Pergamon

Tetrahedron, Vol. 53, No. 22, pp. 7479-7492, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00443-2

Peptide-Unit Assembling *via* Disulfide Cross-linking: A Versatile Approach Which Enables the Creation of Artificial Proteins Comprising Helices with Different Amino Acid Sequences

Shiroh Futaki*

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

Kouki Kitagawa

Niigata College of Pharmacy, Niigata 950-21, Japan

Abstract: A versatile approach was developed to create artificial proteins by assembling α-helical peptide unit *via* selective disulfide cross-linking, which feature should open a way to the construction of the artificial functional proteins having arbitrary combination, arrangement, and orientation of helices. The practicability of this approach was demonstrated through the synthesis of a four-helix-bundle protein composed of 84 amino acid residues.

INTRODUCTION

It has long been thought that peptides do not have a definite structure by themselves and thus fail to work as functional molecules. However, DeGrado[1] showed that a de novo designed 22-residue amphiphilic peptide gathered together in water and acted as if it was a four-helix-bundle protein. Also, Oiki[2] found that a 22- or 23-residue peptide, which corresponds to one of the transmembrane helices of the sodium channel or nicotinic acetylcholine receptor ion channel, respectively, assembled with each other in lipid bilayers to exert ion channel activity. These findings indicate that peptides can self-assemble to act as a protein under the condition which stabilizes the peptide secondary structure; for example, in the former case, the amphiphilic nature of the peptide enables peptides to assemble by hydrophobic interaction. In the latter case, the membrane maintains the helical structure of the peptide corresponding to the transmembrane region of the ion channel.[3] Mutter and his coworkers[4] have established a unique concept of a template assembled synthetic protein (TASP) where the helical peptide is compelled to assemble on a template. Their concept was elegantly applied for preparing functional proteins such as ones possessing ion channel activity.[5] Our idea adopts the assembling of α-helical peptide units by selective inter-unit cross-linking to form a protein structure (Fig. 1). For cross-linking formation, we have developed two approaches using selective disulfide formation[6,7] and S-alkylation.[8] The most salient feature of our approaches is the feasibility of obtaining a

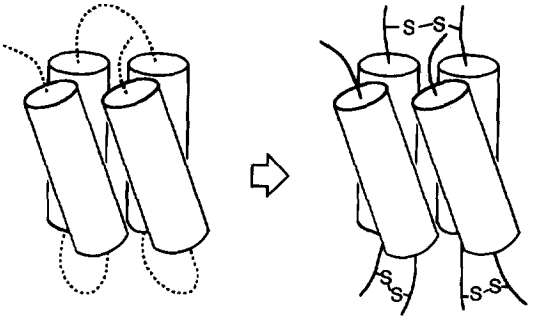

Fig. 1. Schematic representation of a protein constructed with "peptide-unit assembling" *via* selective disulfide cross-linking

protein composed of helices having different amino acid sequences. The selective assembling of peptide units should enable us to organize the combination, arrangement, and orientation (*i.e.*, parallel or antiparallel) of the helices. Also, unnatural amino acids having special functional groups and unnatural back bone surrogates can be introduced into the protein. These features should be indispensable for the design of artificial proteins with a sophisticated function[9] and also useful for the preparation of protein structural models.[10] Here we report the details of the former approach[6] with respect to both the synthetic aspects and characterization of the obtained protein, and discuss the practicability of this approach to create a stable helical protein structure.

RESULTS

Strategy and features for peptide-unit assembling

Our strategy for constructing the α-helical protein structure employs (i) preparation of α-helical peptide units using Fmoc-solid-phase peptide synthesis[11] followed by deprotection and HPLC purification, and (ii) assembling of the peptide units using selective disulfide cross-linking *via* activation of a cysteine by pyridinesulfenylation.[12] As stated above, we can expect to obtain helical proteins composed of helices comprising different amino acids, and to have proteins of arbitrary helical orientation. Another feature of our approach should be the higher purity of the obtained protein. As our approach adopts HPLC purified peptide units for assembling, purification of the intermediates should be easy. Thus we can also expect to obtain highly pure proteins; difficulty during purification is one of the should-be overcome problems for the chemical synthesis of proteins.[4b, 13] Also, the strategy of our "peptide-unit assembling" approach enables us to obtain proteins composed of more than 80 amino acid residues. We should be aware that the chemical synthesis of linear peptides and proteins of such length is still difficult. In order to ascertain that our approach

is practical for preparing four-helix-bundle proteins, we have constructed a four-helix-bundle protein.

Design of peptide units

Four peptide units (**[I]**, **[II]**, **[III]**, **[IV]**) were designed as shown in Fig. 2. An amphiphilic helical sequence was adopted in the center of each peptide unit. On both sides of the helices, flexible linker sequences[14] were placed for inter-unit cross-linking formation. Although it should be possible to obtain helical proteins composed of helices having different amino acid sequences with our approach, we employed the identical α-helical sequence as that of DeGrado's de novo designed, 74-residue artificial protein.[15] The protein is composed of four identical, 14-residue amphiphilic helices which are connected to each other by a β-turn sequence, Pro-Arg-Arg. Characterization of the protein was thoroughly conducted by his group, showing the artificial protein to have a stable α-helical protein structure. We have decided to construct a protein having the same α-helices as DeGrado's because we could estimate the practicability of our "peptide-unit assembling" strategy by comparing the characteristics of both proteins. Also, the compactness of DeGrado's protein was attractive as a working model in terms of convenience of construction. Each N-terminus of the respective peptide units was acetylated and the C-terminus amidated to stabilize helix formation.[1a,16] Also, tyrosine was selected for the C-terminal amino acid in each peptide to facilitate the purification on a gel-filtration column by monitoring the UV absorbance at 275 nm.

