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A Hybrid of Amphiphilic α–Helical Peptides and *meso*-Tetra(α,α,α,α-*o*-carboxyphenyl)-porphyrin. Membrane-Penetrating Porphyrin-4α-Helix Artificial Protein

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Four amphiphilic α-helical peptides were hybridized with α,α,α,α-isomer of *meso*-tetra(*o*-carboxyphenyl)porphyrin to synthesize a picket-fence-type 4α-helix bundle polypeptide. The peptide sequence was designed so that the hybrid polypeptide could penetrate into phospholipid bilayers. Circular dichroism spectra showed that the hybrid folds in a complete four α-helix bundle structure both in methanol and dipalmitoylphosphatidylcholine vesicles.

Strategies for *de novo* design, the construction of three-dimensional structures of polypeptides as artificial proteins have been variously demonstrated.[1)] Especially, four α-helix bundle structures have been focused on modeling by the use of the amphiphilic α-helix motif.[2)] On the other hand, some groups have been trying to embed an atropisomeric porphyrin moiety into membrane environment by various modifications involving the long chain acylation.[3)] The long alkyl chains, however, are too slim to fill a porphyrin plane. On the contrary, the thick α-helical peptide segments in a bundle can occupy the surface of porphyrin ring as shown in Fig. 1A.

Thus, we attempted to design a membrane penetrating polypeptide-porphyrin hybrid **1** (Fig. 1) which is expected to embed stably in the lipid bilayers. For this purpose, we newly developed α,α,α,α-atropisomer of *meso*-tetra(*o*-carboxyphenyl)porphyrin (**3**)[4)] (Fig. 1C) as a template with four definitely oriented anchoring points for peptide segments. The amphiphilic α-helix motif is also employed for the design of 22-peptide (Fig. 1B). Since the bundle must be soluble in the hydrocarbon circumstances, we used a number of Leu residues (four in seven, 4/7) in combination with polar Glu (1/7) and neutral Ala (2/7) residues so that the occupation of hydrophobic circumference reaches about 200 degrees (Fig. 1B). The heptapeptide was repeated three-times to be as long as 30 Å in α-helical conformation. This length is adjusted to the thickness of the hydrocarbon region of the lipid bilayers (30-40 Å). As a reference, we also prepared a starfish-shaped hybrid **2** of the peptides with *meso*-tetra(*p*-carboxyphenyl)porphyrin (**4**) which is free from atropisomerism.

The hybrid protein **1** and **2** were synthesized by the condensation of the protected 21-peptide with porphyrin-Ala derivatives of *o*- and *p*-isomers, respectively. Boc-Ala-Leu-Leu-Glu(OBzl)-Leu-Leu-Ala-OH **5** and its methyl amide **6** were synthesized by the stepwise coupling on a *p*-nitrobenzophenone oxime resin according to the reported procedure.[5)] These peptides were purified by silica gel chromatography and re-precipitation. The purified peptides were identified by FAB-MS (m/z 933 for **5** and 946 for **6** $(M + H)^+$) and amino acid analysis, and the purity was confirmed by a single peak for each peptide on C4 reversed-phase

