Synthesis of a Three-Helix Bundle Protein by Reductive Amination

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An organic trialdehyde, TRIPOD (**2**), was designed as a template for the synthesis of a three-helix bundle protein. Crystallographic data indicate that the aldehyde groups are appropriately spaced to maximize hydrophobic interactions between the chains of the protein. Peptide strands were attached to the template by reductive amination to yield a bundle protein that is 80% helical at pH 4.1. Synthesis and conformational studies of the bundle protein as well as a model compound are detailed. Binding studies with 1-anilino-8-naphthalenesulfonate, a fluorescent dye, suggest a molten globule state for the bundle protein.

Introduction

Polypeptide chains are inherently flexible, and nature utilizes various specific interactions between amino acid residues to force a peptide chain to adopt particular secondary and tertiary structures. Since biological functions of proteins are closely associated with their folded conformations, there is strong interest in developing synthetic strategies to stabilize secondary and tertiary structures of short synthetic peptides. Usually, short peptides exist in a random coil conformation in solution. One of the most promising strategies of supplying folding information uses a rigid template molecule to define the spatial arrangement of amino acid residues or peptide segments.

There are a number of examples of using templates to organize peptide strands with defined secondary structures into a bundled or coiled protein. The template can provide the correct spacing between the strands in the bundle so that maximum stability due to hydrophobic interactions can be achieved. Various inorganic[1] as well as organic molecules have been used as templates.[2] Peptide chains can be attached to a template through stepwise peptide synthesis, although the purity of the final product suffers from accumulated errors in deprotection/coupling reactions. An alternative route is ligation of purified strands to a multifunctional template.[3] This provides synthetic proteins in better yield and purity. It has, however, been difficult to assemble proteins with heteromeric peptide segments by stepwise ligation of multiple peptide segments to a template. We wish to report a new method in which three peptide chains are linked to a trialdehyde organic template by reductive amination of the N-terminus of the peptides to the aldehyde groups on the template (Figure 1). The reductive-amination chemistry offers a convenient route to assemble heteromeric bundle proteins by isolating intermediate proteins after each coupling step.

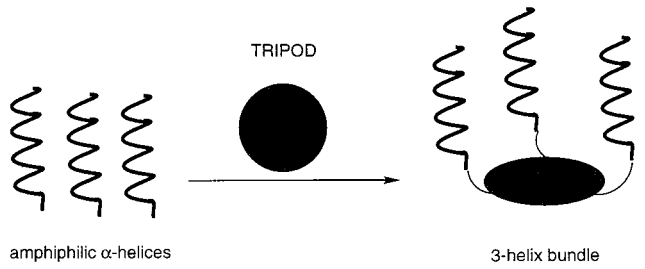
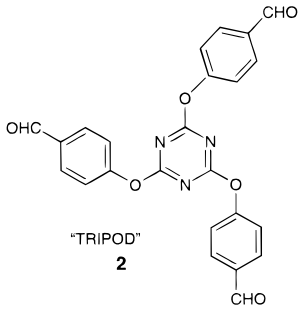


Figure 1.



Results and Discussion

TRIPOD(alanine *tert*-butyl ester)$_3$ (**1**) was synthesized by the reaction of a 3-fold excess of alanine *tert*-butyl ester and TRIPOD (**2**)[4] in the presence of $NaBH_3CN$ (Scheme 1). The reaction was followed by TLC and was complete after 3 h. Compound **1** was isolated and purified from the reaction mixture by silica gel column chromatography.

We have synthesized TRIPOD(glycine *tert*-butyl ester) (**3**), by the reductive amination of 1 equiv of glycine *tert*-butyl ester to TRIPOD. Compound **3** was isolated and purified from the reaction mixture by silica gel column chromatography to yield the desired monoglycinyl TRIPOD in 20% yield. NMR of purified **3** confirmed the