Construction of a four-helix-bundle protein

Each peptide unit (**[I]**-**[IV]**) was prepared by Fmoc-solid-phase synthesis on a Rink amide resin[17] followed by deprotection with 1M TMSBr-thioanisole in TFA in the presence of *m*-cresol and EDT.[18] Purification of the peptides by reversed phase HPLC on a ODS column afforded pure samples. Fidelity of the products was ascertained by LSIMS and amino acid analysis after 6N HCl hydrolysis. Fidelity of other products described later was also basically confirmed in the same way. Assembling of each peptide unit was conducted as shown in Fig. 3. The treatment of **[II]** with 2,2'-dithiodipyridine (3 eq) in 2N AcOH-2-propanol (10:3) at room temperature for 1 h afforded [Cys(Pys)^1]-**[II]** (=**[II](Pys)**).[19] Inter-unit disulfide cross-linking was easily accomplished by mixing **[II](Pys)** with **[I]** (1:1 in molar ratio) for 15 min at room temperature. Purification of the product by HPLC afforded a cross-linked peptide (**[I]**-**[II]**) in a yield of 74%. A cross-linked peptide **[III]**-**[IV]** was also obtained (yield 77%) by activation of Cys^1 in unit **[IV]** by pyridinesulfenylation 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 group from Cys(Acm) in **[III]**-**[IV]** was conducted by the treatment with AgOTf in TFA[20] to afford **[III]**-**[IV](SH)**. Usually DTT treatment is performed after AgOTf treatment for the removal of excess silver ion,[20] however, this step was omitted so as to keep the inter-unit disulfide cross-links intact.[21] The SH group in **[III]**-**[IV](SH)** was then pyridinesulfenylated (**[III]**-**[IV](Pys)**). The Acm group of

Fig. 2. Design of peptide units
(A = Ala, C = Cys, G = Gly, E = Glu, L = Leu, K = Lys, Y = Tyr)

