Pergamon

Tetrahedron, Vol. 53, No. 42, pp. 14263-14274, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00994-0

Synthesis and Structural Characterization of Triple-Helical Peptides Which Mimic the Ligand Binding Site of the Human Macrophage Scavenger Receptor

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Abstract: A synthetic method for triple-helical peptides was developed. Peptides with and without glutamic acid α-thioester at their *N*-termini are prepared by the solid-phase method. These peptides are crosslinked at their *N*-termini one by one to generate trimeric peptides using the activation of the thioester group by silver ions. This method was applied to the synthesis of model peptides, which mimic the binding site of modified low density lipoprotein (LDL) in the human scavenger receptor (SR). These models possessed different spacers, which connect the peptide chains and the crosslinking site. CD and DSC analysis of the peptides revealed that spacer length has a critical effect on the stability of the triple helix. © 1997 Elsevier Science Ltd.

INTRODUCTION

The macrophage SR mediates the endocytosis of modified LDLs, such as oxidized LDL.[1] These ligands, when incorporated in excess, promote the conversion of macrophages into cholesteryl ester-rich foam cells, which may result in arterial sclerosis. The scavenger receptor is trimeric and has a single membrane spanning region at its *N*-terminus, with an inside-out orientation.[2] The extracellular region is characterized by an α-helical coiled-coil and collagen-like domain. The binding site of the modified LDLs is located in the *C*-terminus of the collagen-like domain and composed of about 20 amino acids. Because this region is highly basic, the receptor has rather broad binding specificity toward negatively charged molecules, such as polyribonucleotides, in addition to modified LDLs. An α-helical coiled-coil domain is essential for trimerization,[3] and is located on the *N*-terminal side of the collagen-like domain. Thus, the association of three polypeptide chains at the coiled-coil domain would be a key step in the initiation of triple helix formation by the collagen-like domain.

Model peptides of collagen have been designed by several researchers.[4] Generally, these models consist of the same three peptide chains, which are crosslinked at their *C*- or *N*-termini to stabilize the triple-helical structure. The crosslinking point and the peptide chains are connected by spacers, such as βAla or 6-amino-hexanoic acid (AHA), to add flexibility during triple helix formation. However, the selection of the spacer is empirical and has not yet been systematically examined. In the present study, a new method, which enables the crosslinking of peptide chains one by one to generate trimeric collagen-like peptides, was developed and applied

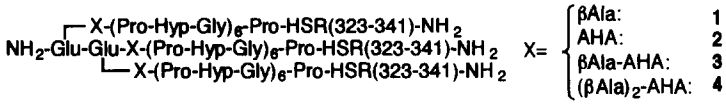

Fig. 1. Structure of triple-helical peptides

to the preparation of the modified LDL binding site in human SR[5] with different spacers as shown in Figure 1. The effect of spacer length on the stability of the triple helices was analyzed using circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC).

RESULTS AND DISCUSSION

Design and synthesis of trimeric peptides

The modified LDL binding site in SR is located in the *C*-terminus of the collagen-like domain.[2] This fact suggests that the *C*-terminal part of the binding site would be more flexible compared to the *N*-terminal. To construct a precise model of the domain, therefore, it is proper to crosslink three peptide chains at their *N*-termini. At present, several methods are known to construct homotrimeric collagen-like peptides, which have a crosslink at the *C*-termini.[4a-4f] Even the syntheses of heterotrimeric collagen models have been recently reported.[4g,4h] In contrast, few reports exist on the synthesis of the *N*-terminally crosslinked trimeric peptide.[4e,4f] Based on the

