Synthesis and Properties of Two Simplified Peptides of PA1-14, the N-Terminal 1-14 Residues of Adrenodoxin Precursor

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(Received December 18, 1992)

Synopsis. Two simplified peptides of PA1-14, which corresponds to the N-terminal 1-14 residues of adrenodoxin precursor, were synthesized and examined by circular dichroism and dye leakage measurements. Both peptides showed a tendency to form α-helices and exhibited moderate dye leakage.

Most mitochondrial proteins are synthesized in the cytoplasm as large precursors with an extension peptide at the N-terminal and then imported into mitochondria. Extension peptides are removed proteolytically during and upon completion of import. They carry the essential information for translocation of the precursors into or across the membrane.[1,2] We previously reported that PA1-14, which corresponds to the N-terminal 1-14 residues of adrenodoxin precursor, showed import inhibition of the precursor into mitochondria.[3] This means that the N-terminal portion of the precursor plays an important role in the import of the precursor into mitochondria. PA1-14 has a rather simple structure, as shown in Fig. 1. Therefore, it would be interesting to investigate whether more simplified peptides also have inhibitory ability.

In the present study, we synthesized two simplified peptides of PA1-14 and compared their properties to those of PA1-14 using CD,[4] membrane perturbation measurements, and antibacterial activity.

Results and Discussion

It is known that extension peptides have high hydrophobic moments and their amphiphilic helicity is important for the import of precursors into mitochondria.[5,6] According to the method of Eisenberg,[7] the hydrophobicity <H> and hydrophobic moment <μH> values of PA1-14 were calculated to be 0.23 and 0.48, respectively.[8] We therefore designed the two peptides (**1** and **2**) shown in Fig. 1 to be similar to PA1-14. Peptide **1** contains three repeated units of Ala-Leu-X (X=Arg, Ser, or Ala) in order to analyze its conformation easily. The chain length, <H> (0.21), and <μH> (0.53) values of this peptide are similar to those of PA1-14, although its sequence is different from that of PA1-14. In peptide **2**, Met at position 1 and Val at position 8 of PA1-14 were replaced by Leu and Ala and its <H> and <μH> values are 0.23 and 0.49, respectively. Two Arg and one Ser residues remain unaltered, because they are thought to be important for inhibitory ability.

The protected derivative of **1** was prepared by the usual solution method and then treated with HF containing anisole. Peptide **2** was synthesized in the same way as the preparation of PA1-14.[8] The crude products, **1** and **2**, were purified by gel filtration with Sephadex G-15 using 30% AcOH and then lyophilized. The purity of the final products was confirmed by HPLC (data not shown). Their elemental and amino acid analyses gave reasonable results.

CD measurements were carried out in Tris–HCl buffer (pH 7.4) and in Tris–HCl buffer (pH 7.4) containing DPPC or DPPC-DPPG (3:1) liposomes at 25 °C. In Tris–HCl buffer, all peptides were found to have a random conformation (data not shown). In Tris–HCl buffer containing DPPC liposomes, PA1-14 and **1** were still random, but the CD curve of **2** showed a small amount of α-helix (Fig. 2a). In Tris–HCl buffer containing DPPC-DPPG (3:1) liposomes, **2** showed an α-helical pattern similar to but shallower than that of PA1-14. Peptide **1** also seemed to contain some α-helix, although its CD curve was somewhat different from that of PA1-14 (Fig. 2b). The helical contents of PA1-14, **1**, and **2** in the presence of DPPC-DPPG (3:1) liposomes were calculated to be 51, 23, and 37%, respectively, using the equation of Holzwarth and Doty, i.e., helical content $(H)=[\theta]_{222}/40000$.[9] Analytical studies on several other extension peptides have shown that these peptides become more α-helical in the presence of phospholipids or SDS;[10,11] this is consistent with the present observation. On the other hand, there are some different findings that α-helical amphipathy is not always essential.[12] This, together with the above results, suggests that α-helicity is not indispensable, but is, rather, one of the favorable factors for the import of precursors into mitochondria.[13]

