Journal of Peptide Science
J. Peptide Sci. **8**: 172–180 (2002)
DOI: 10.1002/psc.381

Synthesis of a Membrane Protein with Two Transmembrane Regions

TAKESHI SATO,[a] TORU KAWAKAMI,[a] KENICHI AKAJI,[a] HIROKI KONISHI,[b] KOJI MOCHIZUKI,[b] TOSHIMICHI FUJIWARA,[a] HIDEO AKUTSU[a] and SABURO AIMOTO[a]*

[a] Institute for Protein Research, Osaka University, Osaka 565-0871, Japan
[b] Faculty of Engineering, Yokohama National University, Kanagawa 240-8501, Japan

Received 6 November 2001
Accepted 3 January 2002

Abstract: A membrane protein with two transmembrane domains was synthesized by means of the thioester method. The F_1F_0 ATP synthase subunit c (Sub.c), which consists of 79 amino acid residues (MW 8257), was chosen as a target. For synthetic purposes, two building blocks, Boc-[Lys^{34}(Boc)]-Sub.c(1-38)-SCH_2CH_2CO-Ala and Sub.c(39-79), were synthesized via solid-phase methods using Boc chemistry. RP-HPLC purification conditions for the transmembrane peptide were examined. As a result, a combination of a mixture of formic acid, 1-propanol and water with a phenyl column was found to be useful for separating the transmembrane peptide. The purified building blocks were condensed in DMSO in the presence of silver chloride, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt), *N*,*N*-diisopropylethylamine to give the product, Sub.c, after removal of Boc groups (yield 16%). The yield of the condensation reaction could be improved to 23% by raising the reaction temperature to 50 °C, and to 26% when a mixture of chloroform and methanol was used as a solvent.

Keywords: membrane protein; thioesters; F_1F_0 ATP synthase subunit c; RP-HPLC

INTRODUCTION

About 30% of the human genome encodes membrane proteins [1], but much of the information concerning the structure and function of these membrane proteins remains to be uncovered. The study of membrane proteins is hampered by difficulties associated with biochemical sample preparation. As an alternative approach to obtaining membrane proteins, chemical synthesis represents a viable candidate, and such chemically synthesized peptides have been utilized in the field of membrane protein research. However, these peptides only have a single transmembrane domain. Membrane proteins with multiple transmembrane domains must be synthesized, for chemical synthesis to be recognized as an acceptable technique for membrane protein study. A strategy that can be employed for the synthesis of a multiple transmembrane protein is the thioester method, which involves the synthesis of a polypeptide using *S*-alkyl peptide thioesters as building blocks, which are subsequently condensed in the presence of silver ion to give a longer, more complete polypeptide [2,3]. The purification of peptide having a transmembrane region(s), however, represents a challenge. RP-HPLC is widely used for the purification of chemically synthesized

Abbreviations: Boc, *t*-butoxycarbonyl; BocOSu, *N*-*t*-butoxycarbonyloxysuccinimide; Bzl, benzyl; Cl-Z, 2-chlorobenzyloxycarbonyl; *c*Hex, cyclohexyl; DCC, dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; HOBt, 1-hydroxybenzotriazole; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; $HOCH_2$-Pam, 4-(hydroxymethyl)phenylacetoamidomethyl; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; NMP, 1-methylpyrolidin-2-one; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

*Correspondence to: S. Aimoto, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871 Japan; e-mail: aimoto@protein.osaka-u.ac.jp
Contract/grant sponsor: Ministry of Education, Science, Sports and Culture, Japan; Contract/grant number: 10179103.

peptides, but difficulties are frequently encountered during the RP-HPLC purification of membrane peptides due to their insolubility in the mobile phases, and irreversible adsorption to the column. Suitable purification conditions must be found for preparing membrane peptide in high purity. In this paper a synthesis of a two-transmembrane protein by the thioester method is reported as an initial step in the synthesis of a multiple transmembrane protein. In addition to the synthesis, RP-HPLC purification of the transmembrane peptide was examined. As a target, F_1F_0 ATP synthase subunit c (Sub.c) was chosen. Sub.c is thought to consist of two membrane-spanning α-helices connected by a polar loop [4].

MATERIAL AND METHODS

General

Boc-Ala-OCH_2-Pam resin was purchased from Applied Biosystems, Inc. (Foster City, CA). Amino acid derivatives were purchased from the Peptide Institute Inc. (Osaka, Japan). Peptide chain elongation was carried out by using a peptide synthesizer 430 A (Applied Biosystems, Inc.) according to the protocol of the system software version 1.40 NMP/*t*-Boc on a 0.5 mmol scale with end capping by acetic anhydride. The first ten amino acids were coupled in a single coupling protocol and the remainder in a double coupling protocol. Ether, used for the precipitation and washing of peptides, was peroxide-free (Nacalai Tesque, Kyoto, Japan). DMSO, used for the condensation, was silylation grade (Pierce, Rockford, IL). The amino acid compositions of peptides were analysed using an L-8500 amino acid analyser (Hitachi Ltd., Tokyo, Japan) after hydrolysis with constant boiling point HCl for amino acid analysis (Nacalai Tesque) at 110 °C for 48 h in an evacuated sealed tube. The peptide molecular mass was determined by MALDI-TOF mass spectrometry using a Voyager™DE (PerSeptive Biosystems, Inc., Framingham, MA). The matrix used was sinapinic acid and peptides were dissolved in a mixture of formic acid and trifluoroethanol.

