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Pseudo-Symmetrical Protein: Construction of a Four-Helix-Bundle Protein with Four Intramolecular Disulfide Cross-Linkings

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Abstract: An 84-residue cyclic protein, which has a four-helix-bundle structure, was constructed by assembling four helical peptides with four intramolecular disulfide cross-linkings.

The construction of α-helical protein motifs is one of the main targets of bioorganic chemists because these motifs are fundamental building blocks for construction of artificial functional proteins.[1)] One of the questions which most often arises during the construction of the four-helix-bundle proteins is if all the helices in the protein are really folded into the proper, globular structure (Fig. 1). To answer such a question, cyclization of the protein will be one of the solutions, which will stabilize the bundle structure and will also limit the movement of the molecule.[2)] We have already reported an approach where four α-helical peptide units are selectively cross-linked with three disulfides to form a four-helix-bundle protein.[3)] The main feature of this approach is the feasibility of constructing proteins composed of helices with different amino acid sequences (i.e., heterogeneous helices). This approach provides a way to construct proteins having more complicated functions than those of de novo designed proteins composed of helices with identical amino acid sequences (i.e., homogeneous helices). Here we report a new strategy for the construction of a pseudo-symmetrical protein where four α-helical peptide units are assembled using four intramolecular disulfide cross-linkings. Its cyclic, pseudo-symmetrical structure should be not only advantageous for obtaining a reliable bundle structure but also convenient for conformational energy calculations.

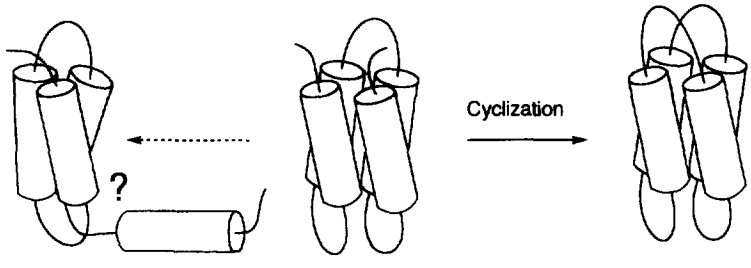


Fig. 1. Schematic Representation of Structures of Four-Helix-Bundle Proteins

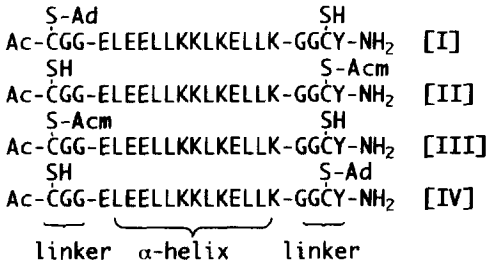
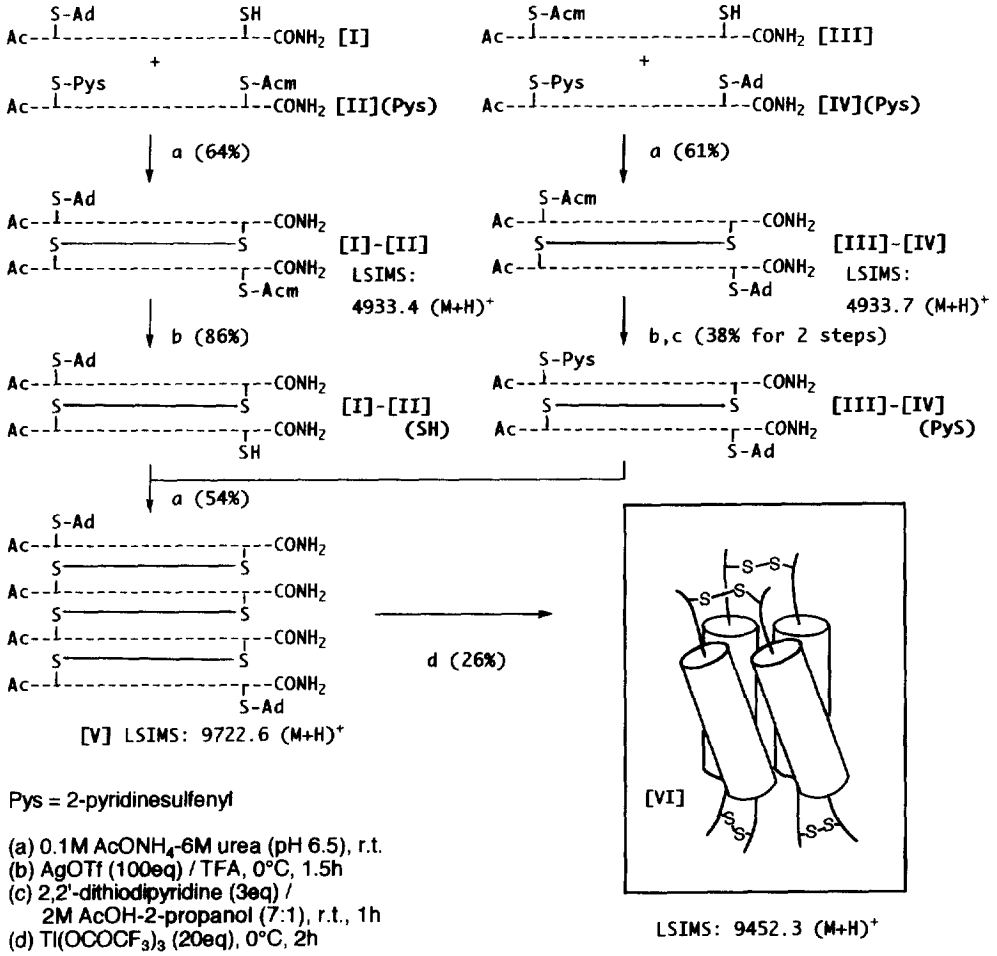