Carboxyfluorescein leakage measurements were conducted in order to gain more insight on the influence of peptides on lipid bilayers, because mild membrane perturbation might be one of the requirements for the import of precursors. The results are shown in Fig. 3. The measurements were carried out at 25 and 42 °C (near the phase transition temperature) and data were recorded at 5 min after the addition of peptides into the mixture. In HEPES buffer (pH 7.4) containing

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1                5                10               14
Met-Ala-Ala-Arg-Leu-Leu-Arg-Val-Ala-Ser-Ala-Ala-Leu-Gly   (PA1-14)
Ala-Ala-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Ser-Ala-Leu-Ala       (1)
Leu-Ala-Ala-Arg-Leu-Leu-Arg-Ala-Ala-Ser-Ala-Ala-Leu-Gly   (2)
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Fig. 1. Amino acid sequences of PA1-14 and simplified peptides.

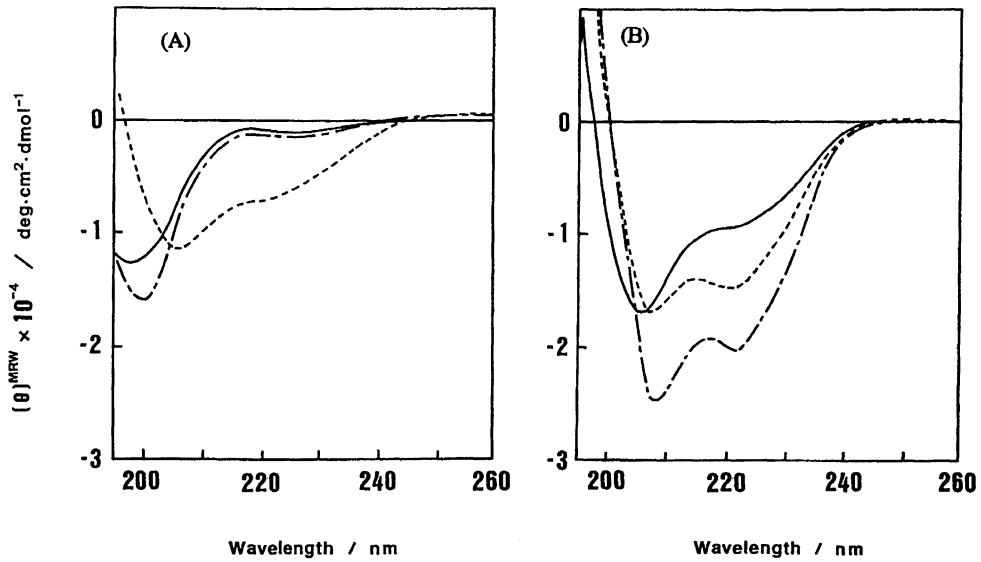

Fig. 2. CD spectra of peptides in Tris–HCl buffer (pH 7.4) containing DPPC liposomes (A) and Tris-HCl buffer containing DPPC–DPPG (3:1) liposomes (B). (A): PA1-14 (–·–), **1** (—), **2** (---); [peptide]=ca. 10^{-5} M. (B): PA1-14 (–·–), **1** (—), **2** (---); [peptide]=ca. 10^{-5} M, [lipid]=ca. 10^{-3} M.

