Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 9, pp 1883–1891, 1997

PII: S0968-0896(97)00119-3
0968-0896/97 $17.00 + 0.00

Embodying a Stable α-Helical Protein Structure through Efficient Chemical Ligation via Thioether Formation

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Abstract—A new approach was developed to embody the α-helical protein structure having an arbitrary combination and arrangement of helices by the successive ligation of a haloacetyl peptide segment with a cysteinyl peptide. A four-helix-bundle protein was efficiently constructed by the repetitive ligation of α-helical peptide segments. The use of HPLC-purified unprotected peptide segments facilitated the purification of the intermediates to afford the highly homogenous desired protein. The use of the bromoacetyl moiety and the chloroacetyl moiety for the ligation was judged to make no difference in practice. A trial of introducing an additional intramolecular disulfide cross-link was also examined. The resulting protein showed high stability in the chaotropic and thermal denaturation and in enzymatic degradation. © 1997 Elsevier Science Ltd.

Introduction

Chemical ligation is a promising method for the preparation of highly pure large molecular weight proteins. Various methods of ligation have been developed. For example, chemical ligation through peptide bond formation by the reaction between a peptide *C*-terminal thiocarboxylic acid (or thioester) and a peptide *N*-terminal amino moiety,[1] between a peptide thioester and a cysteinyl peptide,[2] between a peptide thiocarboxylic acid and *N*-terminal histidinyl pepitide,[3] has been reported. Chemical ligation was also conducted to form protein equivalents through unnatural peptide bond substitution using reactions such as thioether formation,[4,12] thioester formation,[5] disulfide formation,[6,11] pseudoproline formation,[7] and so on. Through these ligation strategies, enzymes with HIV-protease activity[5,6,7b] and some DNA binding proteins[1c,1d] were synthesized. The ligation strategies should be useful not only for the synthesis of natural proteins and their equivalents, but also for creating carriers for drug delivery and constructing multicomponent peptide libraries. Trials of applying the strategy for preparing peptide dendrimers[8] and multiple antigen peptides (MAP)[9] have also been reported.

The ligation strategy is also attractive for de novo design and synthesis of artificial functional proteins.[10] This field has been showing significant progress in manifesting protein regional structure and eventually creating artificial functional molecules. To achieve further progress in the design of the functional proteins, it is necessary to arrange multiple functional groups three-dimensionally within the protein molecules so that they could work together to exert a special function. It is important to establish the strategies to fulfill the above criteria. We have preliminary reported practical approaches for assembling heterogeneous helices to create the four-helix-bundle protein structure by chemical ligation via selective disulfide[11] and the thioether[12] formation. The disulfide bond is sensitive to thiols or reducing agents. When these are present in the buffer, it would be advantageous to have chemically stable thioether linkages in the protein structure for the better handling.

This paper considers: (i) our strategy for the construction of the four-helix-bundle proteins with chemical ligation through thioether formation between an *N*-terminal bromoacetylated peptide segment and a peptide segment having a cysteine at its *C*-terminus, (ii) comparison of the practicability of the chloroacetyl moiety with that of the bromoacetyl one for the ligation, (iii) the trial of introducing an additional disulfide cross-link into the molecule, and (iv) the properties of the resulting four-helix-bundle proteins.

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Abbreviations: Fmoc, 9-fluorenylmethyloxycarbonyl; Ac, acetyl; ^{t}Bu, *t*-butyl; Boc, *t*-butyloxycarbonyl; Acm, acetamidomethyl; Ad, adamantyl; DICDI, diisopropylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; TMSBr, trimethylsilyl bromide; TFA, trifluoroacetic acid; EDT, 1,2-ethanedithiol; AgOTf, silver trifluoromethanesulfonate; DTT, dithiothreitol; AcOH, acetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DMF, dimethylformamide; MeOH, methanol; LSIMS, liquid secondary ion mass spectrometry; CD, circular dichroism.

Results and Discussion

Construction of a four-helix-bundle protein through the chemical ligation between a bromoacetylated peptide segment and a cysteinyl peptide segment

Our strategy was to efficiently construct the protein structure by assembling helical peptides through chemical ligation. We employed the ligation of peptide segments using the reaction of an *N*-terminal bromoacetyl moiety of a segment to a cysteine at the *C*-terminus of the other (Fig. 1). This is one of the most often used ligation techniques and has the advantage that the preparation of haloacetylated peptides is very easy and the ligation itself is efficient and reliable. The α-helical peptide segments involved in the ligation can be prepared conveniently with Fmoc-solid-phase peptide synthesis.[13] By utilizing HPLC-purified peptide segments, we can expect to obtain highly pure proteins. Another advantage of this strategy is that we can expect to obtain proteins composed of heterogeneous helices—this feature is important in designing proteins with a sophisticated function. In order to estimate the practicability and the usefulness of our strategy, a model protein was constructed. To simplify the estimation of the usefulness of our approach we adopted the identical α-helical sequence, as did DeGrado, on the construction of a de novo designed and genetically prepared protein with a four-helix-bundle structure;[14] the properties of the protein were well-characterized by DeGrado and his co-workers, and we may compare the characteristics of these proteins. Segments **1** and **2** were designed as the helical peptide segments (Fig. 2a). In the center of the segments the amphiphilic helical sequence was employed as previously stated, and on both sides flexible linkers were attached. Cys(Acm) (Acm = acetamidomethyl) was employed for the selective thioether formation; the protecting group is stable under acid treatment and is removable with silver trifluoromethanesulfonate (AgOTf) treatment.[15] In addition to Cys(Acm), Cys(Ad)[16] (Ad = adamantyl) was employed for the final introduction of an additional disulfide cross-link into the protein; the effect of this cross-link introduction is described later. Each peptide segment was prepared easily using the Fmoc-solid-phase peptide synthesis followed by the deprotection using trimethylsilyl bromide (TMSBr).[17] The Ad group was almost stable under the deprotection condition. Subsequent purification on HPLC gave highly pure segments. Cross-link formation was accomplished by a reaction between the free SH group of Cys in segment **1** and the bromoacetyl moiety at the *N*-terminus of **2** (molar ratio = 1:1.1) in 6 M guanidine HCl (GnHCl)–0.1 M Tris–HCl (pH 8.0) at room temperature for 18 h to give **3** (Fig. 2b). The Acm group was then removed by AgOTf (100 equiv) treatment in CF_3COOH (TFA) (0 °C, 1.5 h),[15] under which condition the Ad group remained intact. The resulting SH group was reacted with **2** (molar ratio = 1:1.1) as previously stated to give the helix trimer **4**. By repeating the procedure, a protein composed of four α-helical peptide segments **5** was obtained. The decrease in reactivity was anticipated for the ligation among large molecules, such as between **2** (M_r 2413) and the Acm-detached protein from **4** (M_r 7018). However, throughout the synthesis the ligation proceeded smoothly and purification in each step was easy because the the HPLC-purified peptide segments contributed to the reduction of the amount of byproducts (Fig. 3).