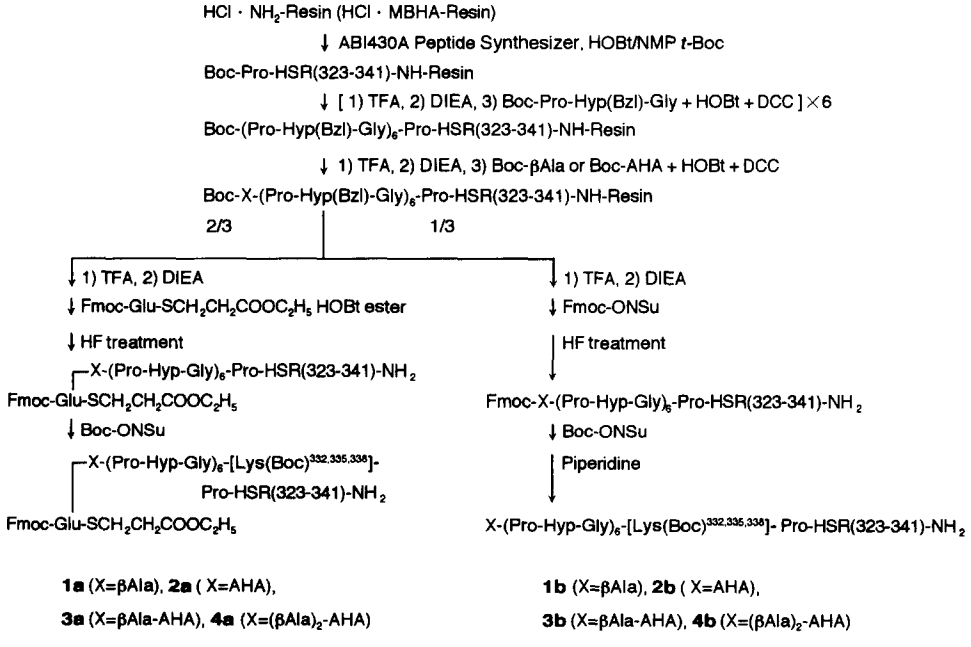

Fig. 2. Synthetic pathway for peptide segments

segment condensation method used for protein synthesis, we developed a new method to synthesize collagen models, which have a crosslink at their *N*-termini. As this method realizes the crosslinking of peptide chains one by one at their *N*-termini, homotrimeric as well as heterotrimeric peptides can be synthesized. To accomplish this strategy, two peptides are required as shown in Figure 2: a peptide which has a glutamic acid α-thioester at its *N*-terminus and the same peptide without the glutamic acid. These peptides were prepared using the solid-phase method. Crosslinking is achieved by the activation of the thioester group of the glutamic acid by silver ions, followed by nucleophilic attack by the terminal amino group of the other peptide as shown in Figure 3. As the carboxyl group of the glutamic acid is selectively activated, the protection of the side chain functional groups is restricted to amino groups. Thus, the minimally protected peptide segments maintain high solubility and reactivity during the condensation reactions. This method can be applicable to the preparation of heterotrimeric collagen models, if a peptide chain with a different sequence is incorporated at the first or second step of the condensation.

A comparison of the human SR with the bovine SR suggested that the binding domain of the modified LDLs in the human receptor consists of the following amino acids (HSR(321-339)): Gly-Tyr-Ala-Gly-Arg-Pro-Gly-Asn-Ser-Gly-Pro-Lys-Gly-Gln-Lys-Gly-Glu-Lys-Gly.[5] Lys^{337} of the bovine SR sequence, which corresponds to Lys^{335} in the human SR, plays an essential role in ligand binding activity.[3, 4f] Thus, the HSR (323-341) sequence containing Lys^{335} was incorporated into our model. The synthesis of peptides with and without the glutamic acid thioester was performed as shown in Figure 2. As an example, the procedure for peptide **2** is described below. Starting with 4-methylbenzhydrylamine (MBHA)-resin (NH_2-resin), the sequence of HSR(323-341) was synthesized using an ABI 430A peptide synthesizer. To the *N*-terminus of HSR(323-341), the collagen sequence $(Pro\text{-}Hyp\text{-}Gly)_6$ was introduced. Due to the spontaneous formation of triple helical structure, this sequence would accommodate some mismatches of three chains at the crosslinking site and provide a proper spatial arrangement of three peptide chains to HSR(323-341). The introduction of $(Pro\text{-}Hyp\text{-}Gly)_6$ was accomplished by manual couplings using the Boc-Pro-Hyp(Bzl)-Gly 1-hydroxybenzotriazole (HOBt) ester. Boc-AHA was then introduced as a spacer using its HOBt ester. The resin obtained was then divided into two portions to simultaneously synthesize the required peptides. Two-thirds of the resin was reacted with the Fmoc-Glu-SCH_2CH_2COOEt γ-HOBt ester to introduce the glutamic acid α-thioester via the γ-carboxyl group. The obtained resin was then treated with HF to yield a crude peptide, which was purified by HPLC. The side-chain amino groups were protected with Boc groups to avoid side reactions during subsequent crosslinking reactions. The peptide thioester **2a** was obtained at a yield of 12% based on the amino groups in the initial resin. The rest of the resin was treated with Fmoc-ONSu and the Fmoc group was introduced to the terminal amino group. The resin was treated with HF to yield a crude peptide, which was purified by HPLC. After the Boc groups were introduced to the side chain amino groups, the Fmoc group was removed by piperidine to obtain a peptide, **2b**, with a free terminal amino group in 12% yield.

