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Peptide-Unit Assembling using Disulfide Cross-Linking: A New Approach for Construction of Protein Models

Shiroh Futaki*

Institute for Medicinal Resources, The University of Tokushima, Shomachi, Tokushima 770, Japan

Kouki Kitagawa

Faculty of Pharmaceutical Sciences, The University of Tokushima, Shomachi, Tokushima 770, Japan

Abstract: *Four α-helical peptide units were assembled by selective disulfide cross-linking using S-pyridinesulfenyl activation of cysteine. A four-helix bundle protein composed of 84 amino acid residues was obtained without difficulty.*

Recently great efforts have been directed toward de novo design of proteins for creation of artificial functional proteins and for the understanding of problems lying on protein foldings. The construction of amphiphilic α-helical protein structure has been extensively studied, and various achievements in constructing α-helix bundle proteins have been reported.[1] Several successful approaches using peptides/proteins that were designed to simulate local three-demensional structure of proteins have also been reported to elucidate how local structure of proteins contributes to manifest protein function as a whole.[2] The accumulated knowledges obtained from design of artificial proteins would greatly contribute to the construction of protein models and eventually to the understanding of function of proteins.[3] And new strategies for construction of much more sophisticated models have been expected.

Here we report a new approach for construction of α-helix bundle protein models using peptide-unit assembling by selective disulfide cross-linking. Our strategy is (i) to construct peptide units using Fmoc (9-fluorenylmethyloxycarbonyl)-solid-phase peptide synthesis,[4] (ii) then to assemble them using selective disulfide cross-linking after activation of a cysteine by pyridinesulfenylation.[5] With this approach, in theory, we should be able to obtain α-helical protein models easily. In order to ascertain that our approach is practical for preparing protein models, we have constructed an equivalent of DeGrado's de novo designed four-helix bundle protein[1a] prepared by gene-manipulation, since the characterization of the protein was thoroughly conducted by his group.

Four peptide units were designed as shown in Fig. 1. In the center of the units we have employed the same α-helical sequences as DeGrado employed[1a] so that we could compare the characteristics of both proteins and confirm the formation of α-helical protein with our approach. On both sides of the helical parts, flexible linkers[2a] were placed. Each N-terminal was acetylated and C-terminal amidated to avoid possible repulsion due to the helix dipole.[6] C-terminal tyrosines were incorporated to facilitate the easy purification with monitoring UV absorbance at 275 nm.

Each peptide unit ([I]-[IV]) was prepared by Fmoc-solid-phase synthesis[4] followed by deprotection with 1M trimethylsilyl bromide-thioanisole in TFA.[7] Purification of the peptides by HPLC afforded pure samples.[8] Next, treatment of [II] with 2,2'-dithiodipyridine (3 eq) in 2N AcOH-2-propanol (10:3) at room temperature for