Fig. 2. Design of Peptide Units
(C = Cys; G = Gly; E = Glu; L = Leu; K = Lys; Y = Tyr; Ac = acetyl)

Scheme



Pys = 2-pyridinesulfenyl

(a) 0.1M $AcONH_4$-6M urea (pH 6.5), r.t.
(b) AgOTf (100eq) / TFA, 0°C, 1.5h
(c) 2,2'-dithiodipyridine (3eq) /
2M AcOH-2-propanol (7:1), r.t., 1h
(d) $Tl(OCOCF_3)_3$ (20eq), 0°C, 2h

First, the four α-helical peptide units were cross-linked with three disulfides, basically following the strategies of our previous reports.[2,3] Namely, four peptide units (**[I]**, **[II]**, **[III]** and **[IV]**) were designed as shown in Fig. 2. Each unit has an amphiphilic α-helical sequence in the middle of the molecule. On each side of the sequence, a Cys residue was placed *via* a Gly-Gly linker. In order to form the selective inter-unit cross-linking formation, selectively cleavable sulfhydryl protecting groups, the acetamidomethyl (Acm) or adamantyl (Ad),[4] were employed. Here the Ad group was the key to the fourth disulfide formation (cyclization). Unit **[I]**,[3] and units **[II]** and **[III]**[2] were synthesized as reported. Unit **[IV]** was prepared using Fmoc-solid-phase synthesis[5] on Rink's amide resin[6] followed by Me_3SiBr[7] and silver trifluoromethanesulfonate (AgOTf)[8] treatments [yield: 15% from the starting resin; liquid secondary ion mass spectrometry (LSIMS): 2499.3 $(M+H)^+$].[9] Construction of the four-helix-bundle protein was achieved as shown in the scheme. Treatment of **[II]** with 2,2'-dithiodipyridine (3 eq) in 2M AcOH-2-propanol (7:1) for 20 min[2,10] afforded [^{1}Cys(Pys)]-**[II]** (=**[II](Pys)**). The inter-unit disulfide cross-linking formation was accomplished by simply mixing **[II](Pys)** with **[I]** (1:1) in 0.1M ammonium acetate containing 6M urea (pH 6.5) at r.t. for 30 min. Purification of the product on HPLC (C_{18} column) afforded a pure cross-linked peptide (**[I]-[II]**). Another cross-linked peptide **[III]-[IV]** was also obtained through pyridinesulfenylation of ^{1}Cys in unit **[IV]** followed by cross-linking formation with unit **[III]** in essentially the same manner as in the case of **[I]-[II]**. Next, unmasking of the Acm groups from Cys(Acm) in **[I]-[II]** and **[III]-[IV]** was achieved by the treatment with AgOTf to afford **[I]-[II](SH)** and **[III]-[IV](SH)**, respectively. By activation of the unmasked Cys in **[III]-[IV](SH)** by pyidinesulfenylation (**[III]-[IV](Pys)**) and coupling with the unmasked Cys in **[I]-[II](SH)** for 1h, the third disulfide cross-linking was formed without problems to give **[V]**.