The crosslinking reactions were carried out by successive condensation of peptide **xa** to **xb** (x=1, 2, 3, 4) as shown in Figure 3. Each coupling reaction was achieved by the application of the thioester method.[6] In the case of peptide **2**, peptide **2a** and peptide **2b** were dissolved in DMSO containing *N*-hydroxysuccinimide

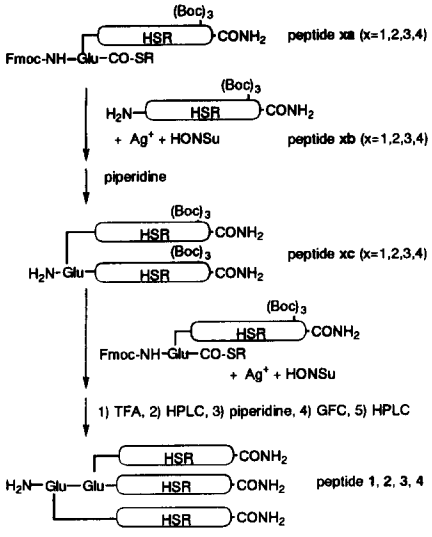

Fig. 3. Synthetic procedure for peptides **1-4**

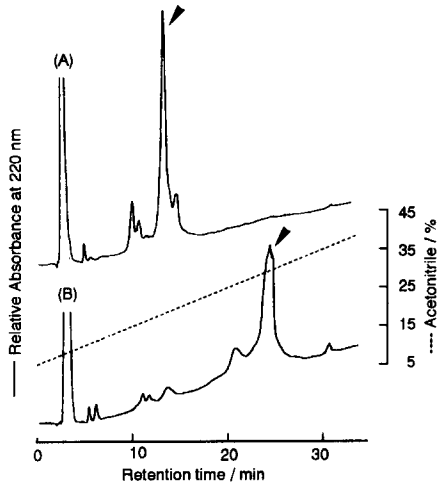

Fig. 4. HPLC profile of TFA-treated reaction mixture; panel A: reaction mixture for preparation of peptide **2c**; panel B: reaction mixture for the preparation of peptide **2**. Column: Cosmosil $5C_{18}$ AR (4.6 x 150 mm) at a flow rate of 1 ml·min^{-1}. Eluent: aqueous acetonitrile containing 0.1% TFA.

(HONSu) and diisopropylethylamine (DIEA). By the addition of $AgNO_3$, the thioester group was easily activated and converted to the HONSu active ester. Monitoring with HPLC revealed that the coupling reaction was almost complete within 12 h without serious side reactions, as shown in Figure 4A. The Fmoc group was then removed by adding piperidine to the reaction mixture. After the product was purified by gel filtration chromatography (GFC) on Superdex 75HR, peptide **2c** was obtained at a yield of 64%. The dimers **1c**, **3c** and **4c** were also prepared in the same manner.

The progress of the coupling reaction of the third chain varied dramatically depending on the spacer length. In the case of peptide **3**, the reaction proceeded under the same conditions as for the dimer peptide **3c**. For the coupling between peptides **2c** and **2a**, it was necessary to use HOBt instead of HONSu to increase the reactivity of the active ester.[7] The HPLC profile of the reaction mixture is shown in Figure 4B. In contrast to peptides **2** and **3**, the condensation reactions for peptides **1** and **4** did not proceed at all at room temperature. This fact might indicate that the peptide chains aggregate into a triple helix-like conformation in DMSO and that the spacers of peptides **1** and **4** cannot provide the appropriate length required to connect the peptide chains. As for peptide **1**, heating of the mixture to 90°C was effective for initiating the condensation. The heating partly melted the structure, thus increasing the flexibility of the spacer to connect the peptide chains. However, the yield of peptide **4** was not increased by the heating, which may indicate that the spacer was not flexible enough to connect the peptide chains even at 90°C. To obtain peptide **4**, complete unfolding of the structure would be required. As the stability of a triple helix in organic solvent is much higher than in water,[8] the peptide chains would partly fold at