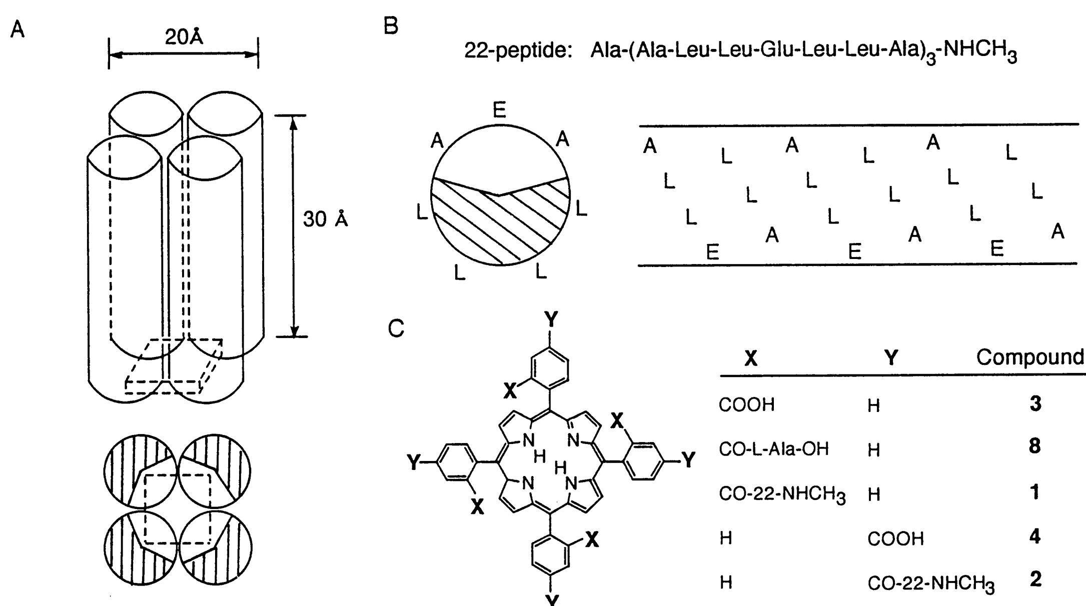

Fig. 1. Structure of the 4α-helix peptide-porphyrin hybrid **1**. (A) Illustration of the designed molecular structure of **1**. The rod and square represent α-helix and tetraphenylporphyrin ring, respectively. The shaded area of α-helices represents the hydrophobic region of the amphiphilic α-helix. The α-helical rods should be organized in twisted relationship, which is not manifested properly in the illustration. (B) The amino acid sequence of the designed 22-peptide and the α-helix wheel and net of the amphiphilic peptide. (C) Structure of *meso*-tetra(*o*-carboxyphenyl)porphyrin **3** and *meso*-tetra(*p*-carboxyphenyl)porphyrin **4** and their derivatives.

HPLC. The 21-peptide **7** was synthesized by the fragment coupling of the heptapeptides **5** and **6** by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/1-hydroxybenzotriazole (HOBt) method. The peptide **7** was purified by Sephadex LH-60 (DMF) chromatography which indicated appropriate molecular weight.

The excess amount (1.5 equiv.) of **7** was used for the condensation with the porphyrin-$(Ala\text{-}OH)_4$ [6] **8** by EDC-HOBt. After purification with Sephadex LH-60 column (DMF), the protected hybrid was obtained in 62% yield. After the deprotection with trimethylsilyl trifluoromethane sulfonate/thioanisole/TFA reagent[7], the crude hybrid **1** was purified with Sephadex LH-60 column (2.0 cm x 80 cm, MeOH) to give the desired protein **1**; 65%; electron spectrum ε (λmax), 416000 (422 nm), 16400 (517 nm), 12200 (559 nm), 6500 (596 nm), 2300 (650 nm); amino acid analysis, Ala (28) 28.0, Glu (12) 11.9, Leu (48) 45.0 for 1.0 mol porphyrin. The hybrid **2** between *meso*-tetra(*p*-carboxyphenyl)porphyrin **4** and the 22-peptide was synthesized by the same method. SDS-polyacrylamide gel electrophoresis of **1** and **2** showed a single band as MW = ca 9500 (calcd. 9813). Both peptides were not eluted from C4 reversed-phase HPLC with any solvent examined probably because of these high hydrophobicity and high molecular weight, but showed rather sharp symmetrical peaks on gel-filtration HPLC (TSK-GEL G3000HXL (7.8 mm x 300 mm) with DMF) at the fraction corresponding to the molecular weight. The α,α,α,α-conformation of **1** must be retained during the coupling and acidic deprotection steps, because the atropisomer of the porphyrin with Ala was not deformed under such conditions.

Circular dichroism (CD) spectra of the hybrid **1** and **2** in MeOH, in the presence of sodium dodecylsulfate (SDS) micelles and dipalmitoylphosphatidylcholine (DPPC) vesicles[8] were measured (Fig. 2).

In MeOH, the hybrid **1** and **2** (10 μM (1 M=1 mol·dm^{-3})) showed CD corresponding to a complete α-helical structure with $[\theta]_{222}$=-38000 deg·cm^{2}·dmol^{-1}, while 22-peptide itself showed half of that. The α-helicity of atropisomeric **1** in MeOH was independent to the concentration, while that of the starfish-shaped **2** decreased with lowering concentration (60% α-helicity[9] at 0.1 μM). The enhanced α-helicity and the concentration independence suggest that the hybrid **1** folds into a parallel four α-helix bundle structure with intramolecular helix-helix interaction due to the constraining effect by the atropisomeric porphyrin template (Fig. 1). The high α-helicity of the reference hybrid **2** could be attributed to the intermolecular helix-helix interaction as proved by the concentration dependence. In the presence of SDS micelles or DPPC vesicles, **1** is still highly α-helical (87% α-helicity) (Fig. 2), while **2** could not keep the α-helical structure (50% in SDS and 35% in DPPC). The rigid hybrid protein **1** may keep the four α-helix bundle structure under the lipophilic environment with placing the hydrophilic face of the amphiphilic α-helix inside and the hydrophobic face outside. A little decrease in α-helicity could be attributed to the repulsion of carboxylate anions in the reversed-micellar type conformation of **1** in the membrane. On the contrary, **2** can not adjust itself to form the bundle conformation by the amphiphilic α-helix motif. The peptide segments on the edge of the porphyrin ring in **2** are separated too far from each other to gather under membrane conditions.