Comparison of the efficiency of ligation with that of chemical ligation between a chloroacetylated peptide and a cysteinyl peptide

Chemical ligation of a haloacetylated peptide and the sulfhydryl group of a cysteine is one of the popular ways of ligation. Generally speaking the reaction rate between bromoacetylated peptide and cysteine is higher than that when chloroacetylated peptide is used. However, we have sometimes encountered the problem that bromoacetylated peptides easily decompose (probably because of hydrolysis) while being stored in a refrigerator or freezer. Also, the possibility of decomposition of the bromoacetyl moiety was suggested in the preparation of bromoacetylated peptide segments, especially during the acid cleavage stage.[9] This feature is not advantageous in terms of sample preparation.

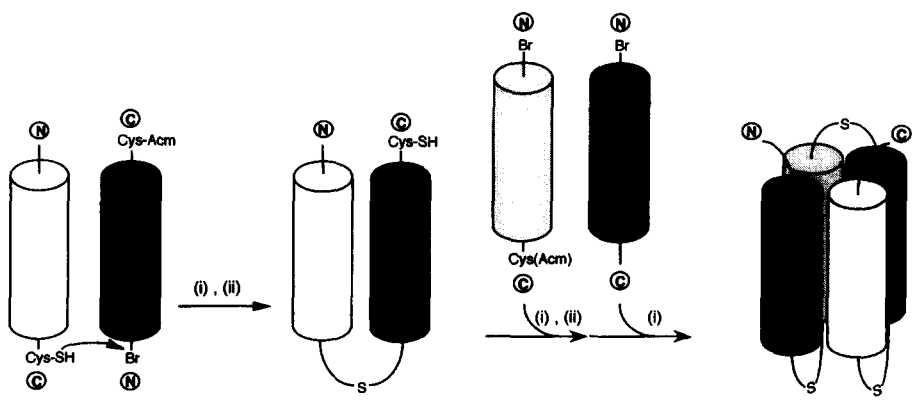

Figure 1. A general scheme for the construction of helical proteins by chemical ligation via thioether formation. The circled N and C denote the *N*- and *C*-terminus sides, respectively. (i) 6 M GnHCl–0.1 M Tris (pH 8.0); (ii) AgOTf/TFA.

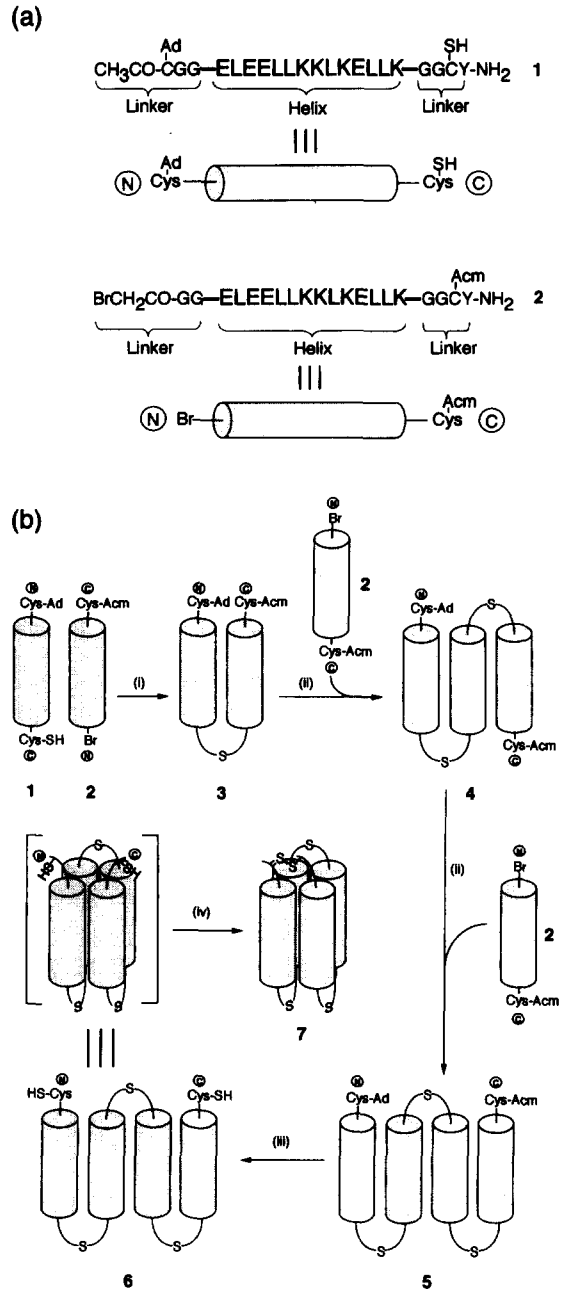

Figure 2. (a) Design of the peptide segments. Amino acids are expressed in the one-letter codes: C = cysteine; G = glycine; E = glutamic acid; L = leucine; K = lysine; Y = tyrosine. The circled N and C denote the *N*- and *C*-terminus sides of the proteins, respectively. (b) Construction of the four-helix-bundle protein. (i) 6 M GnHCl–0.1 M Tris (pH 8); (ii) detachment of Acm group with AgOTf/TFA followed by introduction of **2** in 6 M GnHCl–0.1 M Tris (pH 8); (iii) 1 M CF_3SO_3H–thioanisole/TFA followed by AgOTf/TFA; (iv) air oxidation.