Preparation of Sub.c(39-79) (1)

Starting from the Boc-Ala-OCH_2-Pam resin (0.77 mmol/g, 0.65 g), a protected peptide resin corresponding to the sequence of Sub.c(39-79), Boc-Ala-Ala-Arg(Tos)-Gln-Pro-Asp(OcHex)-Leu-Ile-Pro-Leu-Leu-Arg(Tos)-Thr-Gln-Phe-Phe-Ile-Val-Met-Gly-Leu-Val-Asp(OcHex)-Ala-Ile-Pro-Met-Ile-Ala-Val-Gly-Leu-Gly-Leu-Tyr-Val-Met-Phe-Ala-Val-Ala-OCH_2-Pam resin was obtained (2.9 g). The protected peptide resin (300 mg) was treated with a mixture of anhydrous HF (8.5 ml), anisole (0.75 ml) and 1,4-butanedithiol (0.75 ml) by stirring at 0 °C for 90 min [5,6]. After evaporation of the HF under reduced pressure, ether was added to the mixture, and the resulting precipitate was washed with ether three times and then dissolved in TFA. The solution was passed through a glass filter and precipitated by the addition of cold ether. The precipitate was washed with ether, and mixed with 50% aqueous acetonitrile and freeze-dried to give the crude powder (189 mg).

Preparation of Sub.c(1-38)-SCH_2CH_2CO-Ala (2)

Starting with the Boc-Gly-SCH_2CH_2CO-Ala-OCH_2-Pam resin (0.71 g, 0.50 mmol) [7], 1.7 g of a protected peptide resin corresponding to the sequence of Sub.c(1-38), Boc-Met-Glu(OBzl)-Leu-Asn-Met-Asp(OcHex)-Leu-Leu-Tyr(Br-Z)-Met-Ala-Ala-Ala-Val-Met-Met-Gly-Leu-Ala-Ala-Ile-Gly-Ala-Ala-Ile-Gly-Ile-Gly-Ile-Leu-Gly-Gly-Lys(Cl-Z)-Phe-Leu-Glu(OBzl)-Gly-SCH_2CH_2CO-Ala-OCH_2-Pam resin, was obtained. This peptide resin (300 mg) was treated with a mixture of anhydrous HF (8.5 ml), anisole (0.75 ml) and 1.4-butanedithiol (0.75 ml) with stirring at 0 °C for 90 min. After evaporation of the HF under reduced pressure, ether was added to the mixture, the resulting precipitate was washed with ether three times and then dissolved in TFA. The solution was passed through a glass filter and precipitated by the addition of cold ether. After the precipitate had been washed with ether, it was mixed with 50% of aqueous acetonitrile and freeze-dried to give the crude powder (158 mg).

Solubility Check of a Crude Peptide

Several alcohols, acetonitrile and water were mixed in the ratio of 4:4:2. The crude powder of peptide **1** was added to the solvent (200 µl) at room temperature and allowed to stand for 2 days. The supernatant (50 µl) was removed, evaporated under reduced pressure, suspended in a mixture of acetonitrile and water, and freeze-dried. The amount of peptide was determined by amino acid analysis.

Evaluating RP-HPLC Condition

Analytical RP-HPLC was performed on Cosmosil columns (4.6 × 150 mm, 5C4AR-300, 5TMS, 5Ph

and 5PhAR-300, Nacalai Tesque). 1-Propanol for RP-HPLC was HPLC grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and formic acid was chromatography grade (Nacalai Tesque). The crude powder of peptide **1** (2.0 mg) was dissolved in TFA (50 µl), and the solution (5 µl) injected into the column. Peaks were collected and freeze-dried prior to the amino acid analysis. Each condition was evaluated by the isolated yield as determined by amino acid analysis.

Purification of Sub.c(39-79) and Sub.c(1-38)-SCH_2CH_2CO-Ala

Purification of these segments was performed on a Cosmosil 5PhAR-300 column (10 × 250 mm, Nacalai Tesque), and a linear gradient of formic acid/water (2 : 3) and formic acid/1-propanol (4 : 1) at a flow rate of 3.0 ml/min was employed.

