Artificial Membrane Protein Functionalized with Electron Transfer System

Hisakazu Mihara,*# Kin-ya Tomizaki, Tsutomu Fujimoto, Seiji Sakamoto,† Haruhiko Aoyagi,† and Norikazu Nishino*
Department of Applied Chemistry, Faculty of Engineering, Kyushu Institute of Technology, Tobata, Kitakyushu 804
†*Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852*

(Received November 6, 1995)

An artificial membrane protein bearing a flavin and a porphyrin functionalities was designed and synthesized. The protein took a 4α-helix structure and exhibited an electron transfer function in a bilayer membrane.

Natural proteins that act as electronic devices in membranes such as bacteriorhodopsin have been investigated at the level of atomic resolution.[1] These kinds of proteins contain functional chromophores such as retinal whose functions and positions are organized by polypeptides with defined tertiary structures. Along with these findings, much attention has been focused on the principle how to organize functionalities in the three-dimensional (3D) structure of polypeptides by the *de novo* design method.[2] We have designed artificial proteins with photoactive chromophores such as porphyrin, pyrene, and Ru-trisbipyridine in 3D structures composed of α-helices.[3] Here, we designed an artificial protein with a flavin and a Mn(III)-porphyrin moieties in a 4α-helix bundle structure of polypeptides to be functional as a membrane protein (Figure 1).

To construct the 4α-helix structure, the α,α,α,α-atropisomer of 5,10,15,20-tetrakis(2-aminophenyl)porphyrin (TAPP) was selected as a template for gathering four peptide chains on one side of the porphyrin plane.[3a,4] The porphyrin moiety was expected to act as a reaction center by complexation with Mn(III).[5] The 7-acetyl-10-methylisoalloxazine (AcFla) moiety[3b] as a model flavin functionality was deployed on the side chain of Cys near the center of one helix (G) segment. Two species of hydrophobic helical segments (21-peptides) were taken from the membrane-embedded α-helical segments of bacteriorhodopsin [C and G helices (C(82-100)+Ala_2 and G(204-223)+Ala)].[1] The two helices in the natural protein are oriented in parallel in the membrane. The Lys residue in the G segment, which is the anchoring residue for retinal, was replaced by Cys to bind the flavin in the artificial system. The 21-peptides (C and G) have rather hydrophobic nature, but still retain amphiphilic character when they are in α-helix structures (Figure 1c). The 21-residues in an α-helix form (6 turns) are long enough to span the hydrophobic region of phospholipid bilayer (30 Å). The *N*-terminal amino acids are charged Arg and Glu residues for C and G helices, respectively, and those α-amino groups were glycolylated to be outside the membrane. Therefore, the protein was expected to be stably embedded into phospholipid bilayer by forming hydrophilic pocket inside the bundle structure.

Synthesis of the protein was carried out as follows: Two protected 20-peptides were synthesized by the solid-phase method on *p*-nitrobenzophenone oxime resin.[3c] After (Fmoc-Ala-)(Boc-Ala-)$_3$-TAPP was prepared, selective deprotections of Fmoc and Boc groups and condensations with the protected peptides (G and C) were successively carried out. The obtained protected protein was passed through a Sephadex LH-60 column several times to remove two- and three-segmental compounds. The protein was deprotected with HF and purified with Sephadex LH-60 (dimethylformamide). Mn(III) was introduced into the TAPP moiety with $Mn(OAc)_2$[5] and then 7-bromoacetyl-10-methylisoalloxazine was reacted with the side chain of Cys residue.[3b] UV-VIS and fluorescence spectra indicated the successful introduction of the metal and the flavin moiety.[6]

The protein without Mn showed an typical α-helical circular dichroism (CD) pattern in trifluoroethanol (TFE) ([θ]222=−35000 deg·cm^2·dmol^{-1}) (Figure 2). The protein was also in a highly α-helix structure in hexane/tetrahydrofuran/TFE (10/3/7) as hydrophobic circumstances ([θ]222=−36000 deg·cm^2·dmol^{-1}). The protein was mixed with egg yolk lecithin and sonicated in buffer. The water-insoluble protein could be dissolved in aqueous solution in the presence of the vesicles. The vesicle solution was passed through a column of Sephadex G-75. The