However, when using the chloroacetylated peptide we have not encountered such a problem; in theory, the efficiency of the reaction should decrease. In order to examine the differences during practical synthesis, the above helical protein was constructed through the ligation of chloroacetylated peptide segments and cysteinyl peptides. The chloroacetylated peptide segment **2′** was prepared in a manner similar to the above

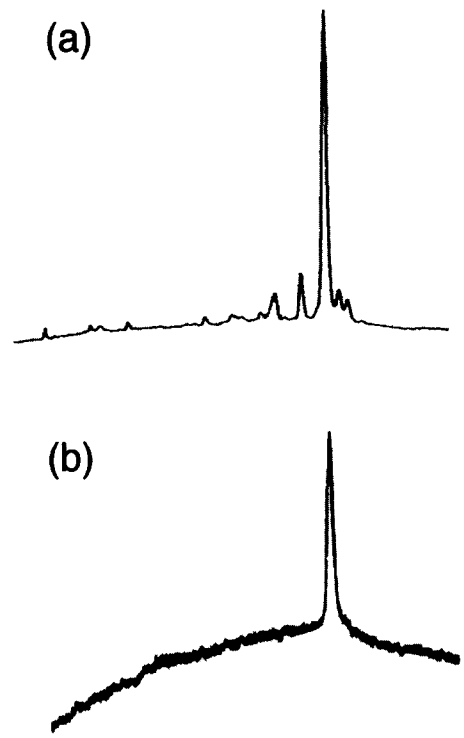

Figure 3. HPLC profiles on the construction of **5**. (a) After an overnight reaction for the ligation. (b) After HPLC purification. Chromatograms between 15 and 40 min are shown [column: YMC R-ODS-5 S-5 120 Å (6 mm × 250 mm); gradient: CH_3CN in 0.1% aqueous TFA of 30–70% for 40 min; flowrate: 1 mL/min; detection: 215 nm].

description. The yield (30%) was better than that of the bromoacetylated one, **2** (18%), which implies that the chloroacetylated derivative could be more stable under the conditions used in its preparation. In the ligation using chloroacetylated segments, each step of the synthesis proceeded without difficulty, and we have attained a yield comparable with that when we employed bromoacetylated segments. In this study overnight reaction conditions were adopted. When the shorter reaction time was necessary, the use of the bromoacetylated segments might give better results. However, the overnight reaction time causes little trouble in performing the experiments. We thus concluded that the use of chloroacetylated peptide is sometimes more advantageous, especially when the decomposition of bromoacetylated moiety is dominant.

Introduction of disulfide which connects both termini of the protein

Disulfide is sometimes introduced into proteins to raise stability against denaturation, especially when the properties of proteins were genetically modified. We have introduced an additional disulfide cross-link on the above obtained protein to examine if the theory holds true on de novo designed artificial proteins. The Ad group of protein **5** was removed by the treatment of 1 M trifluoromethanesulfonic acid (TFMSA)–thioanisole in TFA[18] at 0 °C for 1.5 h, followed by the

removal of the Acm group by the treatment of AgOTf (100 equiv) in TFA at 0 °C for 1.5 h.[15] The deprotected sample **6** was air-oxidized in one of the following two ways: in 0.1 M NH_4HCO_3 (pH 8.0; protein concentration of 0.5 μM) and in a redox buffer containing an equimolar amount of the oxidized and the reduced glutathione[19] (pH 8.5; protein concentration of 20 μM). In the latter buffer, disulfide exchange occurs easily to generate the most thermodynamically stable disulfide form. A distinct peak was observed on HPLC after a 16-h incubation in each buffer (Fig. 4), which corresponds to the desired protein having intramolecular disulfide cross-linking. This fact suggested that the protein folded properly into the four-helix-bundle structure in water so that both termini of the protein came in close proximity to form mainly an intramolecular disulfide bond; intermolecular disulfide formation is usually dominant in the air oxidation of synthetic peptides, especially when the cysteines to be cross-linked are located far from each other within the molecule. HPLC purification on a C_4 column afforded a desired product **7** in good yields [from **6**: 38% (oxidation in 0.1 M NH_4HCO_3), 40% (oxidation in the redox buffer), respectively]. The fidelity of the product was ascertained by liquid secondary ion mass spectrometry (LSIMS)

Characteristics of the four-helix-bundle proteins

The apparent molecular weight of **7** in water was estimated by size-exclusion chromatography using a Sephadex® G-50 fine column [0.1 M phosphate buffer (pH 7.0)] (Fig. 5). The protein was eluted at a retention volume in good agreement with the calculated molecular weight (9.2 versus 9.1 kD). This fact suggests that the protein remains a monomer and does not form aggregates in water.

The CD spectrum of protein **7** was indicative of an α-helical structure with double minima at 208 nm and around 222 nm (Fig. 6a) [$[\theta]_{221}$: −12,000 deg cm²/dmol; protein concentration: 98 μM in 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0)]. The CD spectrum of **5** also showed a similar spectrum with double minima at 208 nm and around 221 nm (Fig. 6a), but the α-helical content [$[\theta]_{222}$: −13,200 deg cm²/dmol; protein concentration: 109 μM in 10 mM MOPS (pH 7.0)] was slightly higher than that of **7**. When the CD spectrum of **7** was taken in MeOH where the hydrophobic interaction between helices is supposed to be weak, the ratio of $[\theta]_{222}/[\theta]_{208}$ changed from 0.94 to 0.77 (Fig. 6b). As was already observed in other helical proteins,[20] the decrease in $[\theta]_{222}/[\theta]_{208}$ implied that the protein adopted a bundle structure. The introduction of the additional disulfide cross-link to **5** might make the helical structure of protein **5** a little strained, which explains why the helical content of **7** is a little smaller than that of **5**. In MeOH the strain could also relax which should cause an increase in its α-helical content. The CD spectrum of **7** in 10 mM MOPS in the presence of 150 mM or 300 mM NaCl (pH 7.0) (protein concentration of 98 μM) was also examined; however, the presence of the salt did not have a significant influence on the spectrum (data not shown). This suggested that the electrostatic interaction could not be critical for the stability of the protein.

