.[10] Interestingly, by studying a peptide with excellent fluorescence quench-

(20) Schlunegger, M, P.; Bennett, M. J.; Eisenberg, D. *Adv. Protein Chem.* **1997**, *50,* 61−122.

(21) Domain swapping as a result of truncations in protein loop regions has been previously reported. For example: (a) Russo, N.; Antignani, A.; D'Alessio, G. *Biochemistry* **2000**, *39*, 3585−3591 (b) Murray, A. J.; Head, J. G.; Barker, J. J.; Brady, R. L. *Nat. Struct. Biol.* **1998**, *5*, 778−791 (c) Green, S. M.; Gittis, A. G.; Meeker, A, K.; Lattman, E. E. *Nat. Struct. Biol.* **1995**, *2*, 746−751.

(22) Peptide **B2** does not form discrete homotrimers as observed by sedimentation equilibrium experiments.

(23) By interpreting the quenching curves, stoichiometry may be determined. See: London, E.; Khorona, H. G. *J. Biol. Chem.* **1982**, *257*, 7003−7011. We chose to use analytical ultracentrifugation as a more direct method to analyze the oligomeric states.

ing properties and a related peptide with acceptable (>1 mM) aqueous solubility properties, a peptide capable of forming discrete trimers was isolated.

There exist many potential methods to study interpeptide (protein–protein) interactions which take advantage of extremely large pools of peptides.[24] Despite the synthesis and screening of relatively few sequences (25 total peptide pairs), a relatively tight self-associating peptide was isolated using this method.[6] Key to this study were: (1) rational redesign of the monomeric **BBA5** scaffold to generate a peptide capable of self-association, (2) the ligation chemistry which proved useful to build a library of both helix and hairpin halves and limit the time required for peptide synthesis, and (3) the use of fluorescence quenching to provide rapid and sensitive information on intermolecular associations. In addition to studying the loop region, future work will focus on the development of methods to generate different oligomeric forms of the $\beta\beta\alpha$ scaffold. We envision these studies will help further our understanding of protein–protein interactions at α–β-type protein interfaces.

Experimental Section

Peptide Synthesis. Peptides were synthesized on either a Milligen 9050 or an Advanced Chemtech 396 automated peptide synthesizer using standard Fmoc/*t*-Bu strategy with HBTU as the coupling reagent. Helical sequences were synthesized on PAL-PEG-PS resin (Applied Biosystems) to afford C-terminal amides. N-terminal cysteine residues were double-coupled using *Boc-Cys(Trt)-OSu* to avoid cysteine racemization.[25] Hairpin sequences were synthesized on TGT resin (Novabiochem) with the first amino acid already coupled to the resin. *Fmoc-nitrotyrosine-OH* (Advanced Chemtech) was used in the synthesis directly without side-chain protection.

Thioester Hairpin Synthesis. 1. Preliminary Cleavage. After solid-phase peptide synthesis, TGT resin was treated with a mixture of AcOH:DCM:MeOH (5:4:1, 10 mL) for 1.5 h. The resin was then filtered and washed with DCM, and the filtrate was poured onto hexanes (300 mL). The solvent was removed in vacuo at which time an additional 100 mL of hexanes was added and removed in vacuo. The side-chain protected peptide was lyophilized from benzene to afford a white solid. Peptide identities were confirmed by ESI-MS.

2. Thioesterification. Protected peptide (80 mg, 52 μmol), HBTU (78 mg, 206 μmol), DIEA (72 μL, 412 μmol), and benzylmercaptan (24 μL, 206 μmol) were added to THF (7 mL) and stirred for 16 h. The reaction mixture was evaporated in vacuo, purified by silica gel flash column chromatography ($CHCl_3$:MeOH, 95:5 followed by 90:10), and lyophilized from benzene (typically 85–95% yield). Identities were confirmed by ESI-MS.

3. Final Cleavage. All of the protecting groups were cleaved from the peptide using 95% TFA, 2.5% triisopropylsilane, 2.5% H_2O for 2 h. The resin was subsequently filtered, the volume of the filtrate was reduced with a stream of N_2, and the peptide was precipitated with ice-cold ether. The peptide was isolated by centrifugation, triturated three times with ether, dissolved in 3:1 H_2O:CH_3CN, and lyophilized to afford a white solid. Each peptide was a single peak by reversed-phase HPLC and gave the appropriate mass by either ESI-MS or MALDI-MS. The hairpins were not purified further prior to native ligation.