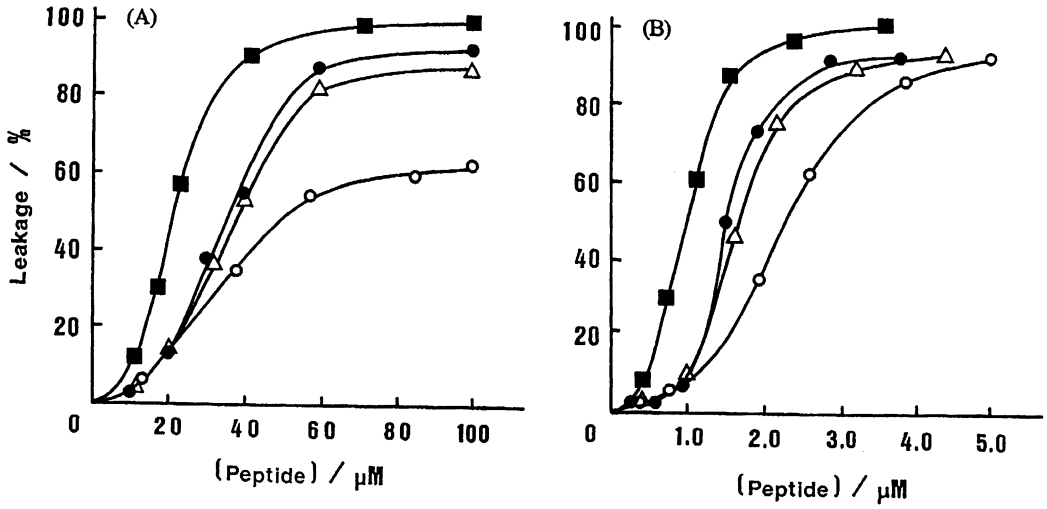

Fig. 3. Peptide-mediated dye leakage from DPPC–DPPG (3:1) vesicles in HEPES buffer (pH 7.4) at 25 °C (A) and 42 °C (B). Excitation wavelength, λ_{ex} 470 nm; emission wavelength, λ_{em} 515 nm. (A): PA1-14 (○), **1** (●), **2** (△), [Leu^1] PA1-14 (■); [lipid]=99.2 μM. (B): PA1-14 (○), **1** (●), **2** (△), [Leu^1] PA1-14 (■); [lipid]=13.5 μM.

DPPC–DPPG (3:1) vesicles, all peptides showed moderate dye leakage and the degree of dye leakage gradually increased with an increase in peptide concentration. The membrane perturbation ability of **1** and **2** was stronger than that of PA1-14 in both cases at 25 and 42 °C (Figs. 3a and 3b, respectively). Recently, we found that increasing the hydrophobicity of PA1-14 caused strong perturbation of mitochondrial membranes, indicating that such peptides, e.g., [Leu^1] PA1-14 are not adequate as an inhibitor.[14] The <H> value of [Leu^1] PA1-14 is 0.26. This peptide showed a stronger perturbation ability than **1** or **2**.

As described previously PA1-14 [30 μM (1 M=1 mol dm^{-3})] inhibited the import of adrenodoxin precursor into bovine adrenocortex mitochondria.[3] In the present study, preliminary inhibitory assays showed that **1** and **2** also possessed this inhibition ability at the same concentration. Peptides **1** and **2**, as well as PA1-14,[13] exhibited no antibacterial activity against Gram-positive bacteria (12 organisms) or Gram-negative bacteria (15 organisms) even at a peptide concentration of 100 μg ml^{-1}. This result indicated that the perturbation ability of **1** and **2** is not strong enough to destroy bacterial membranes. On the other hand, [Leu^1] PA1-14 showed some antibacterial activity against Gram-positive bacteria.[14]

We have reported that the mild membrane perturbation caused by peptides may be necessary for the import of precursors into mitochondria.[8] Peptides **1** and **2** showed import inhibition and antibacterial activity similar to that of PA1-14, although their CD patterns and dye leakage abilities were somewhat different from those of PA1-14. In this connection, it is noteworthy that analogous properties were previously observed for [Leu^1, Ser^4] PA1-14.[14] Furthermore, the effect of [Leu^1, Ser^4] PA1-14 on the mitochondrial membrane potential was much closer to PA1-14 than was [Leu^1] PA1-14 in a respiratory assay, which has been utilized as an indication of the stability of mitochondrial membranes. These results suggest that simple peptides with <H> and <μH> values similar to those of PA1-14 can become candidates for the inhibition of the import of precursors into mitochondria. However, a respiratory assay for **1** and **2** (now in progress) will be necessary to make an exact evaluation.

Experimental

DPPC and DPPG were purchased from Sigma Chemical. 5(6)-Carboxyfluorescein from Eastman Kodak was purified twice by recrystallization from EtOH. Boc-amino acids were obtained from the Peptide Institute. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer after hydrolysis in 6 M HCl in sealed tubes at 110 °C for 24 h.