Next the stability of the protein against chaotropic denaturation was examined by comparing the value of $[\theta]_{222}$ as a measure of helical content (Fig. 7a). Protein **7** showed higher resistance against GnHCl denaturation than protein **5** and an untethered peptide segment; even at 8 M GnHCl concentration the protein retained 84% of the helicity of that in the absence of GnHCl (calculated from $[\theta]_{222}$). On the other hand, the helix content of **7** was smaller than that of **5** when the

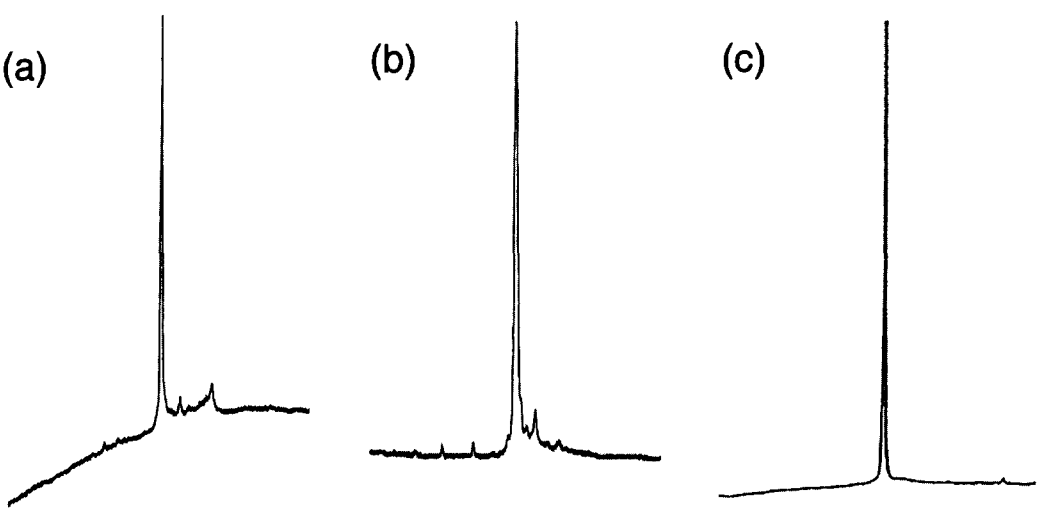

Figure 4. HPLC profiles on the air oxidation of **6** (a) in 0.1 M NH_4HCO_3 (pH 8) for 16 h, and (b) in a redox buffer for 16 h. (c) HPLC-purified **7**. Chromatograms between 20 and 40 min are shown [column: μBondasphere $5C_4$-300 Å (3.9 mm × 150 mm); gradient: CH_3CN in 0.1% aqueous TFA of 30–70% for 40 min; flowrate: 0.8 mL/min; detection: 215 nm].

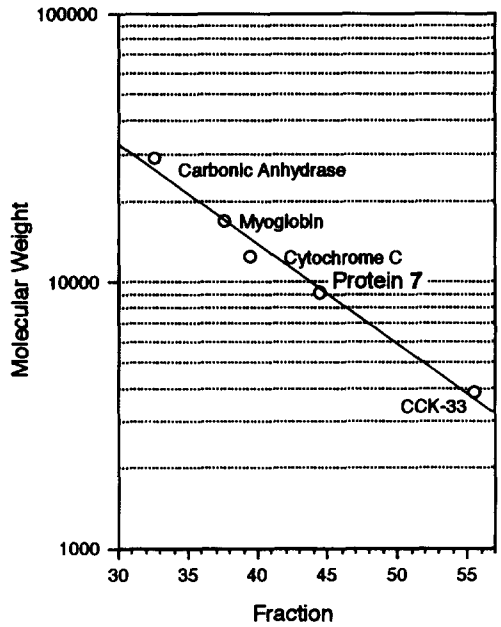

Figure 5. Calibration curve of the four-helix-bundle protein **7** for a Sephadex G-50 fine column.

concentration of GnHCl was below 7 M. The disulfide cross-link formation in the molecule may force the helices to come closer to each other to increase hydrophobic interaction, although the helices may eventually assume a slightly strained structure which gives a lower $[\theta]_{222}$. The extraordinary stability of the protein was also observed against thermal denaturation. When the thermal stability of **7** was examined (Fig. 7b), 80% of $[\theta]_{222}$ was retained at 80 °C as compared with that at 28 °C, and no drastic decrease in the helical content was observed. Even in the presence of 4 M GnHCl, a similar tendency was observed. Also, the stability of **5** in the same buffer was examined and judged comparable with that of **7**, which suggested that, even without the intramolecular cross-linking, the structure of **5** is fairly stable against thermal denaturation.

The CD profile and the way of denaturation of uncross-linked protein **5** were comparable with those of our previously reported artificial protein,[11a,c] where an α-helical peptide segment was assembled by selective disulfide cross-linking. Recently, the importance of the length and amino acid composition of the linkers on