For peptide **1**, 12 mg of crude powder was applied to the column to give purified peptide **1** (5.7 mg, 4.7 µmol, 15% yield based on the Ala content of the starting resin): MS (MALDI-TOF) found: m/z 4433.4. Calcd for $[M + H]^+$ 4433.4 (average). Amino acid analysis: $Asp_{2.1}Thr_{1.0}Glu_{2.2}Pro_{3.4}Gly_{3.4}Ala_6Val_{5.0}Met_{2.9}Ile_{4.5}Leu_{6.7}Tyr_{0.8}Phe_{2.9}Arg_{2.1}$.

For peptide **2**, 6 mg of crude powder was loaded on the column to give purified peptide **2** (1.7 mg, 0.36 µmol, 11% yield based on Gly on the resin): MS (MALDI-TOF) found: m/z 4001.3. Calcd for $[M + H]^+$ 4001.9 (average). Amino acid analysis: $Asp_{2.8}Glu_{1.9}Gly_{7.0}Ala_8Val_{1.0}Met_{4.6}Ile_{4.1}Leu_{6.1}Phe_{1.0}Lys_{1.0}$.

Preparation of Boc-(Lys^{34}(Boc))-Sub.c(1-38)-SCH_2CH_2CO-Ala (3)

Peptide **2** (8.0 mg, 1.7 µmol) and BocOSu (4.3 mg, 20 µmol) were dissolved in DMSO. DIEA was added to the solution followed by stirring for 1 h, and the reaction was monitored by RP-HPLC [Cosmosil 5PhAR300 (4.6× 150 mm, Nacalai Tesque), using a linear increasing gradient of 1–propanol–acetonitrile (1 : 1) in 0.1% aqueous TFA at a flow rate of 0.5 ml/min]. The DMSO was removed by evaporation under reduced pressure, and the reaction mixture was washed with ether. The precipitate was suspended in aqueous acetonitrile and freeze-dried to give partially protected peptide **3**, Boc-[Lys^{34}(Boc)]-sub.c(1-38)-SCH_2CH_2CO-Ala-OH (7.2 mg, 1.7 µmol).

Condensation by the Thioester Method

Peptide **1** (2.3 mg, 0.52 µmol), peptide **3** (2.2 mg, 0.52 µmol) and HOOBt (2.6 mg, 16 µmol) were dissolved in DMSO (400 µl). DIEA (1.8 µl, 10 µmol) and AgCl (0.3 mg, 2.1 µmol) were then added to the solution followed by stirring for 48 h. DMSO was removed under reduced pressure, and the residue was suspended in aqueous acetonitrile and freeze-dried. The reaction mixture was dissolved in TFA containing 5% water, stirred for 90 min, and then purified by RP-HPLC [Cosmosil 5PhAR-300 (4.6 × 150 mm, Nacalai Tesque), using a linear gradient of formic acid/1-propanol (4 : 1) over formic acid/water (2 : 3) at a flow rate of 0.65 ml/min] to give Sub.c (85 nmol) and the isolated yield based on the *C*-terminal building block was determined to be 16%: MS (MALDI-TOF) found: m/z 8256.1. Calcd for $[M + H]^+$ 8257.1 (average). Amino acid analysis: $Asp_{4.9}Thr_{0.8}Glu_{3.9}Pro_{2.4}Gly_{10.2}Ala_{13}Val_{5.1}Met_{7.2}Ile_{7.5}Leu_{12.1}Tyr_{1.4}Phe_{4.0}Arg_{1.7}$.

CD Spectroscopy

Sub.c (0.1 mg) was dissolved in mixtures of TFE–water in different concentrations of TFE. The concentration of Sub.c was determined to be 12 µM by an amino acid analysis. The CD spectrum of the synthesized Sub.c in TFE was recorded on a JASCO J-720 WI (Japan Spectroscopic Co. Ltd). A 1 mm sample cell was used for spectral studies and each measurement was repeated four times at room temperature. For the final ellipticity, the spectrum was corrected by subtracting the reference spectrum.

RESULTS AND DISCUSSION

The amino acid sequence of Sub.c is shown in Figure 1, in which an arrow indicates the site of the segment condensation. In order to make a length of the building block almost equal, the coupling site was chosen in the loop region (between the transmembrane domains). A glycine residue was chosen as the *C*-terminal amino acid residue of the *N*-terminal building block to avoid epimerization. The synthetic route is summarized in Scheme 1.