At the Soret band region, we found induced CD both in **1** and **2** (Fig. 2). Interestingly, they differed in signs, which might be related to the adequate arrangement of the peptide segments on porphyrin ring. It should be noted that amino acid conjugates with the atropisomeric porphyrin showed similar CD.[6]

Furthermore, we demonstrated that the hybrid **1** was stably embedded into phospholipid bilayers. After the mixture of peptides and DPPC was gel-filtrated through a Sephadex G-75 column, more than 90% of **1** was retained in the fractions containing DPPC vesicles but almost nothing for **2** (Both peptides were quantitatively recovered in the case of SDS/peptide.). Thus, the picket-fence-type hybrid **1** could penetrate into

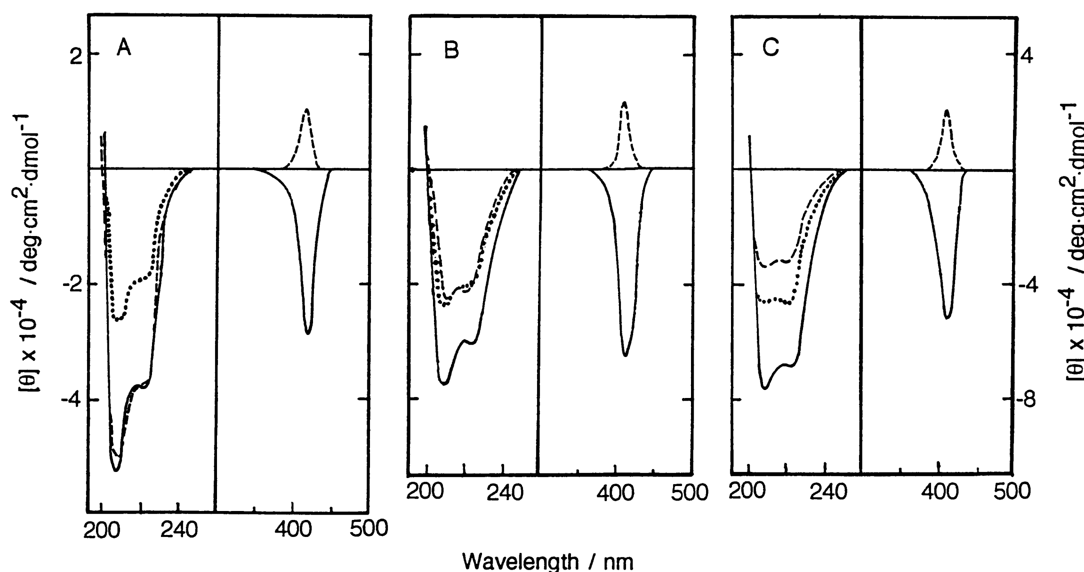

Fig. 2. CD spectra of the *ortho*- (**1**) (——) and *para*-hybrids (**2**) (– – – –), and the 21-peptide (·····). (A) in MeOH; (B) in the presence of SDS micelles (10 mM) in 20 mM Tris·HCl buffer (pH 7.5); (C) in the presence of DPPC vesicles (1 mM) in the same buffer; [peptide] = 10 μM.

the phospholipid bilayers but **2** could not. The starfish-shaped hybrid **2** might be just adsorbed on the surface of DPPC vesicles with placing the hydrophobic side chains inside of the bilayers and the hydrophilic side chains outside.

We selected the picket-fence-type structure of **1** with rigid phenylene connections between the peptides and the atropisomeric porphyrin for the advanced design for membrane-embedding polypeptide. Sasaki and Kaiser[1c] combined four 15-peptides to coproporphyrin I with propylene spacing. The flexibility in the spacing moiety probably allowed the helichrome to exhibit enzyme-like performance. Montal *et al.*[1f] designed a four α-helix bundle polypeptide on a flexible peptide template with ionic channel activity by using natural sequences in a membrane protein. Our template is very much different from theirs. The rigidly-anchoring template is employed expecting that the strong constraining effect into bundle conformation will forbid the perturbation of lipid bilayers, a nature of amphiphilic α-helical peptides such as mastoparan and melittin.[10] The atropisomeric porphyrin will become indispensable in further construction of artificial membrane proteins, for instance, in the mimicry of bacteriorhodopsin, which requires stable embedding in membrane. As the result of this rigidness, the inside of the bundle on the porphyrin in **1** may not accommodate any substrate. Accordingly, this structure will be utilized for more sophisticated design of an artificial protein with electron path by incorporating electronically functionalized side chains on peptide segments in the next step.

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