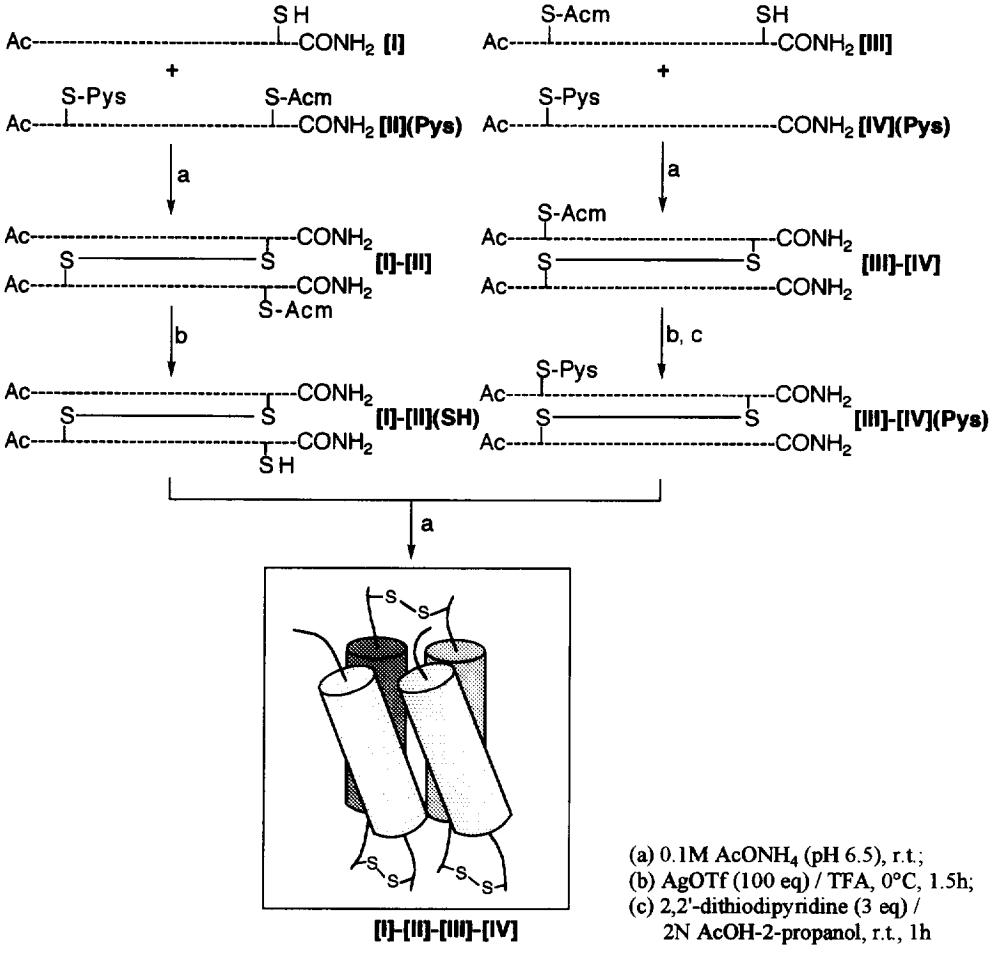

Fig. 3. Construction of the four-helix-bundle protein **[I]-[II]-[III]-[IV]**

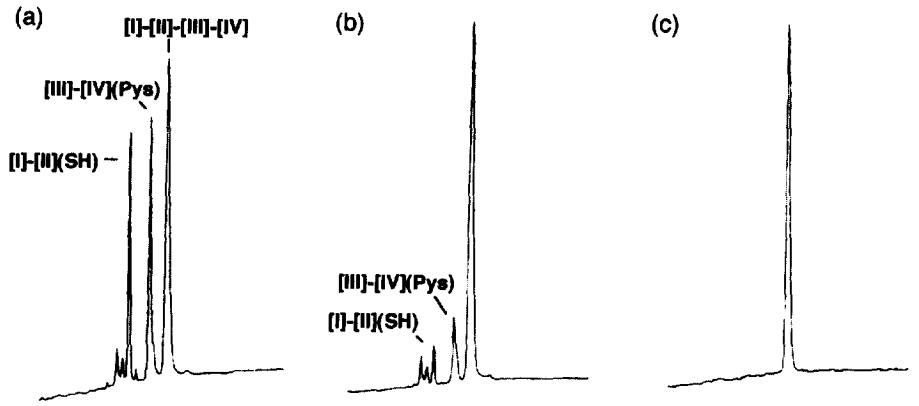

Fig. 4. HPLC on the formation of **[I]-[II]-[III]-[IV]**. (a) 20 min and (b) 2 h after mixing **[I]-[II](SH)** and **[III]-[IV](Pys)**. (c) HPLC purified **[I]-[II]-[III]-[IV]**. HPLC was monitored on a YMC R-ODS-5 120Å column (6 × 250 mm) with a gradient elution of CH_3CN in 0.1% aqueous TFA of 30-70% in 40 min at a flow rate of 1 ml/min (detection: 215 nm). Chromatograms between 15 and 35 min are shown.

[I]-[II] was also removed by AgOTf. The resulting **[I]-[II](SH)** was allowed to react with **[III]-[IV](Pys)** at room temperature for 2 h. Here 0.1M $AcONH_4$ containing 6M urea (pH 6.5) was used as the solvent with the expectation of avoiding the unfavorable aggregation of helices. As shown in Figs. 4a and 4b, the cross-linking formation proceeded without difficulty; only a negligible amount of by-products that came from migration of the disulfide bridges was observed. Considering the fact that the cross-linking is formed between peptides with a molecular weight of nearly 5,000 and the fact that the peptides have already disulfide cross-linking in their own molecules, the reaction was judged to be highly selective and efficient. HPLC purification of the product afforded the highly pure, desired protein **[I]-[II]-[III]-[IV]** composed of 84 amino acid residues (Fig. 4c, yield 57%). The molecular mass of the protein determined by LSIMS [m/z 9389.6 $(M+H)^+$] was in good agreement with the calculated average molecular mass (9390.3). The amino acid composition after 6N HCl hydrolysis was also in good agreement with the theoretical value.

Characterization of the Four-Helix-Bundle Protein

The apparent molecular mass of the protein determined by size exclusion chromatography with a Sephadex® G-50 fine column was in good agreement with the calculated molecular mass (9.3 versus 9.4 kD) (Fig. 5), which indicates that the protein exists as a monomer and does not form larger aggregates.

The CD spectrum of the protein in 10 mM MOPS (pH 7.0) showed two minima, one near 220 nm and the other at 206 nm, indicative of an α-helical structure (Fig. 6). The $[\theta]_{222}$ of the protein was -1.4×10^4 deg·cm^2/dmol. This value might be slightly less than that of DeGrado's protein (-2.0×10^4 deg·cm^2/dmol), however, taking into account the fact that only two-thirds (14 of 21 amino acid residues) of each unit was involved in the α-helix formation, the helical content was judged to be concordant with that of DeGrado's

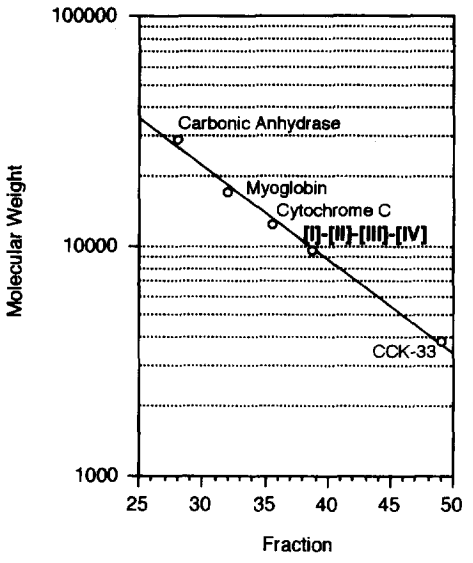

Fig. 5. Calibration curve for a Sephadex G-50 fine column

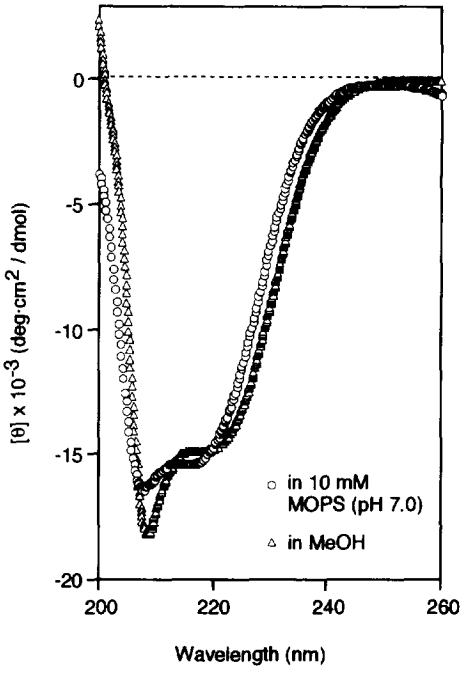

Fig. 6. CD spectra of the four-helix-bundle protein **[I]-[II]-[III]-[IV]**. Concentration: 1.1×10^{-4} M.