Solubility Check

Since RP-HPLC is one of the simplest analytical and preparative methods, we were interested in applying it to the purification of the transmembrane

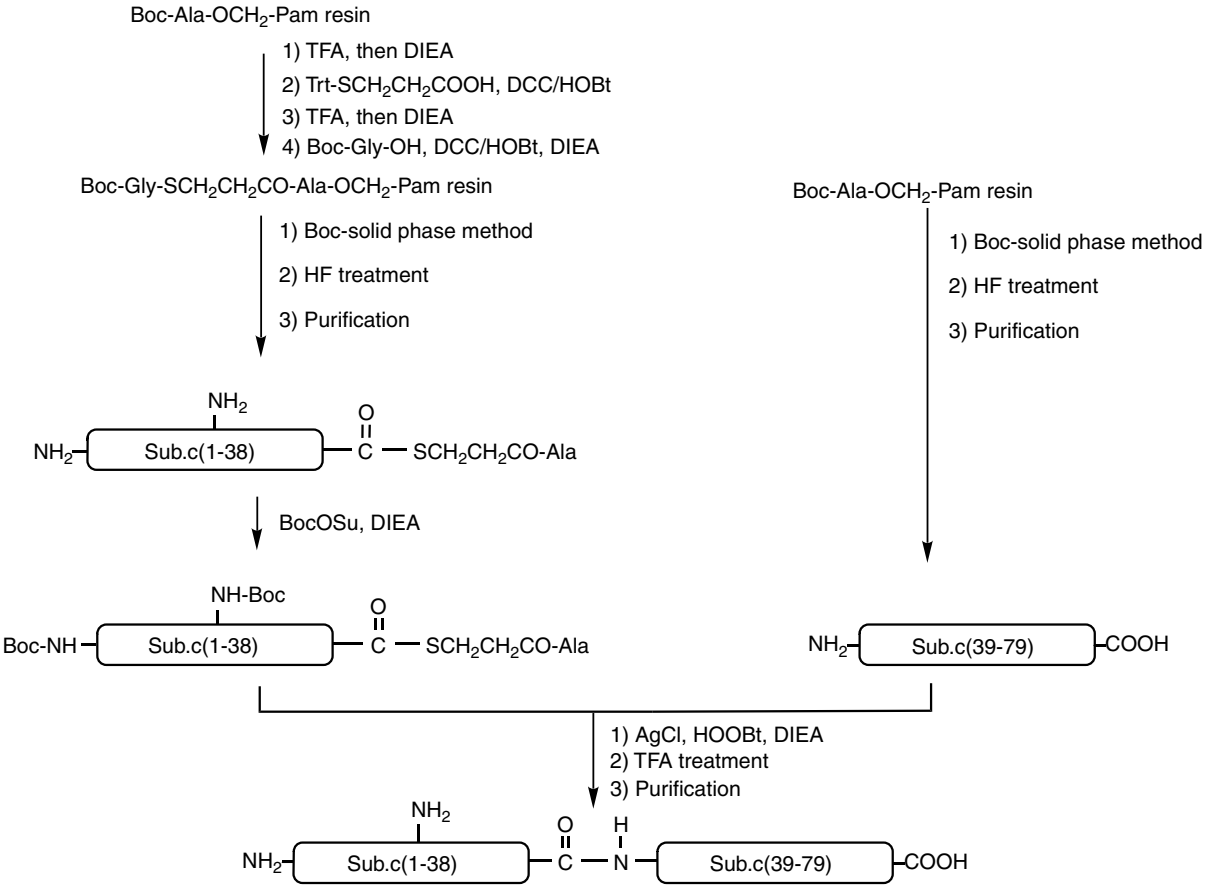
Met-Glu-Asn-Leu-Asn-Met-Asp-Leu-Leu-Tyr-^{10}
Met-Ala-Ala-Ala-Val-Met-Met-Gly-Leu-Ala-^{20}
Ala-Ile-Gly-Ala-Ala-Ile-Gly-Ile-Gly-Ile-^{30}
Leu-Gly-Gly-Lys-Phe-Leu-Glu-Gly↓Ala-Ala-^{40}
Arg-Gln-Pro-Asp-Leu-Ile-Pro-Leu-Leu-Arg-^{50}
Thr-Gln-Phe-Phe-Ile-Val-Met-Gly-Leu-Val-^{60}
Asp-Ala-Ile-Pro-Met-Ile-Ala-Val-Gly-Leu-^{70}
Gly-Leu-Tyr-Val-Met-Phe-Ala-Val-Ala^{79}

Figure 1 Amino acid sequence of F_1F_0 ATP synthase subunit c. The arrow indicates the coupling site.

peptides. A transmembrane peptide, Sub.c(39-79) (**1**), however, could not be purified by RP-HPLC using an ODS column with 0.1% TFA in aqueous acetonitrile. Combinations of C4 or C1 columns with mixtures of 2-propanol as eluents, which are often used for hydrophobic peptides, were not effective, either. Therefore, in a search for a RP-HPLC purification system, especially an eluent system, of the transmembrane peptide, our first objective was to prepare a molecularly dispersed solution of the crude transmembrane peptide with no detergent being present. A number of alcohols, including 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-2-propanol, 1-pentanol and 1-hexanol were tested. Each alcohol was mixed with acetonitrile and water (alcohol : acetonitrile : water = 2 : 2 : 1) and the crude powdered Sub.c(39-79) was added to the solution. The mixture was kept at room temperature and the amount of peptide component in the supernatant was determined by amino acid analysis. In Table 1, the amount of Gly in the supernatant of each mixture is shown. 1-propanol was found to have a relatively higher ability to dissolve the crude powder of Sub.c(39-79).