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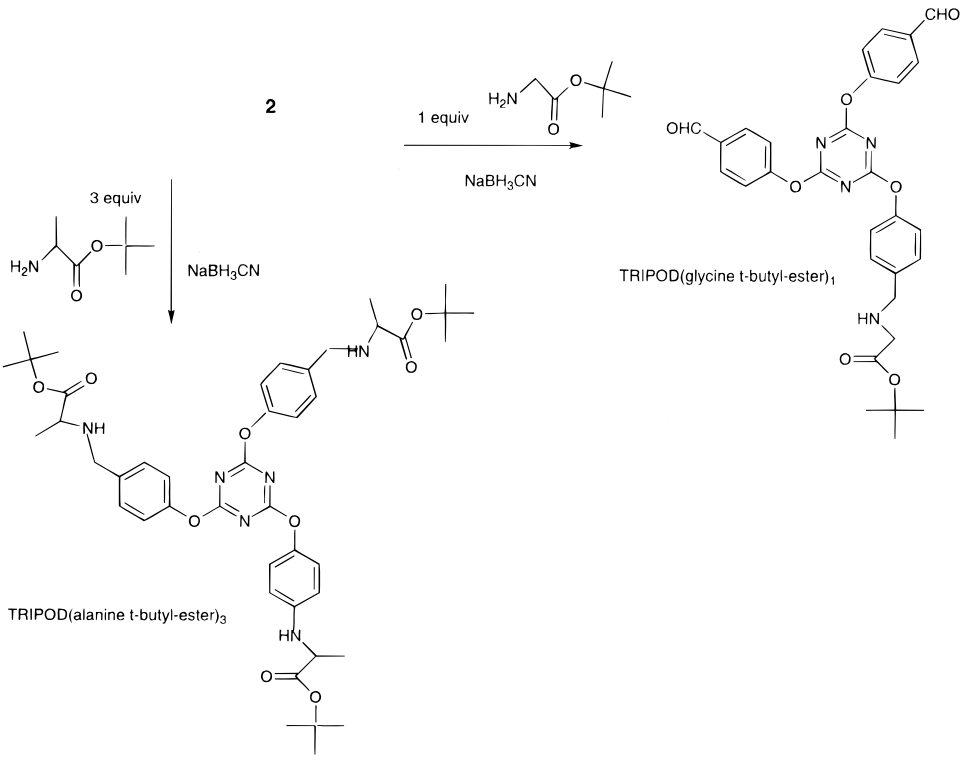
Scheme 1



presence of two intact aldehyde groups that could be reacted with other amino acids or peptides to synthesize a heteromeric protein.

TRIPOD is a good template for the assembly of three-helix bundle proteins. Figure 2 shows the crystal structure of **2**. The distance between the aldehyde carbons is 12.7 Å, which is an ideal distance between amphiphilic α-helices to maximize hydrophobic interactions. We were able to attach a 15-residue peptide to the TRIPOD template to assemble a three-helix bundle protein. The peptide sequence used in this work, AEQLLQEAE-QLLQEL-$CONH_2$, has previously been used by our group in the synthesis of artificial helical proteins.[1a] Figure 3 shows the helical wheel projection of the 15-residue peptide. The peptide has high potential to adopt an amphiphilic α-helical conformation. When attached to the TRIPOD template, three helical segments are expected to interact with each other to form a hydrophobic core and consequently display increased helicity.

The peptide was synthesized using standard Fmoc chemistry. Compound **2** was slowly added to the fully deprotected peptide (9 equiv) in the presence of $NaBH_3$-CN. The reaction was followed by reverse-phase HPLC. Initially, two peaks appeared (retention times = 10.4, 13.8 min). These peaks eventually were replaced by a more hydrophobic peak (retention time = 14.4 min). This third peak was identified as the desired TRIPOD$(pep)_3$ (**4**) on the basis of ion-spray MS analysis. The first two peaks appear to be intermediate mono- and bis-peptide species.

p-Anisaldehyde was attached to the peptide by a similar procedure to synthesize a control compound, pep-*p*-anis (**5**). Compound **5** showed solubility and UV spectroscopic properties similar to those of **4**. The two

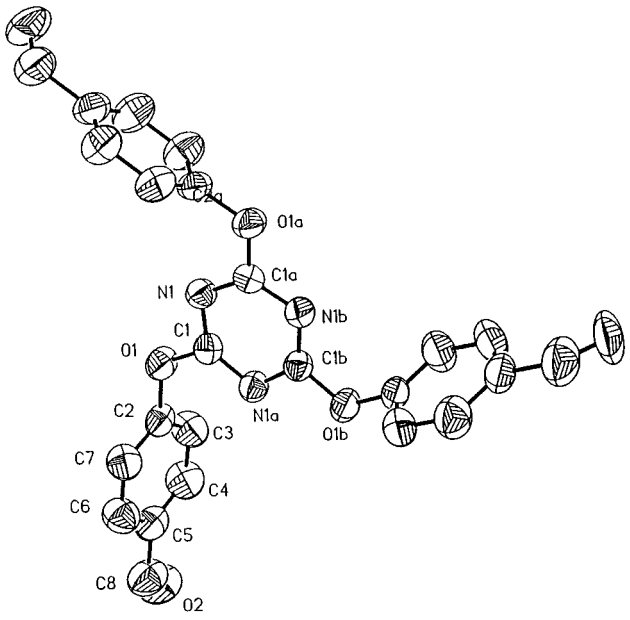


Figure 2. ORTEP of TRIPOD.