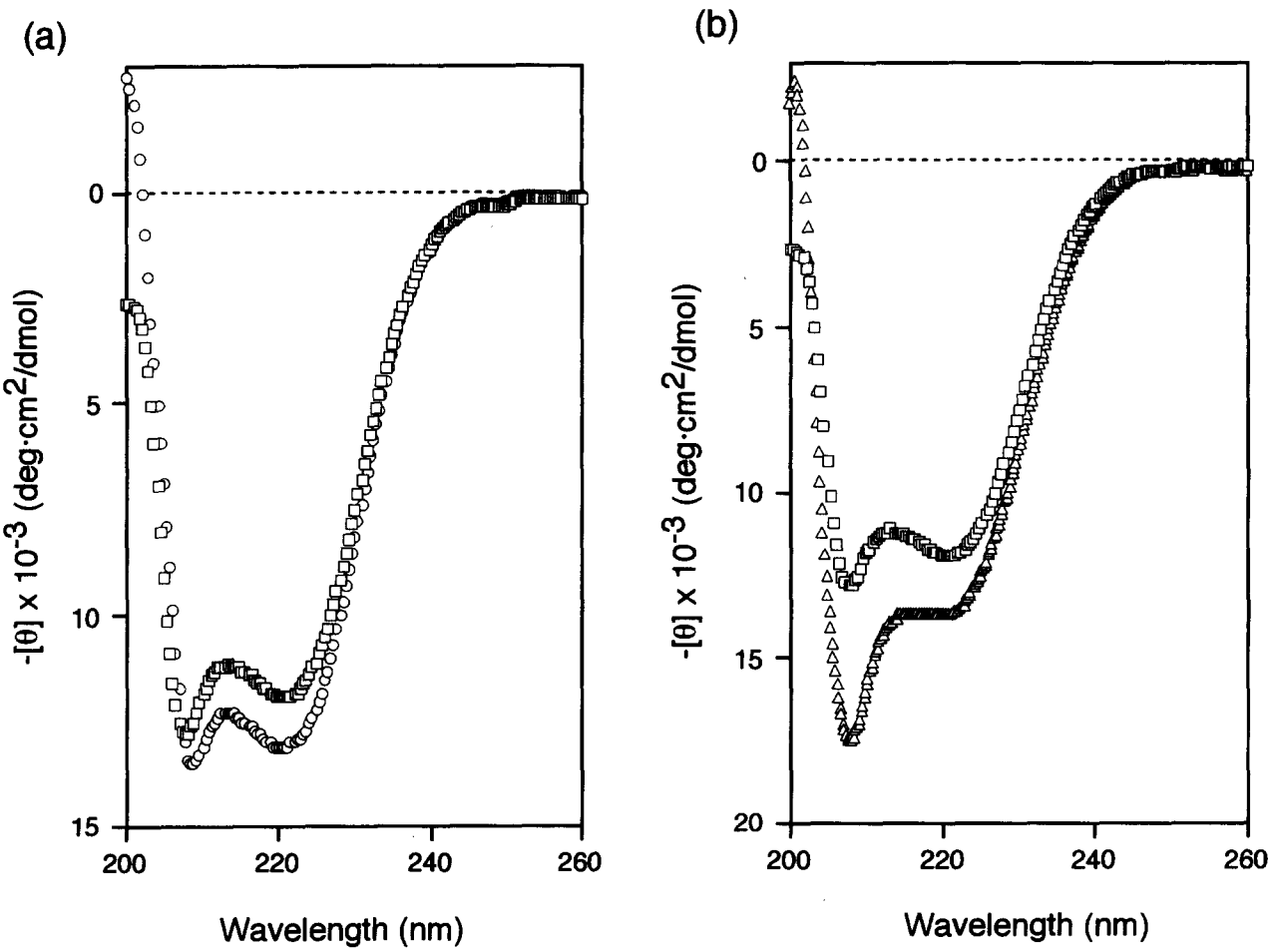

Figure 6. CD spectra of the four-helix-bundle proteins. (a) Open circle: **5** [109 μM in 10 mM MOPS(pH 7.0)]; open square: **7** [98 μM in 10 mM MOPS (pH 7.0)]. (b) Open Square: **7** [97 μM in 10 mM MOPS (pH 7.0)]; open triangle: **7** (86 μM in MeOH).

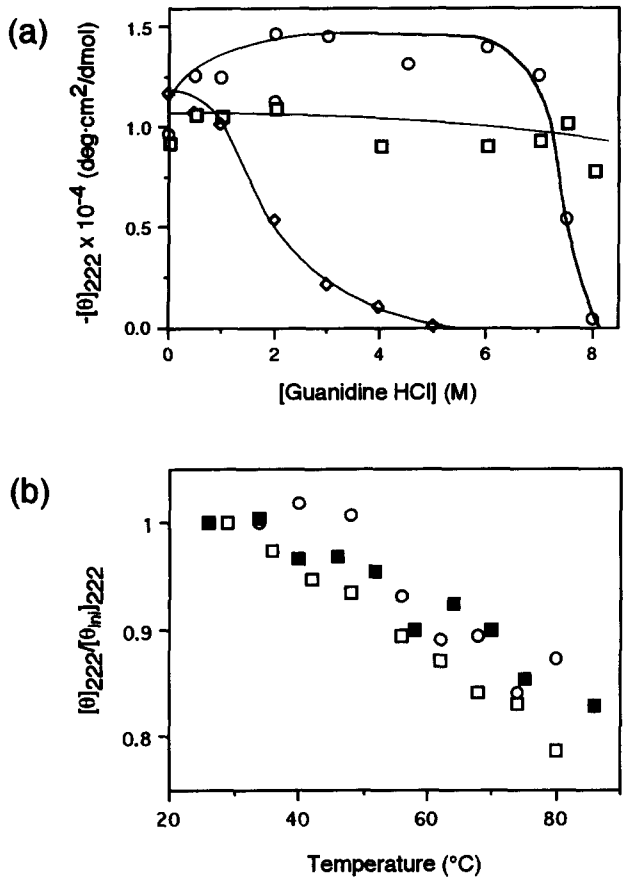

Figure 7. (a) Guanidine denaturation curves for the four-helix-bundle proteins. Open circle: **5** (10 μM); open square: **7** (9 μM); open diamond: [^{1}Ala, ^{20}Cys(Acm)]-**1** (57 μM). All the buffers contain 10 mM MOPS (pH 7.0). (b) Thermal stability of **5** and **7**. Open circle **5** [7 μM in 20 mM MOPS(pH 7.0) containing 4 M GnHCl]; open square: **7** [8 μM in 20 mM MOPS(pH 7.0)]; closed square: **7** [4 μM in 20 mM MOPS(pH 7.0) containing 4 M GnHCl]. Each helical content was compared in the form of $[\theta]_{222}/[\theta_{ini}]_{222}$, where $[\theta_{ini}]_{222}$ denotes $[\theta]_{222}$ at the initial temperature (25–28 °C).

helical stability has been strongly claimed.[21] The linker used here, -Gly-Gly-Cys(S-CH_2CO-Gly-Gly-), may contribute to stabilizing the helical protein structure as effectively as the linker previously reported (-Gly-Gly-Cys-S-S-Cys-Gly-Gly-).[11a,c] The value of $[\theta]_{222}$ corresponding to the α-helical content of **5** and **7** appears a little smaller than that of DeGrado's protein (-2.0×10^4 deg cm^2/dmol),[14] even when taking into account the fact that only two-thirds (14 out of a total of 21 and 20 amino acid residues) in **1** and **2**, respectively, were involved in the α-helix formation. The greater stability against denaturation also should come from the difference in the linker structures.