The Condition of RP-HPLC

Based on data from a solubility check, a mixture containing 1-propanol was tested for its ability as



Scheme 1 Synthetic route for the preparation of Sub.c(1-79).

Table 1 Comparison of Alcohols for Solubilizing the Crude Powder Containing Sub.c(39-79)

Alcohol	Gly contained in supernatant (mmol/ml)
1-propanol	0.098
2-propanol	0.093
1-butanol	0.093
2-butanol	0.080
2-methyl-2-propanol	0.043
1-pentanol	0.054
1-hexanol	0.062

Each alcohol was mixed with acetonitrile and water (alcohol : acetonitrile : water = 2 : 2 : 1) and Sub.c(39-79), in the form of crude powder, was added to the solution. The mixture was kept at room temperature and the amount of peptide component in the supernatant was determined by amino acid analysis. The amount of Gly in the supernatant of each mixture is shown.

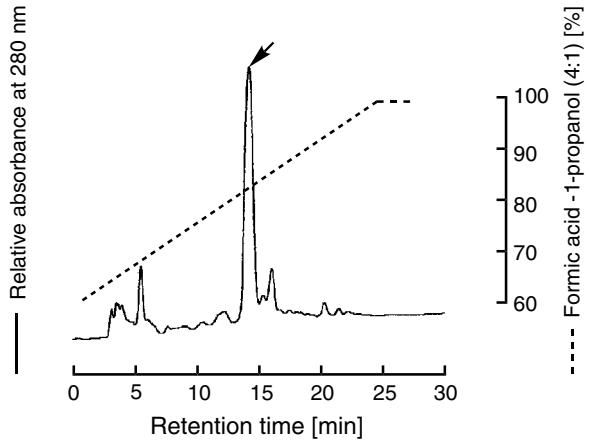


Figure 2 RP-HPLC profile of peptide **1**. Column, Cosmosil 5Ph AR-300, 4.6× 150 mm; linear gradient (25 min) from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1). The desired peptide component was characterized by a retention time of 14 min (indicated by the arrow).

an eluent of RP-HPLC. Since the use of formic acid in an eluent system is also known to be effective for the purification of hydrophobic peptides [8,9], we examined eluents containing formic acid. Ph and C4 columns, which are frequently

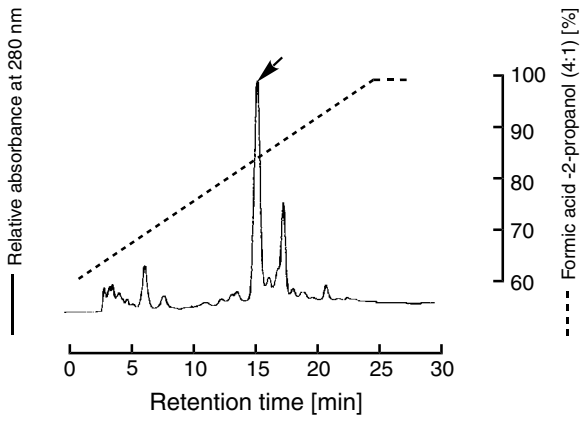


Figure 3 RP-HPLC profile of peptide **1**. Column, Cosmosil 5Ph AR-300, 4.6× 150 mm; linear gradient (25 min) from formic acid–water (2 : 3) to formic acid–2-propanol (4 : 1). The desired peptide component was characterized by a retention time of 15 min (indicated by the arrow).

employed for the separation of hydrophobic peptides, were employed for the evaluation of the eluents. Superior resolution and recovery was obtained using the Ph column, and, therefore, the elution profiles shown in this paper are limited to that of the Ph column. A relatively good resolution and recovery was obtained by employing a linear gradient of formic acid–water (2 : 3) and formic acid–1-propanol (4 : 1) as an eluent system with the Ph column (Figure 2). The desired peptide component was characterized by a retention time of 14 min. For comparison, we examined a linear gradient of formic acid–water (2 : 3) and formic acid–2-propanol (4 : 1) as an eluent system (Figure 3), which was employed by Bollhagen *et al.* for the purification of a transmembrane peptide [6]. No significant difference was observed for the resolution, but the retention time of the desired peptide was shifted back compared with the system described above. A linear gradient of 0.1% TFA in water and 0.1% TFA in 1-propanol–acetonitrile (1 : 1) also showed good separation (Figure 4), which is applicable to the purification of hydrophobic peptides or peptides with protecting groups that are cleavable by acids.