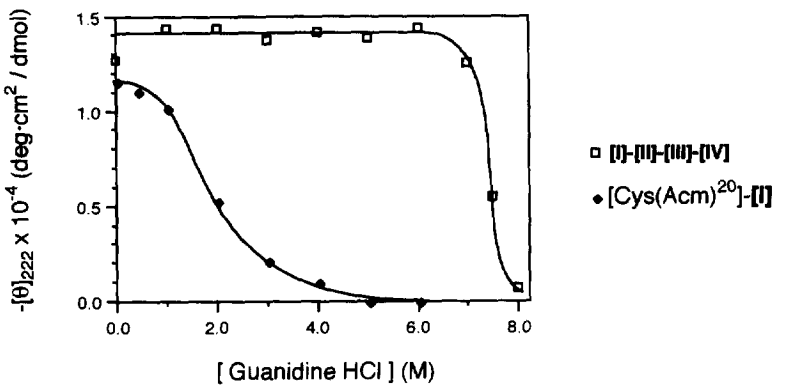

Fig. 7. Guanidine denaturation curves for the four-helix-bundle protein **[I]-[II]-[III]-[IV]** and [Cys(Acm)^{20}]-**[I]** in 10mM MOPS (pH 7.0). Concentration: 1.1 × 10^{-5}M (**[I]-[II]-[III]-[IV]**); 4.4 × 10^{-5}M ([Cys(Acm)^{20}]-**[I]**) (0.10 mg/ml each).

protein. When the CD spectrum was taken in MeOH where hydrophobic interaction between peptide units is weaker, 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,[22] the above decrease implied the protein adopted a bundle structure.

The stability toward guanidine hydrochloride (GnHCl) denaturation was increased by introduction of disulfide cross-linking (Fig. 7). Denaturation curves for the four-helix-bundle protein **[I]-[II]-[III]-[IV]** and an uncross-linked helical peptide ([Cys(Acm)^{20}]-**[I]**) were determined by monitoring the ellipticity at 222 nm as a function of the GnHCl concentration. The midpoint of the denaturation curves occurred at 7.4M GnHCl for the protein **[I]-[II]-[III]-[IV]**, whereas it was 1.8M for [Cys(Acm)^{20}]-**[I]**. The stability toward GnHCl denaturation was also similar to the result that DeGrado obtained (concentration of GnHCl at the midpoint of denaturation: 6.5M).

Also, the stability of the protein in water was examined. When the protein in water (5μM, pH 6.5) was left at 15°C for 7 days, substantially no degradation of the protein was observed as judged by its HPLC profile (data not shown).

DISCUSSION

The aim of this study was to discuss the practicability of our "peptide-unit assembling" approach from both a synthetic and protein-characteristic aspect. Disulfide cross-linking formation was highly successful with the aid of pyridinesulfenyl activation of the cysteines. This activation method was useful, not only

because eventual disulfide cross-linking formation proceeded highly efficiently and specific as was seen in the coupling of **[I]-[II](SH)** with **[III]-[IV](Pys)**, but also because the cross-linking reaction was able to be conducted in water. Thus we were able to employ deprotected and HPLC-purified peptide intermediates for cross-linking, which facilitated the work-ups in the course of the synthesis to obtain a highly pure protein.

The characteristics of the obtained protein was examined in terms of molecular weight estimation with size exclusion chromatography, α-helicity with CD measurement, and stability against guanidine denaturation. These characteristics of our protein were judged to be comparable to those of the DeGrado's genetically prepared 74-residue protein.[15] Thus we concluded that the obtained protein acts as a stable four-helix-bundle protein in water. It is interesting that the concentration of guanidine at the midpoint of the denaturation for the obtained protein (7.4M) was substantially higher than that of DeGrado's protein (6.3M). Recently, the importance of the length and amino acid composition of the linkers has been claimed for the stability of proteins.[23] The linker structure adopted here seems to contribute to sustaining the helix bundle structure against chaotropic denaturation. Also, judging from the fact that the protein was stable in water at room temperature for seven days, the disulfide cross-linking looks sufficient enough for maintaining the four-helix-bundle structure.

In conclusion, we have succeeded in constructing a four-helix-bundle protein composed of 84 amino acid residues without difficulty by "peptide-unit assembling" *via* selective disulfide formation. Considering the fact that direct chemical synthesis of proteins of such length is still difficult, our method is quite applicable to the construction of highly pure artificial proteins. Although we have assembled four identical helices in this study for comparison of the characteristics of the obtained protein with those of a known structure, our approach should be applicable to obtaining proteins composed of helices having different amino acid sequences. Using our approach, we can organize the combination, arrangement, and orientation (*i.e.*, parallel or antiparallel) of the helices. Also, unnatural amino acids having special functional groups and unnatural back bone surrogates can be introduced into the protein. These features should be indispensable for the design of artificial proteins with sophisticated functions and also useful for the preparation of protein structural models. To the best of our knowledge, there is no other approach reported other than by us up to now that can fulfill these features. Finally, we would like to emphasize the fact that construction of spatially designed protein models with an arbitrary helical orientation cannot be achieved by gene-manipulations; thus, our method can provide valuable information on protein functions.

EXPERIMENTAL

General. HPLC was conducted with a Hitachi L-6200 model. Amino acid compositions after acid hydrolysis (6N HCl, 110°C, 24h) were determined with a Hitachi 8500 model amino acid analyzer. Liquid

secondary mass spectra were recorded on a VG ZAB-2SEQ instrument with a 11-250J data system at an accelerating voltage of 8kV. Each expected mass value was expressed in the average chemical mass. CD spectra were recorded on a JASCO J-600 spectropolarimeter at 20°C using a quartz cell with a 0.2 cm path length. Fmoc-amino acid derivatives and the Rink amide resin were purchased from Novabiochem (Läufelfingen, Switzerland) or the Peptide Institute (Osaka, Japan). Sephadex® gel supports were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden), and the HPLC column from YMC (Kyoto, Japan). Retention time on HPLC (R_t) was monitored on a column of YMC R-ODS-5 S-5 120Å (6 × 250 mm) with a gradient of CH_3CN in 0.1% aqueous TFA of 30-70% over 40 min at a flow rate of 1 ml/min (detection: 215 nm).