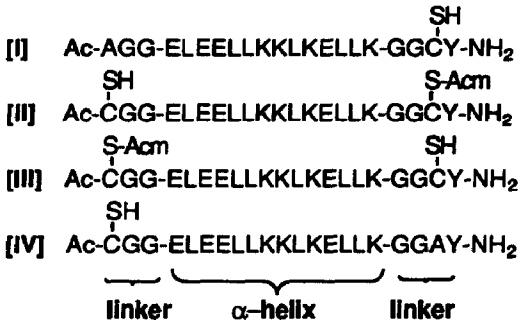
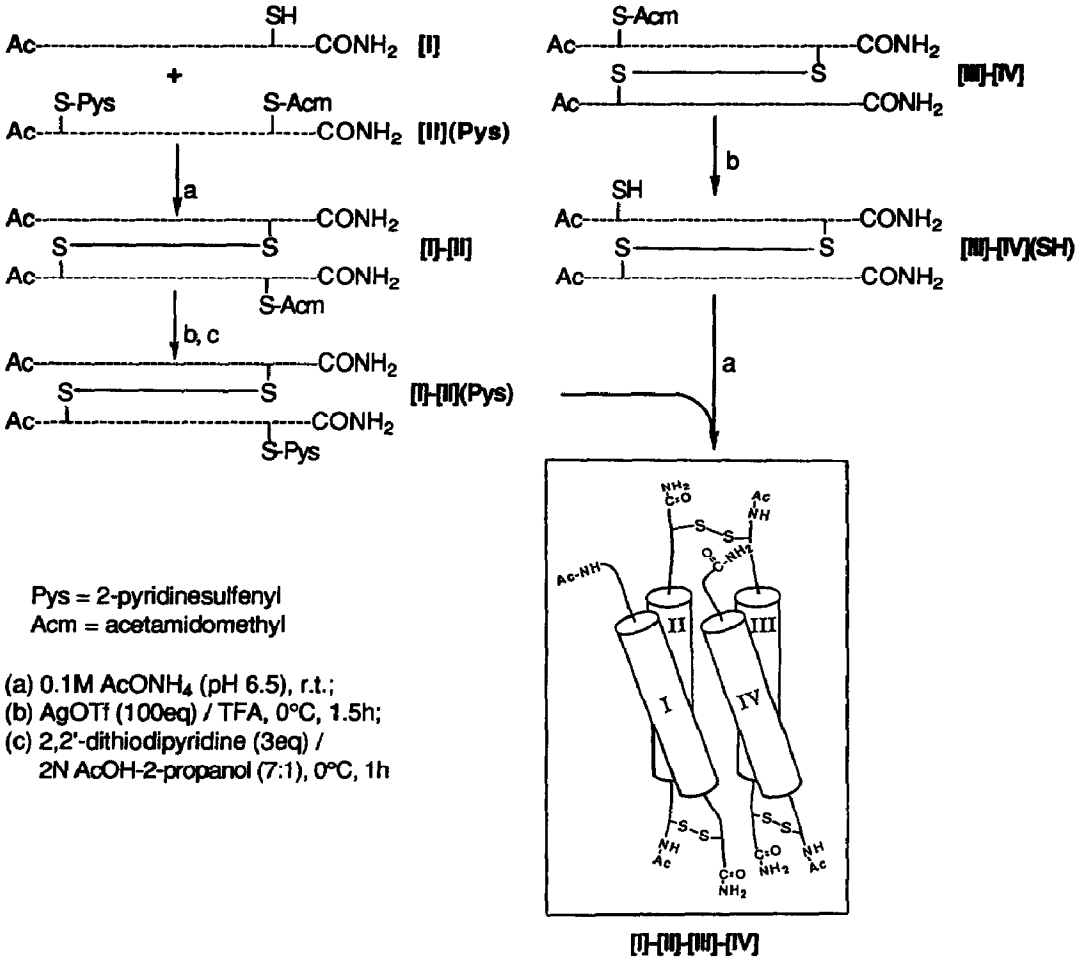


Fig. 1. Design of Peptide-Units
(A = Ala; C = Cys; G = Gly; E = Glu; L = Leu; K = Lys; Y = Tyr)

Scheme



20 min afforded [^{1}Cys(Pys)]-[II] (=[II](Pys)). Inter-unit disulfide cross-linking was accomplished by simply mixing [II](Pys) with [I] (1:1) for 20 min. Purification on HPLC (C18 column) afforded a pure unit-dimer ([I]-[II]) [yield 74%; liquid secondary ion mass spectrometry (LSI-MS): 4767.0 $(M+H)^+$]. The dimer [III]-[IV] was also obtained [yield 77%; LSI-MS: 4767.0 $(M+H)^+$] by activation of ^{1}Cys in unit [IV] by pyridinesulfenylation followed by cross-linking with unit [III] in essentially the same manner as in the case of [I]-[II]. Next, unmasking of the Acm group from Cys(Acm) in [I]-[II] and [III]-[IV] was conducted by the treatment with AgOTf (silver trifluoromethanesulfonate)[9] to afford [I]-[II](SH) and [III]-[IV](SH), respectively. Then, the SH group in [I]-[II](SH) was pyridinesulfenylated ([I]-[II](Pys)) followed by coupling with [III]-[IV](SH) for 2 h to afford a desired unit-tetramer protein [I]-[II]-[III]-[IV] composed of 84 amino acid residues (yield 57%).[10]

With the use of LSI-MS and amino acid analysis, the protein was found to have the expected molecular weight and amino acid composition.[11] The apparent molecular mass of the protein as determined by size-exclusion chromatography with a Sephadex® G-50 fine column [2.5 x 75 cm, 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0) as an eluant] was in good agreement with the calculated molecular mass (9.3 versus 9.4 kD). The CD spectrum of the protein in 10 mM MOPS (pH 7.0) showed two minima, one near 220 nm and another at 206 nm, indicative of an α-helical structure (Fig. 2).[12] When the CD was taken in MeOH where hydrophobic interaction between peptide units is loosen, the minimum at 206 nm shifted to 208 nm and the ratio of $[\theta]_{220}/[\theta]_{206}$ changed from 0.95 to 0.81. As is already observed in two-stranded α-helical coiled-coil proteins,[13] the above decrease implied the protein adopting a bundle structure. The stability toward guanidine hydrochloride (GnHCl) denaturation was increased by introduction of disulfide cross-linking. Denaturation curves for the α-helix tetramer protein [I]-[II]-[III]-[IV] and an uncross-linked monomer ([^{20}Cys(Acm)]-[I]) were determined by monitoring the ellipticity at 222 nm as a function of the GnHCl concentration. Midpoint of the denaturation curves occured at 1.8M and 7.4M GnHCl for the monomer and the tetramer, respectively (Fig. 3).[14]