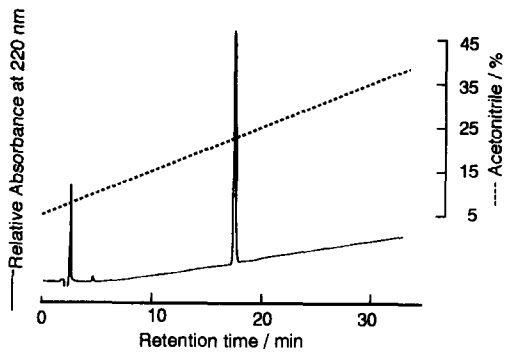

Fig. 5. HPLC profile of peptide **2** after purification; analytical conditions are the same as described in Fig. 4

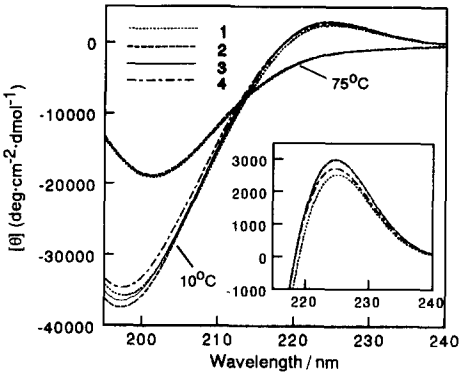

Fig. 6. CD spectra of peptides **1-4** in 50 mM sodium phosphate at pH 7.0

90°C. Thus, from the view point of synthesis, the use of AHA or βAla-AHA as spacers to prepare the triple-helical peptide is the most practical option.

After the coupling reaction was completed, the product was precipitated with ether, washed with AcOEt and dried in vacuo. Boc groups of the crude peptide were removed using 10% 1,2-ethanedithiol (EDT) in trifluoroacetic acid (TFA) for 15 min and the peptide was then purified by HPLC. At this step, the trimer and the hydrolyzed product of the active ester eluted almost at the same position on HPLC, which caused a broadening of the main peak as shown in Figure 4B. Thus, the trimer was further purified by GFC after the removal of the terminal Fmoc group by piperidine and desalted by HPLC in an unfolded state at 65°C. The HPLC elution profile of purified peptide **2** is shown in Figure 5. Other peptides were eluted almost at the same position as peptide **2** with comparable purity. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric data and amino acid analysis data confirmed that the expected peptides were successfully obtained (Table 1). The yields of peptides **1-4** were 10%, 20%, 20%, and 2.1%, respectively, based on the corresponding dimer peptides. Single stranded peptides (NH_2-AHA-(Pro-Hyp-Gly)$_6$-HSR(323-341)-NH_2: **2d**, NH_2-βAla-AHA-(Pro-Hyp-Gly)$_6$-HSR(323-341)-NH_2: **3d**) were also prepared in order to compare thermodynamic parameters for unfolding with triple stranded peptides. The results are summarized in Table 1.

CD spectrum measurement

The CD spectra of peptides **1-4** at 10°C show a maximum elipticity near 224 nm and a large negative peak near 197 nm, suggesting that these peptides form a triple-helical structure (Figure 6).[9] The intensity of $[\theta]_{224}$ is higher in peptides **2** and **3** compared to **1** and **4** as shown in the small box in Fig. 6. In contrast, all peptides show spectra of a melted triple-helix with comparable $[\theta]_{224}$ values at 75°C. From this results, the triple-helix content of peptides, which corresponds to the difference between the intensity of $[\theta]_{224}$ at 10°C and 75°C, is higher in peptides **2** and **3** than in peptides **1** and **4**. As for peptide **1**, the maximum and minimum of the spectra

Table 1. Mass Spectrometric and Thermodynamic Data of Peptides.

peptides	calcd. $(M+H)^+$	obs. $(M+H)^+$	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)	ΔS (cal/mol·K)	ΔG^{**} (kcal/mol)	Tm (°C)
1	10843	10842	81.1	76.1	238	1.6	68.2
2	10969	10973	81.0	81.4	241	0.5	63.1
3	11182	11182	81.7	80.5	244	0.0	61.1
4	11395	11388	-	96.7*	292	-	57.5
2d	3583.0	3581.6	77.1	158*	542	-104	33.4
3d	3654.0	3652.2	82.2	164*	562	-106	34.8

* The values are calculated from temperature scanning of $[\theta]_{224}$ shown in Fig. 7.