The high stability of the obtained proteins implied the potential use of the artificial proteins for drug carriers. When the unligated helical peptide [Ala^1, Cys(Acm)^{20}]-**1**, the protein **5**, and **7** were subjected to enzymatic degradation by lysyl endopeptidase, the order of the stability against the enzymatic digestion was: **7** > **5** > [Ala^1, Cys(Acm)^{20}]-**1**. Protein **7** was estimated to be almost twice as stable as [Ala^1, Cys(Acm)^{20}]-**1**.

Conclusions

In conclusion: (i) a four-helix-bundle protein composed of 81 residues was constructed by efficient chemical ligation through thioether formation, (ii) in terms of the practicality for the ligation, the bromoacetyl and the chloroacetyl moieties did not cause significant differences, and (iii) the introduction of additional disulfide cross-link made the molecule more resistant to chaotropic denaturation and enzymatic degradation. Again, we should emphasize that the easy construction of helix-bundle proteins comprising helices with different amino acid sequences is possible with our approach. We may also manage the combination and arrangement of helices. Unnatural amino acids having special functional groups and unnatural backbone structure can be also introduced into the molecules. These features are advantageous to the design and synthesis of artificial functional proteins. The above feature should also have great potential in developing a new category of the helix-presenting peptide libraries or drug carriers.

Experimental

General

HPLC was conducted with a Hitachi L-6200. Amino acid compositions after acid hydrolysis (6 N HCl, 110 °C, 24 h) were determined with a Hitachi 8500 amino acid analyzer. Liquid secondary ion mass spectra were recorded on a VG ZAB-2SEQ instrument with a 11-250J data system at an accelerating voltage of 8 kV. The expected mass values were expressed in the average chemical masses. 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). Lysyl endopeptidase (EC 3.4.21.50) was obtained from Wako Pure Chemicals (Osaka, Japan). Sephadex gel supports were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden), and the HPLC column from YMC (Kyoto, Japan), Nacalai Tesque (Kyoto, Japan) or Waters (Bedford, MA, U.S.A.). Retention time on HPLC (R_t) was monitored on a column of YMC R-ODS-5 S-5 120 Å (6 mm × 250 mm) with a gradient of CH_3CN in 0.1% aqueous TFA of 30–70% for 40 min at a flowrate of 1 mL/min (detection: 215 nm) (R_{t1}) or on a column of μBondasphere 5C_4-300 Å (3.9 mm × 150 mm) with a gradient of CH_3CN in 0.1% aqueous TFA of 30–70% for 40 min at a flowrate of 0.8 mL/min (detection: 215 nm)(R_{t2}).

Ac-Cys(Ad)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2 (1).

(i) Ac-Cys(Ad)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-(Acm)-Tyr-NH_2. Solid-phase peptide synthesis was carried out manually as reported earlier.[11c,13b] 4-(2′,4′-

Dimethoxyphenyl–Fmoc–aminomethyl)phenoxy resin (Rink amide resin)[22] (606 mg, substitution level 0.33 mmol/g) was used as the peptide anchor. After deprotection of the Fmoc group attached to the resin with 20% piperidine in DMF (20 min treatment time), Fmoc–Tyr(ᵗBu)–OH (230 mg, 0.5 mmol) was introduced with DICDI (78 ml, 0.5 mmol) in the presence of HOBt (68 mg, 0.5 mmol) (2 h reaction time). Repetitive removal of the Fmoc groups and introduction of amino acid derivatives gave the protected peptide resin [Ac-Cys(Ad)-Gly-Gly-Glu(OᵗBu)-Leu-Glu(OᵗBu)-Glu(OᵗBu)-Leu-Leu-Lys-(Boc)-Lys(Boc)-Leu-Lys(Boc)-Glu(OᵗBu)-Leu-Leu-Lys(OᵗBu)-Gly-Gly-Cys(Acm)-Tyr(ᵗBu)-resin] (1.03 g). Here the ᵗBu ester, the Boc, the Ad, and the Acm group were used for side-chain protection of Glu, Lys, Cys[1], and Cys[20], respectively. The *N*-terminal acetylation was conducted with acetic anhydride (5 equiv) in the presence of NMM (5 equiv) at room temperature for 30 min. A part of the above protected peptide resin (200 mg) was treated with 1 M TMSBr–thioanisole in TFA (10 mL) containing 2% (v/v) of *m*-cresol and 2% (v/v) of EDT for 1.5 h under ice-cooling, then for 30 min at 20 °C. 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 (3 mL) and applied to a column of Sephadex G-10 (16 mm × 360 mm), which was eluted with 1 N AcOH. Each fraction (3 mL) was monitored by UV absorption at 280 nm. The fractions corresponding to the first main peak (Nos 9–13) were collected and subjected to lyophilization to give a powder (75.1 mg). The powder was then further purified by HPLC with a column of Cosmosil $5C_{18}$-AR (10 mm × 250 mm) [gradient: CH_3CN in 0.1% aqueous TFA (49–51% for 20 min, then 60% for 5 min); flowrate: 2 mL/min; detection: 235 nm; 7 mg of peptide was applied each time]. The eluate corresponding to the main peak was collected and the solvent was removed by lyophilization to afford a white fluffy powder (25.5 mg, 25% yield from the initial introduction of Tyr on the resin). LSIMS: *m/z* 2570.7 $(M + H)^+$ (expected 2571.1). R_{f1}: 29.2 min.