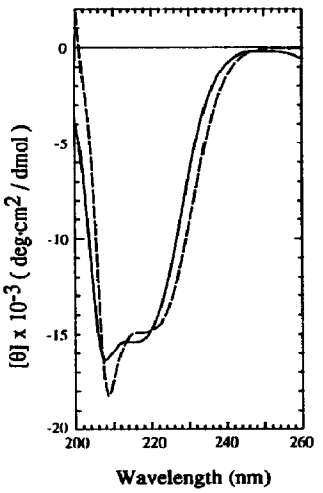


Fig. 2. CD Spectra of the α-Helix Tetramer [I]-[II]-[III]-[IV] in 10 mM MOPS (pH 7.0) (—) and MeOH (----). Concentration: 1.1 x 10^{-4} M.

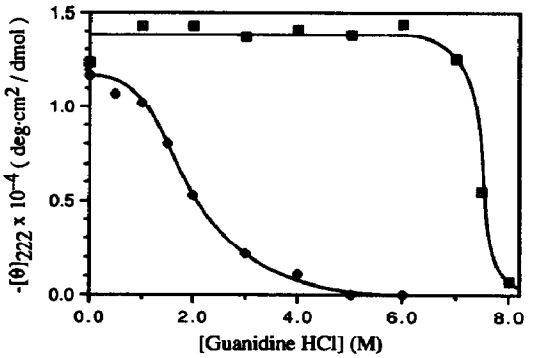


Fig. 3. Guanidine Denaturation Curves for the α-Helix Tetramer [I]-[II]-[III]-[IV] (■) and a Monomer [^{18}Cys(Acm)]-[I] (●) in 10 mM MOPS (pH 7.0). Concentration: 1.1 x 10^{-5} M (Tetramer); 4.4 x 10^{-5} M (Monomer) (0.10 mg/ml each).

These facts suggested that [I]-[II]-[III]-[IV] adopted an α-helical bundle structure in aqueous solution, and the characteristics of the protein were comparable to those of the DeGrado's de novo designed 74 residue protein. Thus we concluded that an α-helical protein of the desired structure was obtained by our "unit-assembling" strategy.

The main features of our approach are as follows: (i) We can construct protein models composed of *more than 80 amino acid residues* without difficulty by "unit-assembling"; chemical synthesis of linear peptides/proteins of such length is still difficult. (ii) We can construct protein models composed of *four different helices*. Moreover, (iii) we can manipulate *combination and arrangement of helices* and also *orientation of helices* (*i.e.*, parallel or antiparallel) willingly. Up to now there is no other approach that can fulfill the above features. The construction of spatially designed protein models with such features cannot be achieved by gene-manipulations; thus, our method can provide valuable information on protein function. Further study of our approach is being conducted in our laboratory.

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8. The yield of each unit calculated from the starting resin was 35-40%, respectively. LSI-MS, $(M+H)^+$: [I] 2332.4; [II] 2434.9; [III] 2435.2; [IV] 2332.3.
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10. Single peak on HPLC [retention time: 23.0 min; column: YMC R-ODS-5 (6x250 mm); solvent system: CH_3CN-H_2O (0.1% TFA) 30-70% in 40 min; flow rate: 1 ml/min; detection: 215 nm].
11. LSI-MS: 9389.6 $(M+H)^+$; Amino acid ratios after 6N HCl hydrolysis: 16Glu 16.00, 16Gly 16.19, 2Ala 2.09, 24Leu 23.29, 4Tyr 3.53, 16Lys 15.87, 6Cys not determined.
12. Considering 14 of 21 amino acid residues of each unit were involved in α-helix formation, the helical content was concordant with that of the DeGrado's protein ($[\theta]_{222}$: -1.4×10^4 versus -2.0×10^4 deg·cm^2/dmol).
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14. The stability toward GnHCl denaturation was also similar to the result that DeGrado obtained (concentration of GnHCl at the midpoint of denaturation: 6.5M).

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