Synthesis of Building Blocks

The peptide resin corresponding to Sub.c(39-79) was synthesized using an automated synthesizer from a Boc-Ala-OCH_2-Pam resin. A thioester resin, Boc-Gly-SCH_2CH_2CO-Ala-OCH_2-Pam resin, was manually synthesized [5], and the peptide chain, corresponding to Sub.c(1-38), was also elongated by

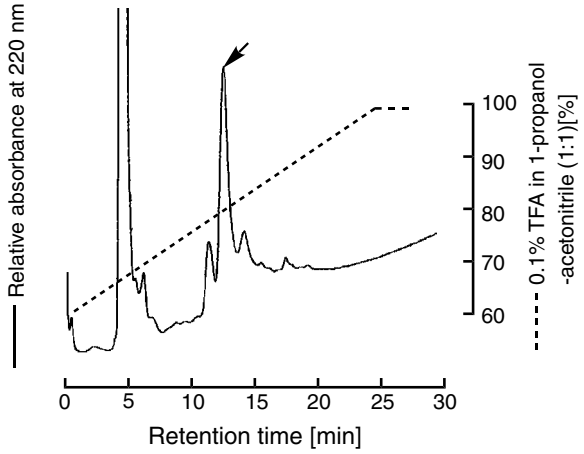


Figure 4 RP-HPLC profile of peptide **1.** Column, Cosmosil 5Ph AR-300, 150×4.6 mm; linear gradient (25 min) from 0.1% TFA water (2 : 3) to 0.1% TFA 1-propanol–acetonitrile (1 : 1). The desired peptide component was characterized by a retention time of 13 min (indicated by the arrow).

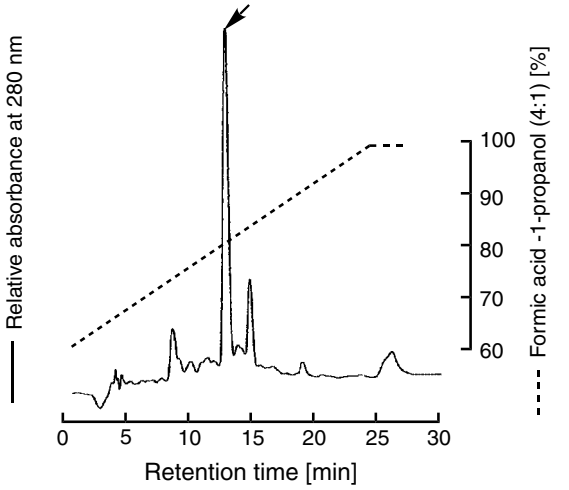


Figure 5 RP-HPLC profile of peptide **1**. Column, Cosmosil 5Ph AR-300, 10× 200 mm; linear gradient (25 min) from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1). The desired peptide component was characterized by a retention time of 13 min (indicated by the arrow).

means of an automated peptide synthesizer via a solid-phase method using Boc chemistry. Peptides were cleaved from the resins by treatment with HF, and purified by RP-HPLC on Cosmosil 5Ph AR-300 (10 × 250 mm) with a linear gradient of formic acid–water (2 : 3) and formic acid–1-propanol (4 : 1). The semi-preparative RP-HPLC profile of peptide **1** is almost same as that of an analytical sample (Figure 5). The major peak at 14 min contained the

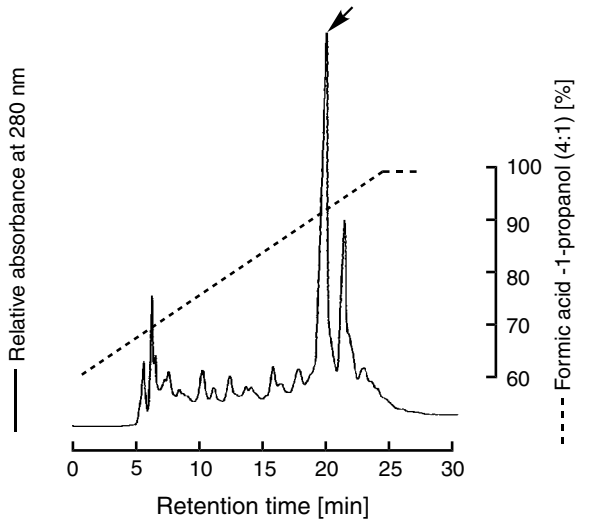


Figure 6 RP-HPLC profile of peptide **3**. Column, Cosmosil 5Ph AR-300, 10× 200 mm; linear gradient (25 min) from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1). The desired peptide component was characterized by a retention time of 19 min (indicated by the arrow).

desired peptide **1**, and the mass number and the amino acid composition were in good agreement with the calculated values. Peptide **1** was obtained in a yield of 15% based on the Ala residue on the resin. The mass number of the content in the 16 min peak was larger than that of peptide **1** by 98 Da, suggesting that it had been trifluoroacetylated, as judged from the mass number. This peak became higher as the time for dissolving the crude peptide in TFA increased. This side reaction could be avoided by shortening the time for dissolving the peptide in TFA. Figure 6 shows the RP-HPLC profile of the peptide, Sub.c(1-38)-SCH_2CH_2CO-Ala (**2**). The peak at 19 min contained peptide **2**, and the mass number and amino acid composition were in good agreement with the calculated values. The yield was 11% based on the Gly residue on the resin. Peptide **2** was treated with BocOSu in DMSO to give the partially protected peptide, Boc-[Lys^{34}(Boc)]-sub.c(1-38)-SCH_2CH_2CO-Ala (**3**). To monitor the reaction, RP-HPLC, using 1-propanol and acetonitrile as the eluent was employed instead of the system using formic acid, since the Boc group is removed under acidic conditions.