(ii) Ac-Cys(Ad)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2. Ac-Cys(Ad)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys(Acm)-Tyr-NH_2 (8.2 mg, 3.2 μmol) was treated with AgOTf (67 mg, 0.26 mmol) in TFA (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 obtained powder was suspended in 50% AcOH (5 mL). Dithiothreitol (DTT) (31 mg, 0.26 mmol) was added. The mixture was stirred overnight in the dark. After centrifugation, the supernatant was applied to a column of Sephadex G-10 (16 mm × 360 mm), which was eluted with 1 N AcOH. Each fraction (3 mL) was monitored by UV absorption at 280 nm. The fractions corresponding to the front main peak were collected and lyophilized to give a powder (6.4 mg). The powder was further purified by HPLC with a column of YMC ODS-AM (20 mm × 150 mm) [gradient: CH_3CN in 0.1% aqueous TFA (50–58% in 16 min, then 70% in 4 min); flowrate: 2.5 mL/min; detection: 230 nm]. The eluate corresponding to the main peak was collected and the solvent removed by lyophilization to afford a white fluffy powder. Yield 5.3 mg (68% for the removal of the Acm group; 17% for the above two steps). LSIMS: *m/z* 2499.8 $(M + H)^+$(expected 2450.0). R_{f1}: 32.4 min.

Br-CH_2CO-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys(Acm)-Tyr-NH_2 (2). The protected peptide resin [H-Gly-Gly-Glu(OᵗBu)-Leu-Glu(OᵗBu)-Glu(OᵗBu)-Leu-Leu-Lys-(Boc)-Lys(Boc)-Leu-Lys(Boc)-Glu(OᵗBu)-Leu-Leu-Lys(Boc)-Gly-Gly-Cys(Acm)-Tyr(ᵗBu)-NH-resin](0.2 mmol) was prepared as stated above. Bromoacetylation was conducted with bromoacetic acid (134 mg, 1 mmol) and DICDI (156 ml, 1 mmol) in the presence of HOBt (135 mg, 1 mmol) (1 h reaction time) to give Br-CH_2CO-Gly-Gly-Glu(OᵗBu)-Leu-Glu(OᵗBu)-Glu-(OᵗBu)-Leu-Leu-Lys(Boc)-Lys(Boc)-Leu-Lys(Boc)-Glu(OᵗBu)-Leu-Leu-Lys(Boc)-Gly-Gly-Cys(Acm)-Tyr(ᵗBu)-NH-resin (1.05 g). Ninety milligrams of the above protected peptide resin was treated with TFA–anisole (10 mL/0.2 mL) for 3 h at 20 °C. The peptide was purified as stated above. A linear gradient of CH_3CN in 0.1% aqueous TFA (41–44% for 20 min, then 60% for 5 min) at a flowrate of 2 mL/min (detection: 235 nm) was used for the HPLC purification using a column of Cosmosil $5C_{18}$-AR (10 mm × 250 mm) (7.5 mg; the yield from the initial introduction of Tyr on the resin was 18%). LSIMS: *m/z* 2412.5 $(M + H)^+$(expected 2412.7). R_{f1}: 21.5 min.

Ac-Cys(Ad)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2

S-CH_2CO-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys(Acm)-Tyr-NH_2 (3). Segment **1** (10.0 mg, 4.0 μmol) was added to the solution of **2** (10.4 mg, 4.4 μmol) in 100 mM Tris–HCl (pH 8.0) containing 6 M guanidine. The mixture was stirred at room temperature overnight. The solution was applied to a column of Sephadex G-10 (16 mm × 150 mm), which was eluted with 1 N AcOH. Each fraction (3 mL) was monitored by UV absorption at 280 nm. The fractions corresponding to the front main peak were collected and lyophilized to give a powder (14 mg). The powder was then further purified by HPLC with a column of Cosmosil $5C_{18}$-AR (10 mm × 250 mm) [gradient: CH_3CN in 0.1% aqueous TFA (47–52% in 25 min, then 70% in 5 min); flowrate: 2 mL/min; detection: 235 nm]. The eluate corresponding to the main peak was collected and the solvent was removed by lyophilization to afford a white fluffy powder. Yield 13.5 mg (70%). LSIMS: *m/z* 4830.4 $(M + H)^+$(expected 4830.9). R_{f1}: 30.3 min.

Ac-Cys(Ad)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2

S-CH_2CO-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2

S-CH_2CO-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys(Acm)-Tyr-NH_2 (4).

(i) Removal of the Acm group from 3. Peptide **3** (13.5 mg, 2.8 μmol) was treated with AgOTf (69 mg, 100 equiv) in TFA (2 mL) in the presence of anisole (50 μL) in an ice bath for 1.5 h. The product was purified as stated above to give a powder (10.8 mg, 81%) (R_{t1}: 30.7 min). Without further purification the product was subjected to introduction of the third peptide segment.

(ii) Introduction of the third peptide segment. The above AgOTf-treated sample (10.8 mg, 2.3 μmol) and **2** (6.0 mg, 2.5 μmol) were dissolved in 100 mM Tris–HCl containing 6 M guanidine (pH 8.0)(1.5 mL). The mixture was stirred at room temperature overnight. The product was purified on Sephadex G-10 as stated above. Yield 15.3 mg (95% for the introduction of **2**; 80% for the above two steps). LSIMS: *m/z* 7090.7 (M + H)$^+$ (expected 7090.6). R_{t1}: 31.2 min.

Ac-Cys(Ad)-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2

S-CH_2CO-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2

S-CH_2CO-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys-Tyr-NH_2

S-CH_2CO-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys(Acm)-Tyr-NH_2 (5).

(i) Removal of the Acm group from 4. Peptide **4** (7.0 mg, 1.0 μmol) was treated with AgOTf (25 mg, 100 equiv) in TFA (2 mL) in the presence of anisole (40 μL) in an ice bath for 1.5 h. The product was purified as stated above. Yield 5.2 mg (76%) (R_{t2}: 31.7 min). Without further purification, the product was subjected to introduction of the final peptide segment.

(ii) Introduction of the final peptide segment. The above AgOTf-treated sample(5.2 mg, 0.75 μmol) and **2** (2.0 mg, 0.82 μmol) were dissolved in 100 mM Tris–HCl containing 6M guanidine (pH 8.0) (0.8 mL). The mixture was stirred at room temperature overnight. The product was purified as stated above. A linear gradient of CH_3CN in 0.1% aqueous TFA (48–53% in 20 min, then 70% in 5 min)at a flowrate of 2 mL/min (detection, 235 nm) was used for the HPLC purification using a column of Cosmosil $5C_{18}$-AR (10 mm × 250 mm). Yield 3.0 mg (43% for the introduction of **2**; 33% for the above two steps). LSIMS: *m/z* 9349.9(M + H)$^+$(expected 9350.3). R_{t2}: 31.4 min.