** The values are calculated at 61°C using $\Delta G = \Delta H_{cal} - T\Delta S$.

shifts approximately 0.3 nm to a higher wavelength compared to the other peptides, indicating that small structural changes are also related to the decrease in the intensity of $[\theta]_{224}$ at 10°C.

Thermal stability by CD and DSC measurement

To analyze the thermal stability of these peptides, $[\theta]_{224}$ of the peptides was monitored as a function of temperature, as shown in Figure 7. Peptides **1**-**4** have approximately a 25 to 35°C higher Tm compared to monomers **2d** and **3d**, which indicates that crosslinking of the peptide chains can reduce entropy loss during the formation of the triple-helical structure and decrease the free energy for folding. Thus, crosslinking effectively increases the thermal stability of a triple-helical structure.

Comparing trimers **1** to **4**, the Tm decreases from 68°C to 58°C, which shows that the stability of the structure is decreased from peptides **1** to **4**. To obtain quantitative data of the stability, excess heat capacity was measured by DSC as shown in Fig. 8. The DSC of peptide **4** was not performed due to insufficient quantities.

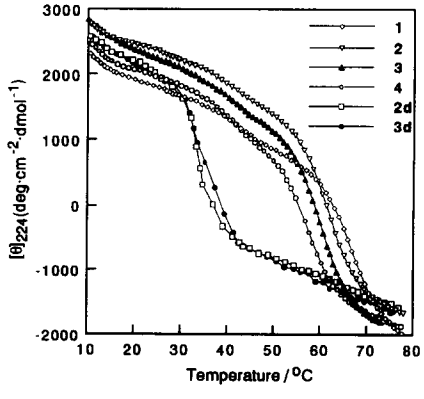

Fig. 7. Thermal transition curves for peptides at a wavelength of 224 nm

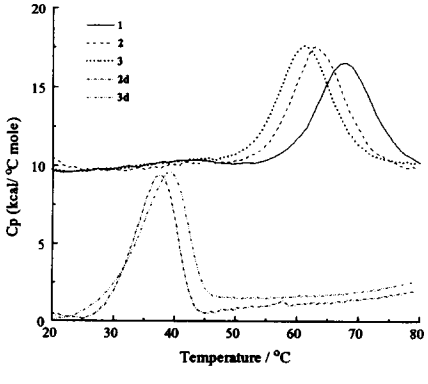

Fig. 8. Excess heat capacity of peptides in 50 mM sodium phosphate pH 7.0

The calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy change (ΔH_{vH}) for the denaturation were directly obtained from the excess heat capacity curves. These values are summarized in Table 1. Trimers **1**, **2**, and **3** underwent large transitions with Tm between 61 and 68°C, which is consistent with the CD data in Figure 7. The ΔH_{cal} values are comparable to ΔH_{vH} of the same peptides, indicating that the transition can be approximated in two states. Based on the the ΔH_{cal} values, ΔS and ΔG (at 61°C) of the peptides are calculated (Table 1). Peptide **1** has a 0.5 and 1.6 kcal·mol^{-1} higher ΔG value compared to peptides **2** and **3**, respectively, suggesting that the structure is destabilized as the spacer lengh increases from peptide **1** to **3**. Since Table 1 shows that ΔS increases as the spacer length becomes longer from peptides **1** to **3**, and that ΔH_{cal} is comparable in these peptides, we concluded that the stability of the structure is mainly regulated by the entropic factor, not by the enthalpic factor. The diminished entropy change is ascribed either to the more restricted motion in the random state or less ordered structure in the triple-helical state. As the conformational space of the peptides in the random state increases with the increase in spacer length, the former factor would predominantly determine the stability.

The CD spectra showed that peptides **1** and **4** have a lower triple helix content than **2** and **3**. In the case of peptide **4**, the decreased helicity is simply attributed to its decreased stability, which results from the larger ΔS of peptide **4** compared to **1**-**3**. However, the reason why peptide **1** has a lower triple helix content than peptides **2** and **3**, although it has the highest stability, cannot be simply explained. One possibility is that the spacer of peptide **1**, which was the shortest and which increased stability by restriction of the random state, also decreased the flexibility of the triple-helical state to such an extent that the amino acids near the crosslinking point could not form a triple helix. Further structural information, obtained by other methods such as NMR, is required to clarify this point. The present data shows that excess elongation or reduction in spacer length causes a decrease in the triple helix content. Thus, AHA or βAla-AHA are suitable spacers for the connection of the three peptide chains.