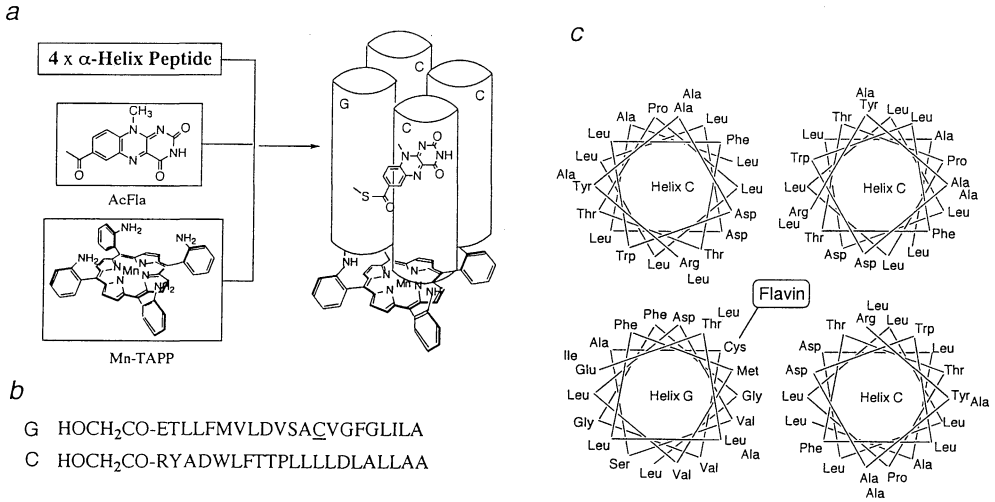

Figure 1. Construction of the artificial membrane protein. The protein was designed and synthesized with the hydrophobic helix segments derived from the transmembrane regions of bacteriorhodopsin (C- and G-helices) on the functional template Mn(III)-TAPP (***a***, ***b***). The flavin moiety was introduced on the side chain of the Cys residue in the G-helix. The peptide segments were shown in the α-helical wheel drawing (***c***).

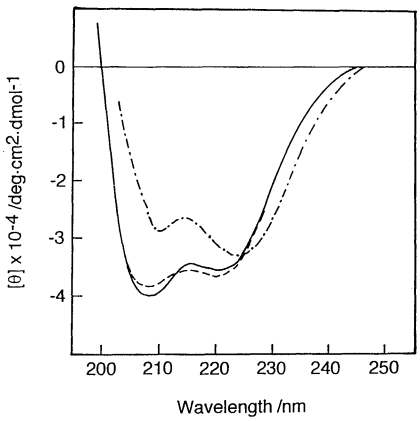

Figure 2. CD spectra of the protein under various conditions. CD spectra were measured in TFE (———), hexane / tetrahydrofuran / TFE (10 / 3 / 7) (– – – –) and in egg-yolk lecithin (0.5 mM in 20 mM TrisHCl, pH 7.4) membrane (– · – · –). [protein] = 3.0 μM at 25 °C. 1 M = 1 mol·dm^{-3}.

protein was quantitatively (>90%) recovered in the fractions containing vesicles, indicating that the protein was stably embedded into the phospholipid bilayer. The protein in the membrane showed a CD spectrum with double minima at 225 and 210 nm ([θ]225=−34000 deg·cm^2·dmol^{-1}), which were deviated from an usual CD for α-helix. The distorted CD spectrum of bacteriorhodopsin in purple membrane was attributed to the αII-type helix conformation,[7] though the decrease in ellipticity at 210 nm might be due to the effect of light scattering of the vesicles. The membrane protein has large potential taking a 4α-helix bundle structure in a membrane as well as in a solution. This was supported by FT-IR of the protein in a cast film of egg yolk lecithin; 1653 and 1540 cm^{-1} for amide I and II bands, respectively, characteristic for α-helix. Furthermore, Trp residues in C-helices showed the fluorescence emission at 343 nm in TFE, while they had the emission at 332 nm in the membrane, indicating that the Trp residues were located under more hydrophobic circumstances in the membrane such as hydrocarbon region than in the protein structure in TFE.