Cl-CH_2CO-Gly-Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Gly-Cys(Acm)-Tyr-NH_2 (2′). The title peptide was obtained in the same manner as **2**. Cl–CH_2COOH was used for *N*-terminal chloroacetylation instead of Br–CH_2COOH. Yield: 30% from the starting resin. LSIMS: *m/z* 2368.0 (M + H)$^+$ (expected 2368.2). R_{t1}: 20.6 min.

Construction of the four-helix-bundle protein 5 using the chloroacetylated peptide segment 2′. Construction of **5** using **2′** was conducted as in the case using **2**. The yield in each step of ligation is given in the following. Preparation of **3**, 67%; removal of the Acm group from **3**, 66%; Preparation of **4**, 95%; removal of the Acm group from **4**, 85%; preparation of **5**, 40%. The fidelity of the products were confirmed by LSIMS. These products were also confirmed to have the identical retention times on HPLC with samples prepared with **2**.

Removal of the Ad and the Acm groups from 5. Protein **5** (2.0 mg, 0.21 μmol) was treated with 1 M CF_3SO_3H–thioanisole in TFA (0.4 mL) in the presence of *m*-cresol (10 μL) and EDT (10 μL) in an ice bath for 1.5 h. TFA was removed under N_2 stream and ether (1 mL) was added. The resulting powder was collected by centrifugation. The powder was then treated with AgOTf (5.5 mg, 21 mmol) in TFA (0.6 mL). TFA was removed under N_2 stream and ether (1 mL) was added. The resulting powder was collected by centrifugation. DTT (3.2 mg, 21 mmol) in 1 N AcOH (0.5 mL) was added to the powder, and the mixture stirred at room temperature overnight. Insoluble material was removed by centrifugation. The supernatant was applied to a column of Sephadex G-10 (16 mm × 150 mm), which was eluted with 1 N AcOH. Each fraction (3 mL) was monitored by UV absorption at 280 nm. The fractions corresponding to the front main peak were collected and lyophilized to give a powder of **6**. Yield 1.3 mg (68%). LSIMS: *m/z* 9144.7 (M+H)$^+$ (expected 9145.0). R_{t2}: 28.6 min.

Disulfide formation by air oxidation. Protein **6** (1.3 mg, 0.14 μmol) was dissolved in 0.1 M NH_4HCO_3 (260 mL, peptide concentration: 0.5 μM) and was kept standing at 20 °C for 16 h. The solution was then lyophilized. The resulting powder was further purified by HPLC [column: μBondasphere (3.9 mm × 150 mm); gradient: CH_3CN in 0.1% aqueous TFA (45–50% in 20 min, then 65% in 5 min); flowrate: 0.8 mL/min; detection: 215 nm] to give a pure product **7** (0.75 mg, 58%). LSIMS: *m/z* 9142.6 (M + H)$^+$(expected 9142.9). R_{t2}: 24.5 min.

Disulfide formation in a redox buffer. Protein **6** (0.95 mg, 0.10 μmol) was dissolved in a redox buffer containing 0.1 M Tris–HCl, 0.2 M NaCl, 1 mM reduced glutathione, and 1 mM oxidized glutathione (pH 8.5) (5 mL, peptide concentration: 30 μM). The solution was kept standing at 20 °C for 16 h and lyophilized. The resulting powder was dissolved in 1 M AcOH (0.5 mL) and subject to a column of

Sephadex G-25 (10 mm × 50 mm), which was eluted by 1 N AcOH. Each fraction (3 mL) was monitored by UV absorption at 280 nm. The fractions corresponding to the front main peak were collected and lyophilized to give a powder (0.64 mg, 68%). Then the powder was further purified by HPLC with a column of μBondasphere (3.9 mm × 150 mm) [gradient: CH_3CN in 0.1% aqueous TFA (42-47% in 20 min, then 65% in 5 min); flowrate: 0.8 mL/min; detection: 230 nm]. The eluate corresponding to the main peak was collected and the solvent was removed by lyophilization to afford a white fluffy powder of **7**. Yield 0.56 mg (59%). The sample had an identical retention time on HPLC with the sample obtained by air oxidation (R_{t2}: 24.5 min).

Size-exclusion chromatography

Size-exclusion chromatography of protein **7** was conducted using a column of Sephadex G-50 fine (25 mm × 720 mm). The column was eluted with a buffer of 100 mM potassium phosphate buffer (pH 7.0) at a flowrate of 0.8 mL/min. Each fraction (6 mL) was monitored by measuring the absorbance at 280 nm. The molecular weights of the standard proteins used for calibration were: carbonic anhydrase, 29,000; myoglobin, 17,000; cytochrome *c*, 12,400; CCK-33 (nonsulfate), 3800.

CD measurements

Buffers and the concentration of the peptides are noted in the figure legends. [Ala[1], Cys(Acm)[20]]-**1** was prepared in the same manner as **1**. The molecular ellipticity ([θ]) was calculated on the basis that total numbers of amino acids in [Ala[1], Cys(Acm)[20]]-**1**, **5**, and **7** are 81, 81 and 21, respectively.

Stability of the constructed proteins against lysyl endopeptidase digestion

A quantity of 10 μg of a protein were dissolved in 100 mM Tris (pH 9.5) (20 μL). A solution of the lysyl endopeptidase (0.02 AU/mL in distilled water) (0.5 μL) was added to the protein solution. The mixture was incubated at 37 °C. The degree of the degradation of each protein was monitored by HPLC using a column of YMC Protein-RP (6 mm × 250 mm). The remaining proteins after 5 h were 52% for **7**, 43% for **5**, and 27% for [Ala[1], Cys(Acm)[20]]-**1**, respectively.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (nos 06780468 and 08772021) from the Ministry of Education, Science and Culture of Japan.

(*Received in Japan* 21 *April* 1997; *accepted* 30 *May* 1997)

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