Trimeric models that incorporate the sequence of bovine SR and type I, IV collagen have previously been prepared.[4c-4b] In these peptides, the specific sequence is stabilized at both ends; one side by crosslinking and the other side by Pro-Hyp-Gly repeats. The triple helix stabilized at both ends is thought to retain a higher Tm than ones with a one-sided stabilization.[10] Although the difference in the length of the (Pro-Hyp-Gly) repeats does not allow a simple comparison to be drawn, the fact that peptides **2** and **3** retain a high Tm indicates that one-sided stabilization is also a good choice in the design of triple-helical peptides.

In summary, we have developed a convenient method for the synthesis of trimeric collagen-like peptides, which may also be used to prepare heterotrimeric peptides. By optimization of the spacer length between peptide chains and the crosslinking point, triple-helical peptides with enhanced thermal stability compared to monomeric peptides were successfully prepared. Modified LDL binding activity of the peptides are now under study.

Materials and Methods

Peptide segments for crosslinking were prepared by a peptide synthesizer 430A (Applied Biosystems Inc.,

Foster City, CA) using the 0.5 mmol scale double coupling protocol of the benzotirazole active ester method. Boc-Pro-Hyp(Bzl)-Gly was prepared according to the reference with modifications.[11] HPLC purification of the peptides was performed by CCPM-II (TOSOH, Tokyo) using aqueous acetonitrile containing 0.1% TFA as an eluent. The column used was Cosmosil $5C_{18}AR$ (20 x 250 mm, Nacalai Tesque, Kyoto) at a flow rate of 7 $ml \cdot min^{-1}$ for preparative purpose and (4.6 x 150 mm) at a flow rate of 1 $ml \cdot min^{-1}$ for analytical purposes. The detection was carried out at 230 nm or 220 nm. Amino acids were analyzed using a Hitachi L8500 amino acid analyzer after acid hydrolysis of a peptide with constant boiling point HCl at 110°C for 48 h. Mass measurement was performed using a MALDI-TOF mass spectrometer Voyager ™RP (PerSeptive Biosystems, Framingham, MA). The weight of the purified peptide was calculated based upon the amino acid analysis data. Mass numbers were calculated as averages.

Fmoc-Glu-SCH_2CH_2COOEt

To the ice chilled solution of Fmoc-Glu(OBu^t)·H_2O (2.0 g, 4.5 mmol) and HONSu (0.62 g, 5.4 mmol) in DCM (15 ml), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.95 g, 5.0 mmol) was added. After the solution was stirred at room temperature for 3 h, the solvent was evaporated in vacuo. The oily residue was dissolved in ethyl acetate, washed with 1 M HCl and aqueous NaCl solution and dried over Na_2SO_4. After the removal of the solvent, Fmoc-Glu(OBu^t)-ONSu was obtained as an oil (3.3 g). This oil and 3-mercaptopropionic acid ethyl ester (581 μl, 4.5 mmol) were dissolved in DCM (10 ml) and reacted overnight in the presence of DIEA (390 μl, 2.2 mmol). The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate and successively washed with 1 M HCl (x2), 5% aqueous $NaHCO_3$, aqueous NaCl and then dried over Na_2SO_4. The solvent was evaporated to give an oil, which was treated with TFA (6 ml) at room temperature for 1 h. TFA was removed by evaporation and the residual oil was purified by silicagel column chromatography using $CHCl_3$-CH_3OH-CH_3COOH (100:2:1) as a solvent to give Fmoc-Glu-SCH_2CH_2COOEt (1.7 g, 3.5 mmol). mp. 61-63°C. $[\alpha]_D$= -26.0° (c=0.90, methanol). Found: m/z 508.8 $(M+Na)^+$ (monoisotopic). Calcd for $(M+Na)^+$: 508.1 (monoisotopic).

*Fmoc-Glu([Lys(Boc)^{332,335,338}]-X-(Pro-Hyp-Gly)$_6$-Pro-HSR(323-341)-NH_2)-SCH_2CH_2COOEt (X= βAla (**1a**), AHA (**2a**), βAla-AHA (**3a**), (βAla)$_2$-AHA (**4a**))*