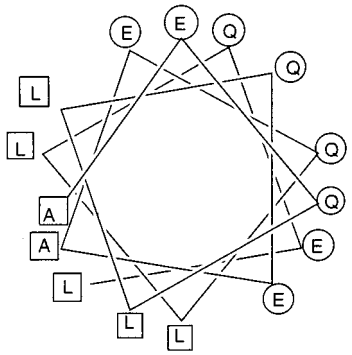


Figure 3. Animo acid sequences: A = alanine, E = glutamic acid, Q = glutamine, L = leucine. Hydrophobic residues are circled.

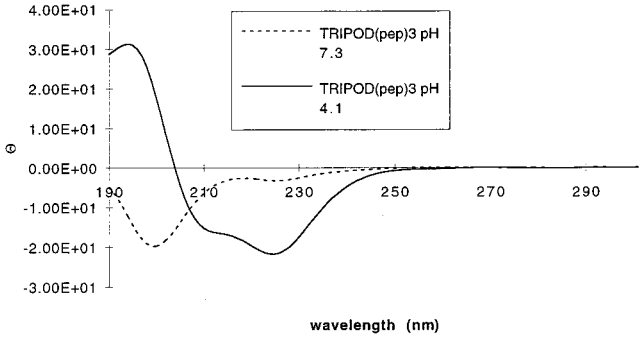


Figure 4. CD spectra of TRIPOD(pep)$_3$.

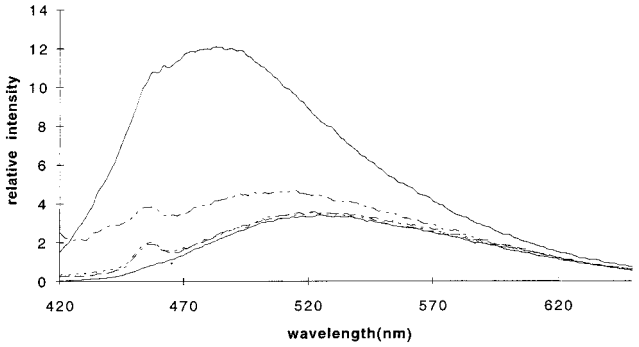


Figure 5. (a) ANS (—); TRIPOD(pep)$_3$, pH 7.0 (— - —); (c) ANS + TRIPOD(pep)$_3$, pH 6.0 (- — -); (d) TRIPOD(pep)$_3$, pH 5.0 (— — -); TRIPOD(pep)$_3$, pH 4.0 (—).

compounds were soluble in neutral and basic aqueous solution and form precipitates at pH <4. TRIPOD(pep)$_3$ is most stable in the neutral pH range, between pH 6 and 8, and is stable at room temperature for at least 1 day under these conditions. At more acidic or basic pH, there is irreversible decomposition of the TRIPOD core after several hours. At pH 5, TRIPOD(pep)$_3$ is stable between −4 and 50 °C, at which point the compound precipitates out of solution.

Circular dichroism (CD) spectroscopy was used to analyze the structure of the three-helix bundle formed and of the control compound **5**. CD spectra of these two compounds were independent of their concentrations, consistent with monomeric species under the experimental conditions. Compound **5** is not very helical (9% at pH 6.95) at neutral pH, but becomes more helical as the pH decreases (28% at pH 4.0) due to protonation of the glutamic acids. This is consistent with previous observations of artificial proteins that contain the same sequence.[1a] The α-helicity of **4**, the three-helix bundle protein, also shows a similar pH dependence. It ranges from approximately 11% helical at pH 7.3 to almost 86% helical at pH 4.1 (Figure 4). This large difference in helicity between pep-*p*-anis and TRIPOD(pep)$_3$ can be attributed to the association of three helices organized by the TRIPOD.[5]