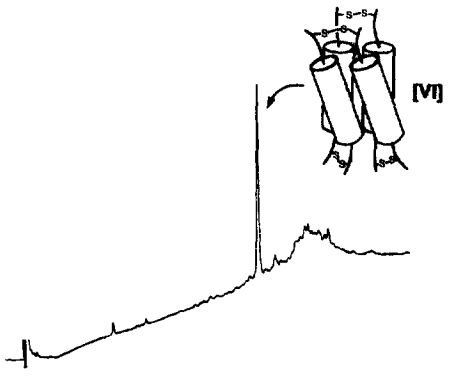


Fig. 3. Formation of **[VI]** by $Tl(OCOCF_3)_3$ Treatment. HPLC profile after a 2h-treatment is shown [Column: μBondasphere 5C4 300Å (3.9x150 mm); Eluent: A= CH_3CN-0.1%TFA, B=H_2O-0.1%TFA, A%=30-70% in 40 min; Flow Rate: 0.8 ml/min; Detection: 215 nm].

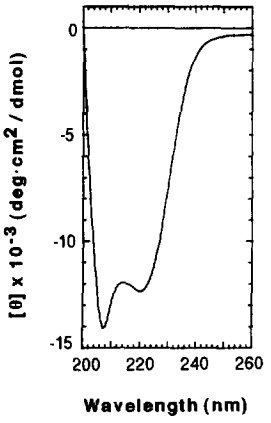


Fig. 4. CD Spectrum of **[VI]** in 10mM 3-(*N*-Morpholino)propanesulfonic Acid (MOPS) (pH 7.0). Concentration: 34μM

Formation of the fourth cross-linking was simultaneously achieved with detachment of two Ad moieties in [V] by treatment with thallium(III) trifluoroacetate [$Tl(OCOCF_3)_3$] (20 eq; peptide concentration: 80μM) in trifluoroacetic acid (TFA) containing 1% anisole (0°C, 2h).[11] As shown in Fig. 3, the fourth disulfide formation was very successful with an isolation yield of 26% to give the desired four-helix-bundle protein [VI]. Considering that these two Cys residues were located 84 residues apart in the molecule and that intermolecular disulfide formation is, in general, much more dominant over intramolecular disulfide formation in such cases, the yield seems exceptionally good. One possible explanation for the high yield may be that, even in TFA, each peptide still holds the helical conformation to some extent. The strong tendency to form a helical structure might restrict the movement of the helical region of the protein and, eventually, the possibility of both Cys residues located in close proximity became high. The CD spectrum of the protein was indicative of an α-helical structure with a double minima at 207 nm and 221 nm ($[\theta]_{222}$: -1.3×10^4 deg·cm^2/dmol, protein concentration: 34μM) (Fig. 4).

In conclusion, our strategy has opened the way to construct artificial four-helix-bundle proteins with four intramolecular disulfide cross-linkings. The pseudo-symmetrical structure should be advantageous not only for stabilizing the helical structure but also for the conformational energy calculation. Also, it may be possible to utilize this molecule as a new chemical receptor or drug carrier. In addition, to our knowledge, this is the largest cyclic protein of which synthesis has been reported. Further characterization of the protein is under way in our laboratory.

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