Ac-Ala-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2 [I]. Solid-phase peptide synthesis was manually carried out, basically as reported earlier.[24] 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (Rink amide resin)[17] (435 mg, substitution level 0.46 mmol/g) was used as the peptide anchor. After deprotection of the Fmoc group attached to the resin with 20% piperidine in DMF (treatment time: 20 min), Fmoc-Tyr(^{t}Bu)-OH (230 mg, 0.5 mmol) was introduced with DICDI (78μl, 0.5 mmol) in the presence of HOBt (68 mg, 0.5 mmol) (reaction time: 2h). Repetitive removal of the Fmoc groups followed by introduction of amino acid derivatives gave the protected peptide resin [Ac-Ala-Gly-Gly-Glu(O^{t}Bu)-Leu-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Leu-Leu-Lys(Boc)-Lys(Boc)-Leu-Lys(Boc)-Glu(O^{t}Bu)-Leu-Leu-Lys(Boc)-Gly-Gly-Cys(MBzl)-Tyr(^{t}Bu)-NH-resin] (990 mg). Here the ^{t}Bu ester, Boc group, and MBzl group were used for the side chain protection of Glu, Lys, and Cys, respectively. Acetylation of the N-terminal was conducted with acetic anhydride (94 μl, 1 mmol) in the presence of NMM (110 μl, 1 mmol). Then, 125 mg of the above protected peptide resin was treated with 1M TMSBr-thioanisole in TFA (7.5 ml) in the presence of *m*-cresol (300 μl) and EDT (150 μl) for 1.5 h under ice-cooling, then for 30 min at room temperature. After the resin was removed by filtration, the filtrate was evaporated *in vacuo*. Ether (40 ml) was added to the resulting oil to give a powder, which was collected by centrifugation. The powder was dissolved in 50% AcOH (5 ml) and applied to a column of Sephadex® G-10 (2.5 × 60 cm), which was eluted with 1N AcOH. Each fraction (6ml each) was monitored by UV absorption at 280 nm. The fractions corresponding to the first main peak (Nos. 18-22) were collected and subjected to lyophilization to give a powder (53.0 mg). The powder was then further purified by HPLC with a column of YMC D-ODS-5 S-5 120Å (20 × 250 mm) [gradient: CH_3CN in 0.1% aqueous TFA (46-47% in 20 min, then 60 % in 7 min); flow rate: 5 ml/min; detection: 235 nm; 5 mg of peptide was applied each time.]. The eluate corresponding to the main peak was collected and lyophilized to afford a white fluffy powder (17.9 mg, 30% yield from the initial introduction of Tyr on the resin). LSIMS: m/z 2332.4 $(M+H)^+$ (expected 2332.3). Amino acid ratios in a 6N HCl hydrolysate: 4Glu 4.00; 4Gly 4.20; 1Ala 1.08; 6Leu 6.25; 1Tyr 1.08, 4Lys 4.16; 1Cys not determined. R_t: 22.1 min.

Ac-Cys-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys(Acm)-Tyr-NH_2 [II]. The above peptide was basically prepared as stated above. Cys(MBzl) and Cys(Acm) were used for the side chain protecting groups of Cys^1 and Cys^{20}, respectively. Yield from the initial introduction on Tyr on the resin: 29% and LSIMS: m/z 2434.9 $(M+H)^+$ (expected 2435.3). Amino acid ratios in a 6N HCl hydrolysate: 4Glu 4.00; 4Gly 4.80; 6Leu 5.86; 1Tyr 0.88, 4Lys 4.08; 2Cys not determined. R_t: 20.4 min.

Ac-Cys(Acm)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2 [III]. The above peptide was basically prepared as stated above. Cys(Acm) and Cys(MBzl) were used for the side chain protecting groups of Cys^1 and Cys^{20}, respectively. The yield from the initial introduction on Tyr on the resin: 38%. LSIMS: m/z 2435.2 $(M+H)^+$ (2435.3). Amino acid ratios in a 6N HCl hydrolysate: 4Glu 4.00; 4Gly 4.63; 6Leu 5.78; 1Tyr 1.18, 4Lys 3.88; 2Cys not determined. R_t: 19.6 min.

Ac-Cys-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Ala-Tyr-NH_2 [IV]. The above peptide was basically prepared as stated above. Cys(MBzl) were used for the side chain protecting groups of Cys. The yield from the initial introduction on Tyr on the resin: 35%. LSIMS: m/z 2332.3 $(M+H)^+$ (expected 2332.3). Amino acid ratios in a 6N HCl hydrolysate: 4Glu 4.00; 4Gly 4.43; 1Ala 1.24; 6Leu 5.87; 1Tyr 1.23; 4Lys 3.75; 1Cys not determined. R_t: 20.8 min.

Ac-Cys(Pys)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys(Acm)-Tyr-NH_2 [II](Pys). 2,2'-Dithiodipyridine (3.3 mg, 15 μmol) in 2-propanol (0.3 ml) was added to a solution of **[II]** (12.2 mg, 5 μmol) in 2N AcOH (1ml). The mixture was stirred at room temperature for 1 h. The solution was applied to a column of Sephadex® G-10 (1.5 × 15 cm) which was eluted with 1N AcOH. Each fraction (3ml) was monitored by UV absorption at 280 nm. The fractions corresponding to the front main peaks (Nos. 6-10) were collected and lyophilized. The obtained powder (8.7 mg) was further purified by HPLC with a column of YMC D-ODS-5 S-5 120Å (20 × 250 mm) [gradient: CH_3CN in 0.1% aqueous TFA (45-50% in 30 min, then 60 % in 10 min); flow rate: 5 ml/min; detection: 235 nm; 4 mg of peptide was applied at a time]. The fractions corresponding to the main peak were collected and lyophilized to give a white fluffy powder. Yield 3.8 mg (30%). LSIMS: m/z 2545.6 $(M+H)^+$ (expected 2546.1). R_t: 23.8 min.