Condensation by the Thioester Method

Condensation of peptides **1** and **3** was carried out in the presence of silver chloride, HOOBt and DIEA in DMSO. The reaction mixture was stirred for

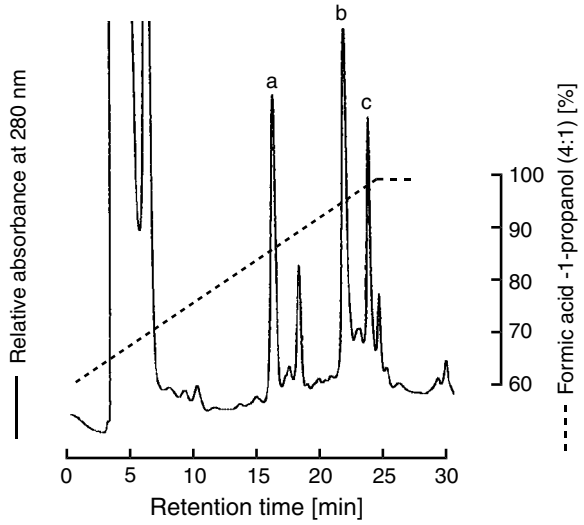


Figure 7 RP-HPLC profile of the TFA treated reaction mixture. Condensation by the thioester method was carried out in DMSO at room temperature for 48 h. Column, Cosmosil 5Ph AR-300, 4.6×150 mm; linear gradient (25 min) from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1). Peaks **a**, **b** and **c** were found to be the *C*-terminal building block, the thioester hydrolysed *N*-terminal building block, and the desired product, respectively.

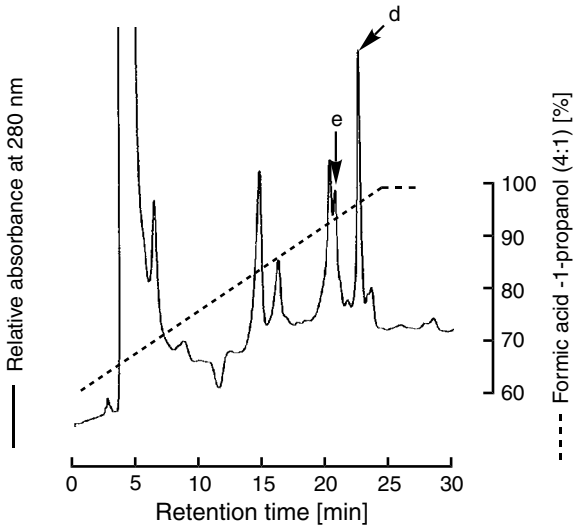


Figure 8 RP-HPLC profile of the TFA-treated reaction mixture. Condensation by the thioester method was carried out in chloroform–methanol (2 : 1) for 48 h at room temperature. Column, Cosmosil 5Ph AR-300, 4.6 × 150 mm; linear gradient (25 min) from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1). The content of peak **d** was found to be the desired product. The content of peak **e** had a larger mass number by 14 units than expected for the thioester hydrolysed *N*-terminal building block.

48 h at room temperature, and then treated with TFA to remove Boc groups. The RP-HPLC profile of the TFA-treated reaction mixture is shown in Figure 7. Three main sharp peaks at (**a**) 16 min, (**b**) 21 min and (**c**) 23 min were collected and checked by mass analysis. The mass number of the material corresponding to peak **c** was in good agreement with the calculated value of the desired product. The amino acid composition was also in good agreement with the calculated value. The yield of the condensation was 16% based on the *C*-terminal building block. The condensation reaction was also performed at 50 °C and the yield of the reaction was improved to 23%. A mixture of chloroform and methanol (2 : 1), which is typically used for sample preparation of membrane proteins, was also examined as a solvent for the condensation. The RP-HPLC profile of the TFA-treated reaction mixture is shown in Figure 8. The mass number and the amino acid composition of the content of peak **d** was in good agreement with the calculated values of the desired product. The yield was found to be 26% based on the *C*-terminal building block. The content of peak **e** had a larger mass number by 14 units than would have been expected for the thioester hydrolysed *N*-terminal building block.