Fluorophore-Labeled Helix Synthesis. 1. Alloc Removal from Dap Residue.[26] Protected PAL-PEG resin (1.5 g, 0.22 mmol) was swelled in $CHCl_3$:AcOH:morpholine (90:5:5, 10 mL) while purging with N_2. $Pd(PPh_3)_4$ (250 mg) was added to the reaction vial and vortexed for 2 h. The resin was then filtered, washed with DCM (25 mL), cocktail A (25 mL DMF, 225 mg diethyldithiocarbamic acid·$3H_2O$, 250 μL Et_3N), cocktail B (25 mL DMF, 125 μL Et_3N), and finally DCM (25 mL).

2. Coupling of Anthranilic Acid or Benzoic Acid. The peptide resin was split into two parts in an approximate ratio of 2.5:1. The larger portion was reacted with a 4-fold excess of benzoic acid, HOBt, HBTU, and an 8-fold excess of DIEA for 1 h. The smaller portion of resin was reacted with a 4-fold excess of Boc-anthranilic acid, HBTU, and an 8-fold excess of DIEA for 1 h. The above reactions were performed in parallel on a manual peptide synthesis station using N_2 bubbling for mixing.

3. Final Cleavage. The derivatized resin was cleaved with 95% TFA, 2.5% ethanedithiol, 1.5% triisopropylsilane, and 1% H_2O for 2 h. The crude peptide was isolated as for the hairpin. Each desired peptide was determined to be the major component of a reverse-phase HPLC chromatogram and gave the appropriate mass by either ESI-MS or MALDI-MS. The helices were not purified further prior to native ligation.

General Procedure for Chemoselective Ligation of Hairpins and Helices. Peptide hairpin (2 mg, 1.6 μmol) and peptide helix (4.8 mg, 3.2 μmol) were added to a mixture of acetonitrile and 200 mM pH 7.5 phosphate buffer (1:1, 2 mL), benzylmercaptan (40 μL), and benzenethiol (40 μL). The reaction was agitated for 16 h at room temperature, cooled to 4 °C, and reacted with methyl methanethiolsulfonate (132 μL) for 30 min. The reaction mixture was extracted with ice-cold ether (3 mL). This ether layer was back-extracted with water and combined with the aqueous layer. The crude ligated peptide was then purified by reversed-phase HPLC and lyophilized. Each $\beta\beta\alpha$ peptide gave the expected mass by either ESI-MS or MALDI-MS and was found to be a single peak (>95% pure) by analytical reverse-phase HPLC.

Fluorescence Experiments. The approximate concentrations of each peptide were determined on the basis of the following estimated extinction coefficients: anthranilic acid, $\epsilon = 2000\ M^{-1}\ cm^{-1}$ at 315 nm; 2-nitrophenol, $\epsilon = 3500\ M^{-1}\ cm^{-1}$ at 424 nm. Excitation was performed at 315 nm, and emission was scanned from 350 to 550 nm. Each scan was baseline-corrected and volume-corrected prior to tabulating the data at 424 nm. All experiments were performed at 25 °C in pH 7.5, 50 mM phosphate buffer. For the titration experiments involving a cuvette, a 1 cm path length was used. For experiments using the 96-microwell plate reader, each well had a total volume of 350 μL. For each $\beta\beta\alpha$ pair, the fluorophore-bearing peptide was added to six of the wells such that its final concentration would be 5 μM. To each of these six wells was added $\beta\beta\alpha$ quencher peptide such that the final concentration of quencher peptide would be: 0, 5, 10, 25, 50, and 75 μM. For $\beta\beta\alpha$ peptides containing hairpin **1**, the experiment was repeated with control quencher peptide **CON-q**. The data for each peptide pair were compared by normalizing the fluorescence at zero concentration of quencher to that of peptide **A1** at zero concentration of its quencher.

Acknowledgment. We thank Kevin McDonnell for insightful discussions. This research was supported by NSF Grant CHE-9996335. The Multiuser Facility for the Study of Complex Macromolecular Systems (NSF-0070319), the Francis Bitter Magnet Laboratory, and the Department of Chemistry Instrumentation Facility (NIH 1S10RR13886) are also gratefully acknowledged.

JA004292F

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