Preparation of the disulfide cross-linked peptide [I]-[II] by the reaction between [I] and [II](Pys). **[I]** (7.4 mg, 3.2 μmol) in 0.1M $AcONH_4$ (pH 6.5, 3.2 ml) was mixed with the solution of **[II](Pys)** (8.1 mg, 3.2 μmol) in 0.1M $AcONH_4$ (pH 6.5, 3.2 ml). The mixture was stirred for 15 min at room temperature. The product was then basically purified in the same way as in the case of **[II](Pys)**. Gradient elution of CH_3CN in 0.1% aqueous TFA (45-48% in 30 min, then 60 % in 5 min; flow rate: 5 ml/min; detection: 235 nm) was used for HPLC purification. Yield 11.0 mg (74%). LSIMS: m/z 4767.0 $(M+H)^+$ (expected 4767.7). Amino acid ratios in a 6N HCl hydrolysate: 8Glu 8.00; 8Gly 8.41; 1Ala 1.15; 12Leu 12.02; 2Tyr 1.99, 8Lys 7.97; 2Cys not determined. R_t: 25.4 min.

Ac-Cys(Pys)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Ala-Tyr-NH_2 [IV](Pys). 2,2'-Dithiodipyridine (30.8 mg, 60 μmol) in 2-propanol (1.2 ml) was added to a solution of **[IV]** (46.7 mg, 20 μmol) in 2N AcOH (4 ml). The mixture was stirred at room temperature for 1 h. The product was basically purified in the same way as in the case of **[II](Pys)**. Gradient elution of CH_3CN in 0.1% aqueous TFA (46-49% in 30 min, then 60 % in 10 min; flow rate: 5 ml/min) was used for HPLC purification. Yield 18.4 mg (38%). LSIMS: m/z 2439.3 $(M+H)^+$ (expected 2441.3). R_t: 23.3 min.

Preparation of the disulfide cross-linked peptide [III]-[IV] by the reaction between [III] and [IV](Pys). **[III]** (9.3 mg, 3.8 μmol) in 0.1M $AcONH_4$ (pH 6.5, 3.8 ml) was mixed with the solution of **[IV](Pys)** (9.3mg, 3.8 μmol) in 0.1M $AcONH_4$ (pH 6.5, 3.8 ml). The mixture was stirred for 15min at room temperature. The product was basically purified in the same way as in the case of **[II](Pys)**. Gradient elution of CH_3CN in 0.1% aqueous TFA (43-48% in 30 min, then 60 % in 5 min; flow rate: 5 ml/min) was employed for HPLC purification. Yield 13.8 mg (77 %). LSIMS: m/z 4767.3 $(M+H)^+$ (expected 4767.7). Amino acid ratios in a 6N HCl hydrolysate: 8Glu 8.00; 8Gly 8.47; 1Ala 1.20; 12Leu 12.13; 2Tyr 2.07, 8Lys 8.05; 2Cys not determined. R_t: 21.1 min.

Preparation of [III]-[IV](Pys).

(i) **[III]-[IV](SH)**: Deprotection of the Acm group of **[III]-[IV]**. **[III]-[IV]**(7.9 mg, 1.7 μmol) was treated with AgOTf (43 mg, 0.17 mmol) in TFA (0.8 ml) in the presence of anisole (20 μl) in an ice bath for 1.5 h. Ether (40 ml) was added and the resulting powder was collected by centrifugation. The powder was then dissolved in 50% AcOH (2 ml) and applied to a column of Sephadex® G-10 (1.5 × 15 cm), which was eluted with 1N AcOH. Each fraction (3ml) was monitored by UV absorption at 280 nm. The fractions corresponding to the front main peak was collected and lyophilized to give a powder (6.4 mg, R_t: 22.1 min). Without further purification, the powder was subject to pyridinesulfenylation.

(ii) **[III]-[IV](Pys)**: Pyridinesulfenylation of **[III]-[IV](SH)**. The above obtained product (6.4 mg, 1.4 μmol) was dissolved in 2N AcOH (650 μl). 2,2'-Dithiodipyridine (0.91 mg, 4.1 μmol) in 2-propanol (90 μl) was mixed with the solution. The mixture was stirred at room temperature for 1h. The product was purified by gel-filtration with a column of Sephadex® G-10 (1.5 × 15 cm) (eluate: 1N AcOH; detection: 280 nm). Fractions corresponding to the front main peak were collected and lyophilized to give a powder. The product was sufficiently pure judged by HPLC and used for the cross-linking reaction without further purification. Yield 5.6 mg (88% for the pyridinesulfenylation; 72% for the above two steps). LSIMS: m/z 4804.9 $(M+H)^+$ (expected 4804.9). R_t: 23.5 min.

Successful construction of the four-helix-bundle protein [I]-[II]-[III]-[IV].

(i) **[I]-[II](SH)**: Deprotection of the Acm group of **[I]-[II]**. **[I]-[II]** (10.7 mg, 2.3 μmol) was accomplished by AgOTf (59 mg, 0.23 mmol) in TFA (1.1 ml) in the presence of anisole (20 μl) in an ice bath for 1.5 h. Ether (40 ml) was added and the resulting powder was collected by centrifugation. The powder was then

dissolved in 50% AcOH (2 ml) and applied to a column of Sephadex® G-10 (1.5 × 15 cm), which was eluted with 1N AcOH. Each fraction (3ml) was monitored by UV absorption at 280 nm. The fractions corresponding to the front main peak was collected and lyophilized to give a powder (9.0 mg, R_t 24.3 min).