In the case of peptide **2a**, Boc-Pro-Ala-Gly-Arg(Tos)-Pro-Gly-Asn-Ser(Bzl)-Gly-Pro-Lys(Cl-Z)-Gly-Gln-Lys(Cl-Z)-Gly-Glu(OBzl)-Lys(Cl-Z)-Gly-Ser(Bzl)-Gly-NH-resin was prepared by the synthesizer, starting from the MBHA-resin hydrochloride (0.33 mmol). Pro-Hyp-Gly repeats were manually introduced using Boc-Pro-Hyp(Bzl)-Gly HOBt ester (0.67 mmol) for six times. After Boc-AHA was introduced using the same procedure, the obtained resin (**5**) was divided into two portions. One-third of resin **5** was stored for the preparation of peptide **2b** . The rest of the resin was reacted with the Fmoc-Glu-SCH_2CH_2COOEt γ-HOBt ester (0.5 mmol). The obtained resin (1.4 g) was treated with 10% anisole-HF at 0°C for 90 min to give a crude

product (640 mg). This peptide was applied to the HPLC column (20 mg at a time) and the main fraction was collected to yield Fmoc-Glu(AHA-(Pro-Hyp-Gly)$_6$-Pro-HSR(323-341)-NH_2)-SCH_2CH_2COOEt (110 mg, 26 μmol, 12%). To the solution of this peptide (230 mg, 26 μmol) in DMSO (1 ml), Boc-ONSu (200 mg, 950 μmol) and DIEA (170 μl, 950 μmol) were added and the reaction mixture was stirred for 1.5 h. Ether was added to precipitate the product, which was collected by centrifugation to give peptide **2a** (110 mg, 26 μmol, 12% based on amino group on the MBHA-resin). Found: m/z 4350.5 $(M+H)^+$. Calcd for $(M+H)^+$: 4350.8. Amino acid analysis of peptide **2a**: $Asp_{1.03}Ser_{1.95}Glu_{2.36}Pro_{6.40}Gly_{13}Ala_{1.07}Lys_{2.46}Arg_{0.81}$. Peptides **1a**, **3a**, and **4a** were prepared by the same procedure for peptide **2a**. The yields were 4%, 7%, and 4% for peptides **1a**, **3a**, and **4a**, respectively.

*[Lys(Boc)^{332,335,338}]-X-(Pro-Hyp-Gly)$_6$-Pro-HSR(323-341)-NH_2 (X= βAla (**1b**), AHA (**2b**), βAla-AHA (**3b**), (βAla)$_2$-AHA (**4b**))*

In the case of peptide **2b**, one-third of the resin **5** (110 μmol) prepared during the synthesis of **2a** was reacted with Fmoc-ONSu (0.36 g, 1.1 mmol) after TFA treatment. The obtained resin (520 mg) was treated with 10% anisole-HF at 0°C for 90 min to give the crude peptide (240 mg). This peptide was purified by HPLC (20 mg at a time) to give Fmoc-AHA-(Pro-Hyp-Gly)$_6$-Pro-HSR(323-341)-NH_2 (53 mg,14 μmol). The purified peptide (53 mg, 14 μmol) and Boc-ONSu (23 mg, 110 μmol) were dissolved in DMSO (250 μl). DIEA (19 μl, 110 μmol) was added and the solution was stirred for 1 h at room temperature. Ether was added to precipitate the product, which was collected by centrifugation and lyophilized from a dioxane suspension to give Fmoc-[Lys (Boc)^{332,335,338}]-AHA-(Pro-Hyp-Gly)$_6$-Pro-HSR(323-341)-NH_2. This peptide was dissolved in DMSO (300 μl) and piperidine (15 μl) was added and the solution was stirred for 1.5 h at room temperature. Ether was added and the product was obtained as an precipitate. The peptide was dissolved in DMSO (300 μl) and DIEA (30 μl) was added. The solution was kept 10 min at room temperature. Ether was added to form a precipitate, which was collected by centrifugation to obtain peptide **2b** (50 mg, 13 μmol, 12% based on amino groups on MBHA resin). Found: m/z 3883.2 $(M+H)^+$. Calcd for $(M+H)^+$: 3883.3. Amino acid analysis of peptide **2b**: $Asp_{1.05}$ $Ser_{2.06}Glu_{2.11}Pro_{7.36}Gly_{13}Ala_{1.07}Lys_{2.25}Arg_{0.90}$. Peptides **1b**, **3b**, and **4b** were prepared using the same procedure described for peptide **2b**. The yields were 7%, 20%, and 13% for peptide **1b**, **3b**, and **4b**, respectively.