This side reaction can be explained by formation of a methyl ester due to the methanol in the solvent. Considering that the thioester is a type of active ester and methanol, a primary alcohol, has a high reactivity for the esterification of carboxyl acid, this side reaction is unavoidable in this solvent system. Therefore, 2-propanol, instead of methanol, was examined as a component of the solvent mixture. Esterification could be avoided by using this secondary alcohol, but the yield of the condensation reaction decreased to 14% (Figure 9). Other secondary or tertiary alcohols were not tested, the data from the solubility experiment indicated that the ability of these alcohols to dissolve the transmembrane peptide was low. In view of their high solubility, HFIP [10] and phenol [11] were also examined. Using HFIP, however, the condensation reaction, for unknown reasons, failed to proceed, and phenol did not dissolve the reactants.

Structural Analysis of Synthesized Sub.c

To verify whether the synthesized Sub.c folds into the structure mentioned above, we performed structural analysis by CD spectroscopy. The CD spectra of Sub.c in TFE–water mixture from 100%

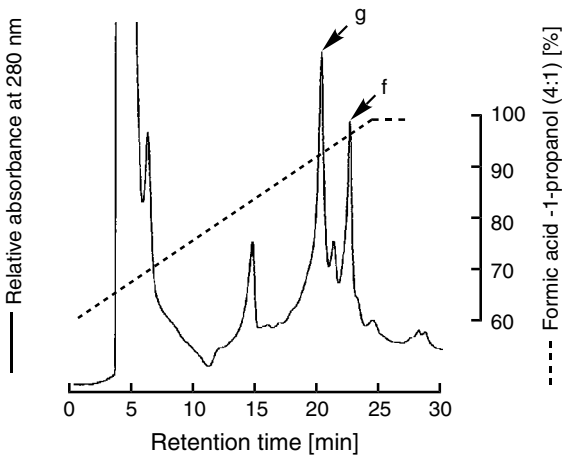


Figure 9 RP-HPLC profile of TFA-treated reaction mixture. Condensation by the thioester method was carried out in chloroform–2-propanol (2 : 1) for 48 h at room temperature. Column, Cosmosil 5Ph AR-300, 4.6 × 150 mm; linear gradient (25 min) from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1). The content of peaks **f** and **g** were the desired product and the thioester hydrolysed *N*-terminal building block, respectively. The esterification mentioned in the text was avoided in this system.

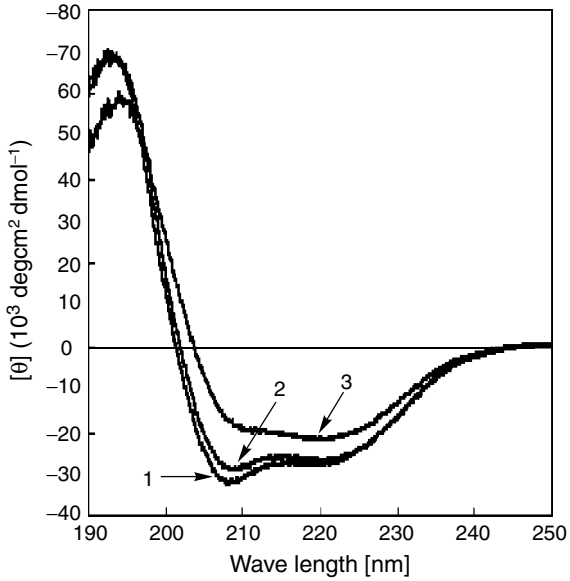


Figure 10 CD spectra of synthesized Sub.c in TFE–water mixtures of different TFE concentrations. The spectra 1, 2 and 3 represent CD spectra of Sub.c in 100% TFE, 40% TFE and 30% TFE respectively. The concentration of Sub.c in each solution was determined to be 12 μM by amino acid analysis.

TFE to 10% TFE (100%, 80%, 60%, 50%, 40%, 30%, 20% and 10% TFE were used as solvents) were obtained. Sub.c was found to fold into an α-helical structure in the TFE–water mixture. However, the titration with water produced changes in the shapes of the spectra. There were two patterns of shapes, one with a range of TFE from 100% to 40%, and the other from 30% to 10%. The spectra of Sub.c in 100%, 40% and 30% TFE are shown in Figure 10. The result implies some structural change in Sub.c has occurred in the range from 40% to 30% TFE. Further structural analysis using synthesized peptides containing each transmembrane region of Sub.c is now in progress.

CONCLUSION

The main goal of this research was to synthesize a membrane protein, with two transmembrane regions, by means of the thioester method. As a result, Sub.c was successfully synthesized. Also, an effective condition for separation of a transmembrane peptide by RP-HPLC was found. Since a number of membrane proteins are thought to contain multiple transmembrane regions, the results described here provide a contribution to research on membrane proteins.

Acknowledgements

This research was supported, in part, by a Grant-in-Aid for Scientific Research on Priority Areas No. 10179103 from the Ministry of Education, Science, Sports and Culture, Japan.

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