(ii) **[I]-[II]-[III]-[IV]**: Disulfide bond formation between **[I]-[II](SH)** and **[III]-[IV](Pys)**. **[I]-[II](SH)** (2.31 mg, 0.5 μmol) was added to the solution of **[III]-[IV](Pys)** (2.36 mg, 0.5μmol) in 0.1M $AcONH_4$ containing 6M urea (pH 6.5, 1 ml). The mixture was stirred for 2 h at room temperature. The product was basically purified the same as in the case of **[II](Pys)**. Gradient elution of CH_3CN in 0.1% aqueous TFA (45% for 10 min, 45-55% for 30 min, then 60 % for 5 min; flow rate: 5 ml/min) was used for HPLC purification. Yield 5.9 mg (57% for the coupling of **[I]-[II](SH)** and **[III]-[IV](Pys)**; 48% for the above two steps). LSIMS: m/z 9389.6 $(M+H)^+$ (expected 9390.3). Amino acid ratios in a 6N HCl hydrolysate: 16Glu 16.00; 16Gly 16.19; 2Ala 2.09; 24Leu 23.29; 4Tyr 3.53, 16Lys 15.40; 6Cys not determined. R_t: 23.0 min.

Size exclusion chromatography. Size exclusion chromatography of the **[I]-[II]-[III]-[IV]** was conducted using a column of Sephadex® G-50 fine (2.5 × 73 cm). The column was eluted with a buffer of 50 mM MOPS (pH 7.0) at a flow rate of 1.6 ml/min. The eluent was monitored by measuring the absorbance at 280 nm. Molecular weight of the standard proteins used for calibration: carbonic anhydrase, 29,000; myoglobin, 17,000; cytochrome c, 12,400; CCK-33 (non-sulfate), 3,800.

CD measurements. Buffers and the concentration of the peptides are noted in the figure legends. $[Cys(Acm)^{20}]$-**[I]** was basically prepared the same as **[I]**. The Acm group was employed instead of the MBzl group for the side-chain protection of Cys^{20}. The concentration of **[I]-[II]-[III]-[IV]** was calculated from UV absorbance using the molar extinction coefficient of 5360 $M^{-1}cm^{-1}$ (The molar extinction coefficient per one tyrosine residue was judged to be 1340 $M^{-1}cm^{-1}$,[25] and **[I]-[II]-[III]-[IV]** contains four tyrosine residues in its molecule).

Stability of [I]-[II]-[III]-[IV] in water. Five μM of **[I]-[II]-[III]-[IV]** in H_2O (pH 6.5) as left at 15°C for 7 days. No significant change was observed on HPLC (data not shown).

Abbreviations. Fmoc=9-fluorenylmethyloxycarbonyl, ^{t}Bu=*t*-butyl, Boc=*t*-butyloxycarbonyl, Acm=acetamidomethyl, MBzl=*p*-methoxybenzyl, Pys=pyridinesulfenyl, DICDI=diisopropylcarbodiimide, HOBt=1-hydroxybenzotriazole, Ac_2O=acetic anhydride, NMM=*N*-methylmorpholine, TMSBr=trimethylsilyl bromide, TFA=trifluoroacetic acid, EDT=1,2-ethanedithiol, AgOTf=silver trifluoromethanesulfonate, DTT=dithiothreitol, AcOH=acetic acid, $AcONH_4$=ammonium acetate, MOPS=3-(*N*-morpholino)propanesulfonic acid, DMF=dimethylformamide, MeOH=methanol, LSIMS=liquid secondary mass spectrometry, CD=circular dichroism, HPLC=high performance liquid chromatography, ODS=octadecylsilyl.

Acknowledgments. The authors are grateful to Professor M. Niwa, The University of Tokushima, Japan for his kind discussion. Thanks are also extended to Mr. T. Yagami, National Institute of Health Sciences, Japan for LSIMS measurement and to Dr. Kenichi Akaji, Kyoto Pharmaceutical University, Japan for amino acid analysis. This work was supported by Grant-in-Aids for Scientific Research from the Ministry of Education, Science and Culture of Japan and by the Sasakawa Scientific Research Grant from The Japan Science Society. A part of this work was also supported by the Nissan Science Foundation.

REFERENCES AND NOTES

1. a) Ho, S. P; DeGrado, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 6751-6758. b) Osterhout, J. J. Jr.; Handel, T.; Na, G.; Toumadje, A.; Long, R. C.; Connolly, P. J.; Hoch, J. C.; Johnson, W. C. Jr.; Live, D.: DeGrado, W. F. *Am. Chem. Soc.* **1992**, *114*, 331-337.
2. a) Oiki, S.; Danho, W.; Montal. M. *Proc. Natl. Acad. Sci., USA* **1988**, *85*, 2393-2397. b) Oiki, S.; Danho, W.; Madison, V.; Montal, M. *Proc. Natl. Acad. Sci., USA* **1988**, *85*, 8703-8708. c) Oiki, S.; Madison, V.; Montal. M. *PROTEINS: Structure, Function, and Genetics* **1990**, *8*, 226-236.
3. Other examples of channel formation by the self-assembling of peptides in the membranes are: a) Lear, J. D.; Wasserman, Z. R.; DeGrado, W. F. *Science* **1988**, *240*, 1177-1181. b) Åkerfeldt, K. S.; Lear, J. D.; Wasserman, Z. R.; Chung, L. A.; DeGrado, W. F. *Acc. Chem. Res.* **1993**, *26*, 191-197. c) Agawa, Y.; Lee, S.; Ono, S.; Aoyagi, H.; Ohno, M.; Taniguchi, T.; Anzai, K.; Kirino, Y. *J. Biol. Chem.* **1991**, *266*, 20218-20222. d) Iwata, T.; Lee, S.; Oishi, O.; Aoyagi, H.; Ohno, M.; Anzai, K.; Kirino, Y.; Sugihara, G. *J. Biol. Chem.* **1994**, *269*, 4928-4933. And the references cited therein.
4. a) Mutter, M.; Vuilleumier, S. *Angew. Chem. Int. Ed. Engl.* **1989**, *28*, 535-555. b) Mutter, M.; Tuchscherer, G. G.; Miller, C.; Altmann, K.-H.; Carey, R. I.; Wyss, D. F.; Labhardt, A. M.; Rivier, J. E. *J. Am. Chem. Soc.* **1992**, *114*, 1463-1470. c) Tuchscherer, G.; Dömer, B.; Sila, U.; Kamber, B.; Mutter, M. *Tetrahedron* **1993**, *49*, 3559-3575. d) Tuchscherer, G.; Mutter, M. *J. Peptide Sci.* **1995**, *1*, 3-10.
5. a) Montal, M.; Montal, M. S.; Tomich, J. M. *Proc. Natl. Acad. Sci., USA* **1990**, *87*, 6929-6933. b) Grove, A.; Tomich, J. M.; Montal, M. *Proc. Natl. Acad. Sci., USA* **1991**, *88*, 6418-6422. c) Grove, A.; Mutter, M.; Rivier, J. E.; Montal, M. *J. Am. Chem. Soc.* **1993**, *115*, 5919-5924.
6. Futaki, S; Kitagawa, K. *Tetrahedron Lett.* **1994**, *35*, 1267-1270.
7. Futaki, S.; Ishikawa, T.; Niwa, M.; Yagami, T.; Kitagawa, K. *Tetrahedron Lett.* **1996**, *37*, 201-204.
8. Futaki, S.; Ishikawa, T.; Niwa, M.; Kitagawa, K.; Yagami, T. *Tetrahedron Lett.* **1995**, *36*, 5203-5206.
9. a) Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1989**, *111*, 380-381. b) Ghadiri, M. R.; Granja, J. R.; Buehler, L. K. *Nature*, **1994**, *369*, 301-305. c) Bryson, J. W.; Betz, S. F.; Lu, H. S.; Suich, D. J.; Zhou, H. X.; O'Neil, K. T.; DeGrado, W. F. *Science* **1995**, *270*, 935-941. d) Mihara, H.; Tanaka, Y.; Fujimoto, T.;