It has been demonstated that many designed proteins do not adopt a nativelike structure but instead are "molten globules".[6] These intermediate folding states are compact and have a high degree of secondary structure but lack well-defined tertiary interactions. The structural fluctuation causes the hydrophobic core of the protein to be accessible to binding of hydrophobic dyes. We examined the degree of structural fluctuation of **4** by a dye-binding experiment with a hydrophobic fluorescent dye, 1-anilino-8-naphthalenesulfonate (ANS). ANS is known to bind to the hydrophobic core of the molten globule state of proteins. The binding causes a blue shift in the fluorescence maximum with a concomitant increase in the fluorescence intensity of ANS. The hydrophobic core of a native protein is inaccessible to ANS due to its rigid structure.[7]

The ANS binding experiments were carried out at constant ANS concentration and varying pH (Figure 5).[8] The addition of **4** at high pH has very little effect, which is consistent with an unfolded state of the protein. As the pH is decreased to 5, where the bundle becomes moderately helical, there is a slight increase in emission and the maximum is shifted to lower wavelength. At pH 4, the emission spectrum of ANS showed a large change upon addition of **4**: the fluorescence intensity is increased approximately 4-fold as compared with the bundle at high pH and the emission maximum is shifted from 526 to 482 nm. Reported values of λ_{max} of ANS when bound to various molten globules are 470−492 nm. The dye-binding experiments support the molten globule state of **4** and are consistent with the broad packing energy surface determined for a similar three-helix bundle protein assembled on a metal template.[1]

In conclusion, we have demonstrated a novel method of ligating peptide strands to an organic template for the synthesis of three-helix bundle proteins. The method can be extended for the synthesis of heteromeric bundle proteins by stepwise reductive amination. TRIPOD-(pep)$_3$, the model homotrimer, has been shown to be random coil at neutral pH and approximately 86% helical at pH 4.1. The three-helix bundle protein appears to have an exposed hydrophobic core to which ANS binds, indicating a molten globule state of the protein.

Experimental Section

General Comments. An ABI model 430A automated peptide synthesizer was used with standard FMOC cycles. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, using TMS as an internal standard.

Pep (NH$_2$-AEQLLQEAEQLLQEL-CONH$_2$). Pep was synthesized using conventional solid-phase Fmoc chemistry on Rink resin.[10]

The yield of resin-bound peptide was 88%, as determined by weight gain of the resin. The final Fmoc was removed with 20% piperidine/DMF. Pep was cleaved from the resin with 2% H_2O/TFA for 2 h at room temperature, filtered, and then precipitated in anhydrous diethyl ether. Pep was purified by G-15 column in 2% NH_4HCO_3 followed by semipreparative reversed-phase HPLC purification (44% CH_3CN isocratic, 3 mL/min, 220 nm).

ESMS: 877.4 $[M + 2H]^{2+}$, 888.0 $[M + Na + H]^{2+}$, 896.5 $[M + K + H]^{2+}$. Amino acid analysis: Q/E 8.68 (8), A 2.00 (2), L 4.75 (5)

(5) TRIPOD(alanine *tert*-butyl ester)$_3$ showed a very small CD signal at 222 nm (278 mol deg cm^2 dmol^{-1}), indicating that there is negligible contribution to the CD spectrum by the template itself.

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(8) The emission spectrum of ANS was not pH dependent in the range studied, pH 4−7. TRIPOD(alanine *tert*-butyl ester)$_3$ did not bind ANS to the TRIPOD core. The studies also indicate that the addition of pep has very little effect on the emission of ANS and does not shift the maximum at all.

TRIPOD(alanine *tert*-butyl ester)$_3$ (1). To a solution of 100 mg (2.27×10^{-4} mol) TRIPOD in 5 mL of 50% CH_3CN/MeOH with a small amount of $NaBH_3CN$ was added 370 mg (2.04×10^{-3} mol, 3×) alanine *tert*-butyl ester. The reaction was monitored by TLC (90% EtOAc−10% EtOH). The reaction mixture was stirred for 3 h, concentrated, and purified on a silica column in 90% EtOAc/10% EtOH to recover 43 mg (5.19×10^{-5} mol, 23% yield).