*Dimer peptides **1c-4c***

In the case of peptide **2c**, **2a** (23 mg, 5.3 μmol) and **2b** (15 mg, 3.9 μmol) were dissolved in DMSO (190 μl) containing HONSu (8.5 mg, 74 μmol) and DIEA (2.6 μl, 15 μmol). $AgNO_3$ (2.5 mg, 15 μmol) was added and the solution was stirred at room temperature in the dark. After the solution was kept overnight, piperidine (10 μl) was added to the reaction mixture and the solution was stirred for 1.5 h. Ether was added to form a precipitate (62 mg), which was purified by GFC on Superdex 75 HR (10 x 300 mm, Pharmacia Biotech) using 50% aqueous acetonitrile containing 0.1% TFA as an eluent at a flow rate of 0.3 ml·min^{-1} to give peptide **2c** (20 mg,

2.5 μmol, 64% based on peptide **2b**). Peptides **1c**, **3c**, and **4c** were prepared using the same procedure described for peptide **2c**. The yields were 84%, 85%, and 67% for peptides **1c**, **3c**, and **4c**, respectively.

Trimer peptides 1-4

In the case of peptide **2**, peptide **2a** (20 mg, 4.6 μmol) and peptide **2c** (10 mg, 1.3 μmol) were dissolved in DMSO (100 μl) containing HOBt (2.6 mg, 19 μmol) and DIEA (2.2 μl, 13 μmol). After $AgNO_3$ (2.2 mg, 13 μmol) was added, the solution was stirred at room temperature for 5 h in the dark. Ether was added and the precipitate was collected by centrifugation. This peptide was treated with 5% EDT-TFA (250 μl) for 10 min at room temperature. After TFA was removed by a nitrogen stream, ether was added to form a precipitate which was purified by HPLC. The obtained peptide was dissolved in DMSO (90 μl) and piperidine (5 μl) was added. After the solution was kept at room temperature for 40 min, ether was added to precipitate the product, which was purified by GFC on Superdex 75HR using 50% aqueous acetonitrile containing 0.1% TFA as an eluent to give peptide **2** (3.0 mg, 270 nmol, 20% based on peptide **2c**). Amino acid analysis of peptide **2**: $Asp_{3.11}Ser_{5.04}Glu_{8.59}Pro_{26.4}Gly_{39}Ala_{3.17}Lys_{8.48}Arg_{2.87}$. Peptide **3** was prepared as described above, using HOBt instead of HONSu. Peptides **1** and **4** were prepared in the same manner as described above except that the reaction was carried out in 1-methyl-2-pyrrolidinone (NMP) at 90°C in the presence of *p*-nitrophenol instead of HONSu. The yields of peptides **1**, **3**, and **4** were 10%, 20%, and 2.1%, respectively.

CD spectrum measurement

CD spectra were recorded using a J-720 spectropolarimeter (Jasco, Tokyo) with a 1.0 mm path length cell. Samples were prepared at a concentration of 0.2 mg·ml^{-1} in 50 mM sodium phosphate buffer pH 7.0. For equilibrium melting transitions, the temperature was increased at the rate of 1°C·min^{-1} and $[\theta]_{224}$ was monitored. The concentration of the peptide was 0.4-0.7 mg·ml^{-1}. The van't Hoff enthalpy of peptides **2d** and **3d** was determined by a previously described method.[7]

DSC measurement

The peptides were dissolved in 50 mM sodium phosphate buffer, pH 7.0, at a concentration of 0.4 to 0.7 mg·ml^{-1} and sealed in the cell of a differential scanning calorimeter, MC-2 (MicroCal Inc., MA). The heating rate was 1°C·min^{-1} and the temperature was increased from 10°C to 80°C. The scanning was repeated three times with 50 min intervals to confirm the reversibility of the structure. The calculations of the thermal parameters were carried out using the software Origin provided by the MicroCal Inc.

ACKNOWLEDGMENTS

We wish to express our thanks to Professor Saburo Aimoto and Dr. Toru Kawakami of the Institute for

Protein Research, Osaka University for allowing us the use of the peptide synthesizer and mass spectrometer. We also thank Dr. Toshiki Tanaka of the Biomolecular Engineering Research Institute for valuable suggestions about the purification of the trimeric peptides and Ms. Matsumi Doe of the Analytical Laboratory, Faculty of Science, Osaka City University for amino acid analyses. This work was supported in part by Grants-in-Aid for Scientific Research No. 08780833 from the Ministry of Education, Science and Culture.

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(*Received in Japan* 3 *July* 1997; *accepted* 15 *August* 1997)