Nishino, N. *J. Chem. Soc., Perkin 2* **1995**, 1915-1921. e) Cheng, R. P.; Fisher, S. L.; Imperiali, B. *J. Am. Chem. Soc.* **1996**, *118*, 11349-11356. And the references cited therein.

10. a) Carr, C. M.; Kim, P. S. *Cell* **1993**, *73*, 823-832. b) Morii, T.; Yamane, J.; Aizawa, Y.; Makino, K.; Sugiura, Y. *J. Am. Chem. Soc.* **1996**, *118*, 10011-10017. And the references cited therein.
11. Atherton, E.; Sheppard, R. C. *Solid phase peptide synthesis, a practical approach*; IRL Press: Oxford, 1989.
12. a) Grassetti, D. R.; Murray, J. F. Jr. *Arch. Biochem. Biophys.* **1967**, *119*, 41-49. b) Akaji, K.; Fujino, K.; Tatsumi, T.; Kiso, Y. *Tetrahedron Lett.* **1992**, *33*, 1073-1076.
13. Dawson, P. E.; Kent, S. B. H. *J. Am. Chem. Soc.* **1993**, *115*, 7263-7266.
14. O'Shea, E. K.; Rutkowski, R.; Stafford, W. F. III; Kim, P. S. *Science* **1989**, *245*, 646-648.
15. Regan, L.; DeGrado, W. F. *Science* **1988**, *241*, 976-978.
16. Shoemaker, K. R.; Kim, P. S.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *Nature* **1987**, *326*, 563-567.
17. Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787-3790.
18. Fujii, N.; Otaka, A.; Sugiyama, N.; Hatano, M.; Yajima, H. *Chem. Pharm. Bull.* **1987**, *35*, 3880-3883.
19. Here, the **(SH)** and **(Pys)** on the compound names indicate that the compounds have reactive functional groups (-SH or -Pys) on their molecule. This may be the unusual way of expression, however, the easy understanding of the properties of the compounds is first considered. The **(SH)** on the uncross-linked peptide units (**[I]**, **[II]**, **[III]**, **[IV]**) was omitted for simplification.
20. Fujii, N.; Otaka, A.; Watanabe, T.; Okamachi, A.; Tamamura, H.; Yajima, H.; Inagaki, Y.; Nomizu, M.; Asano, K. *J. Chem. Soc., Chem. Commun.* **1989**, 283-284.
21. After the AgOTf/TFA treatment followed by gel-filtration using 1N AcOH as an eluate, the sulfhydryl group of Cys was probably present in the mixture of the -SH and the $-S^-Ag^+$ forms. Even when the sulfhydryl group in **[III]-[IV]** was present in the $-S^-Ag^+$ form, the S^- could nucleophilically attack one of the sulfur atoms of 2,2'-dithiodipyridine to give **[III]-[IV](Pys)**. Also, even if the sulfhydryl group of **[I]-[II](SH)** was in the $-S^-Ag^+$ form, that S^- could also attack the sulfur atom in the Pys-activated cystein residue in **[III]-[IV](Pys)** to afford the desired protein, **[I]-[II]-[III]-[IV]**.
22. Zhou, N. E.; Kay, C. M.; Hodges, R. S. *J. Biol. Chem.* **1992**, *267*, 2664-2670.
23. a) Chou, K.-C.; Maggiora, G. M.; Scheraga, H. A. *Proc. Natl. Acad. Sci., USA* **1992**, *89*, 7315-7319. b) Suzuki, N. and Fujii, I. In *Peptide Chemistry 1996*; Kitada, C. Ed.; Protein Research Foundation: Osaka, 1997; pp. 437-440.
24. Futaki, S.; Taike, T.; Akita, T.; Kitagawa, K. *Tetrahedron* **1992**, *48*, 8899-8914.
25. Edsall, J. T.; Wyman, J.: *In Biophysical Chemistry*; Academic Press: New York, 1958; Vol. 1, pp. 427.

(*Received in Japan* 28 *February* 1997; *accepted* 17 *April* 1997)