H-Ala-Ala-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Ser-Ala-Leu-Ala-OH (1). Boc-Ala-Ala-Leu-Arg(Tos)-Ala-Leu-Arg(Tos)-Ala-Leu-Ser(Bzl)-Ala-Leu-Ala-OBzl (302 mg, 0.16 mmol), prepared by the solution method, was treated with HF (19 ml) containing anisole (1 ml) at 0 °C for 1 h. After evaporation of the HF at 0 °C, the residue was washed with ether. The crude product was dissolved in a small amount of 30% AcOH and passed through a Sephadex G-15 column (2.0×108 cm) using 30% AcOH. The fraction containing product was lyophilized to give a white fluffy powder; yield, 179 mg. Purity was confirmed by HPLC. Amino acid ratio in acid hydrolysate: Ser 0.88 (1), Ala 6.00 (6), Leu 3.89 (4), Arg 1.88 (2). Found: C, 48.91; H, 7.78; N, 17.51%. Calcd for $C_{57}H_{105}O_{15}N_{19}\cdot 3CH_3CO_2H\cdot 7/2H_2O$: C, 49.14; H, 8.12; N, 17.28%.

H-Leu-Ala-Ala-Arg-Leu-Leu-Arg-Ala-Ala-Ser-Ala-Ala-Leu-Gly-OH (2). Boc-Leu-Ala-Ala-Arg(Tos)-Leu-Leu-Arg(Tos)-Ala-Ala-Ser(Bzl)-Ala-Ala-Leu-Gly-OBzl (291mg, 0.15 mmol), prepared by the solution method, was treated as described above; yield, 132 mg. Amino acid ratio of acid hydrolysate: Ser 0.93 (1), Gly 1.03 (1), Ala 6.00 (6), Leu 4.12 (4), Arg 1.89 (2). Found: C, 45.56; H, 7.71; N, 16.55%. Calcd for $C_{59}H_{108}O_{16}N_{20}\cdot 3CH_3CO_2H\cdot 4H_2O$: C, 45.87; H, 7.58; N, 16.46%.

CD Measurements. CD measurements were made on a JASCO J-600 spectropolarimeter using a cell of 1 mm path length as described previously.[13] Spectra in 20 mM Tris–HCl buffer (pH 7.4) were measured at a peptide concentration of 100 μM. DPPC–DPPG (3:1) vesicles (a mixture of uni- and multi-lameller vesicles) were prepared by sonication at 50 °C for 30 min in 20 mM Tris–HCl buffer (pH 7.4). The peptides were dissolved at a concentration of 10 μM in 20 mM Tris–HCl buffer containing 0.9 mM DPPC–DPPG (3:1) versicles. All the measurements were performed at 25 °C. CD spectra are expressed as mean residue ellipticities as shown in Fig. 2.

Carboxyfluorecein Leakage.[15] To 2 ml of 0.1 M NaCl/5 mM HEPES buffer (pH 7.4) in a cuvette was added 50 μl of the vesicles containing 100 mM carboxyfluorescein to give a final concentration of 10—100 μM lipid. The cuvette was placed in the heated (25 or 42 °C) cuvette holder of the fluorometer. After the cuvette was kept at the temperature for 3 min, to the mixture was added 20 μl of an appropriate dilution of the peptide in HEPES buffer. The fluorescence intensity was recorded at 5 min after the addition of the peptide. To determine the fluorescence intensity derived from 100% dye release, 10 μl of Triton X-100 solution (20% in HEPES buffer) was added to dissolve the vesicles. The percentage of dye leakage caused by the peptide was evaluated by the equation, $100(F-F_0)/(F_t-F_0)$, where F is the fluorescence intensity achieved by the peptide, and F_0 and F_t are the intensities without the peptide and with Triton X-100 treatment, respectively. The results are shown in Fig. 3.

Antibacterial Assay. The antibacterial assay was done by the dilution method using Mueller Hinton agar (Difco) medium as described previouly.[13]

We thank Drs. Hiroshi Nishikawa and Setsuko Ando, Fukuoka University, for the CD measurements, Dr. Shin-ichi Sato, Research Laboratories, Yoshitomi Pharmaceutical Ind., Ltd., for the antibacterial assay, and Dr. Shigeki Furuya, Kyushu University, for the import-inhibitory assay.

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4) Abbreviations: AcOH, acetic acid; CD, circular dichroism; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPPG, dipalmitoyl-DL-α-phosphatidylglycerol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate. Amino acid symbols denote L-configuration except glycine.

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