IR (KBr pellet): 3472, 3119, 2979, 1727, 1577, 1385, 1218, 844 $cm^{-1.}$ ^1H NMR (DMSO) δ 1.17 (d, 9H, 6.95 Hz), 1.43 (s, 18H), 3.11 (q, 3H, 6.81 Hz), 3.57 (d, 3H, 13.54 Hz), 3.73 (d, 3H, 13.54 Hz), 7.16 (d, 6H, 8.50 Hz), 7.34 (d, 6H, 8.50 Hz). ^{13}C NMR (DMSO) δ 18.59, 27.64, 50.02, 55.83, 79.94, 120.98, 128.80, 138.15, 149.98, 173.03, 174.29. ESMS 829.7 M^+, 415.4 $[M + 2H]^{2+}$

TRIPOD(glycine *tert*-butyl ester) (3). To a solution of 100 mg (2.27×10^{-4} mol) TRIPOD in 5 mL of MeOH with a small amount of $NaBH_3CN$ was added 30 mg (1.59×10^{-4} mol) of glycine *tert*-butyl ester. The reaction mixture was stirred for 30 min, concentrated, and purified on a silica column in 100% EtOAc to recover 18 mg (3.23×10^{-5} mol, 20% yield).

^1H NMR (CD_3CN) δ 1.44 (s, 9H), 3.18 (s, 2H), 3.73 (s, 2H), 7.09 (d, 2H, 8.60 Hz), 7.35 (d, 8H, 8.60 Hz), 7.93 (d, 6H, 8.60 Hz), 9.96 (s, 2H). ESMS: 557.1 M^+.

TRIPOD(pep)$_3$ (4). To a solution of 3 mg (1.7×10^{-6} mol) of HPLC-purified pep in 1 mL of absolute MeOH containing a small amount of $NaBH_3CN$, was added 1.8×10^{-7} mol of dilute TRIPOD (stock solution is 8 mg/mL) slowly over a few hours. The reaction was monitered by reversed-phase HPLC on an analytical column (38% CH_3CN−80% CH_3CN linear gradient in 20 min, 1 mL/min, 230 nm). TRIPOD had a retention time of 11.4 min; pep, 11 min; and TRIPOD(pep)$_3$, 14.4 min. The reaction was left overnight. The yield of TRIPOD(pep)$_3$ was approximately 60% on the basis of HPLC. TRIPOD(pep)$_3$ was purified by HPLC, collected directly into dry ice/acetone to prevent decomposition, and immediately lyophilized.[11]

ESMS: 5676 $[M + Na]^+$, 5692 $[M + K]^+$. Amino acid analysis: Q/E 8.00 (8), A 1.20 (1), L 4.60 (5)

***p*-Anisaldehyde Modified Peptide (pep-*p*-anis) (5)**. To a solution of 2 mg (1.1×10^{-6} mol) of HPLC-purified pep in 1 mL of absolute MeOH was added an excess of $NaBH_3CN$. Approximately a three times excess of *p*-anisaldehyde was added slowly. The reaction was monitored by reversed-phase HPLC on an analytical column (38% CH_3CN−80% CH_3CN linear gradient in 20 min, 1 mL/min, 230 nm). *p*-Anisaldehyde had a retention time of 5.8 min; pep, 11 min; and pep-*p*-anis, 12.4 min. After 2 h, the reaction was 25% complete. There were no side products observed. The reaction was left standing overnight at room temperature to complete the reaction. The product was purified by G-15 column in 2% NH_4HCO_3 to remove excess $NaBH_3CN$. No further purification was necessary.

ESMS: 949.1 $[M + Na + H]^{2+}$, 938.1 $[M + 2H]^{2+}$. Amino acid analysis: Q/E 8.00 (8), A 1.27 (1), L 5.24 (5)

Fluorescence Binding Experiments. In a typical experiment, 3 mL of sample (approximately 4×10^{-7} M protein) was placed in a cuvette. ANS was added so that there was 1 mol of ANS/1 mol of amino acid residue (ANS concentrations were approximately 2×10^{-5}). The sample was excited at 395 nm and the emission spectrum was recorded from 420 to 650 nm.

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Supporting Information Available: ^{13}C NMR spectra of **1** and crystallographic details, crystallographic experimental procedures, and tables of positional and anisotropic positional parameters, bond lengths and bond angles for TRIPOD (13 pages). This material is contained in 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.

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(11) There is degradation if the product is not frozen directly off of the HPLC. There is also degradation during Sephadex G-15 gel column purification. The product can be isolated with very high purity if frozen directly off of the HPLC.