The function as an electron path of the artificial membrane protein was examined using the protein-embedded lecithin vesicles. Mn(III)-TAPP in the protein was efficiently reduced to Mn(II)-TAPP (λmax 440 nm) with both *N*-benzyl-1,4-dihydronicotinamide (BzlNAH) and *N*-hexyl-1,4-dihydronicotinamide (HexNAH) added outside the vesicles under anaerobic conditions[5] (Figure 3). The pseudo-first-order rate constants k_{obsd} calculated from the initial rates were proportional to the concentration of *N*-alkyldihydronicotinamides. The apparent second-order rate constants k^{2nd} were 4.8 and 6.7 M^{-1}s^{-1}, respectively, for BzlNAH and HexNAH. In contrast, the protein lacking the flavin showed much lower activity (k^{2nd} 0.8 and 1.3 M^{-1}s^{-1}, respectively) and the system containing the non-bonding flavin (an equivalent amount to the protein added outside the vesicles) showed moderate activity (k^{2nd} were 2.7 and 3.7 M^{-1}s^{-1}, respectively). As a consequence, the electron transfer function of the protein was accelerated 6 times by the intramolecularly organized chromophores in the protein. The natural reductant NADH could also reduce the Mn(III)-TAPP moiety in the protein, though the rate constant was ~1/10 compared with the model NADH compounds. The protein was possibly embedded in two different directions in the membrane; one has the porphyrin facing outside the vesicle and the other's porphyrin is inside the vesicle. Because the direct reduction of Mn-TAPP by the alkyldihydronicotinamides was much slower, it can be considered

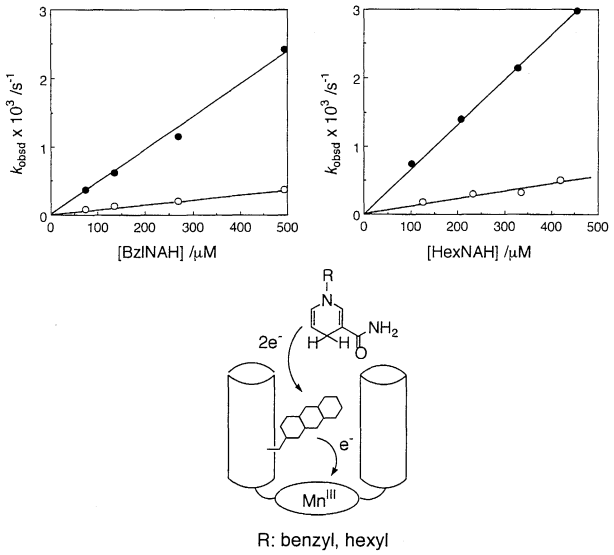

Figure 3. Profiles of the pseudo-first order rate constants (k_{obsd}) as a function of the concentration of BzlNAH and HexNAH; (●) the artificial protein and (○) the protein lacking the flavin. The dihydronicotinamide was added to the membrane-embedded protein solution ([protein] = 3.0 μM in egg-yolk lecithin 0.5 mM) at various concentrations under anaerobic conditions at 20 °C, and then the decrease in the absorbance of Mn(III)-TAPP at 470 nm was monitored. Illustration of the electron transfer reaction from dihydronicotinamides to Mn(III)-TAPP *via* the flavin moiety was shown.

that the accelerated reaction was responsible for the electron mediation activity of the flavin. Furthermore, the distance between the dihydronicotinamides, the flavin and the porphyrin must be critical for the reaction rate. The flavin moiety was anchored at the 13th residue from the *N*-terminal (ca. 15Å from the porphyrin). This would be a considerable point for further design of such proteins with improved activity. It should be noted that the artificial membrane protein is designed employing natural protein fragments with artificial functional groups. The general strategy described here would provide information on the significance of the organized functionalities in proteins.

References and Notes

Present address: Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226.

1 R. Henderson, J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing, *J. Mol. Biol.*, **213**, 899 (1990).

2 S. F. Betz, D. P. Raleigh, and W. F. DeGrado, *Curr. Opin. Struct. Biol.*, **3**, 601 (1993).

3 a) H. Mihara, N. Nishino, R. Hasegawa, and T. Fujimoto, *Chem. Lett.*, **1992**, 1805; b) H. Mihara, K. Tomizaki, N. Nishino, and T. Fujimoto, *Chem. Lett.*, **1993**, 1533; c) H. Mihara, Y. Tanaka, T. Fujimoto, and N. Nishino, *J. Chem. Soc., Perkin Trans. 2*, **1995**, 1133.

4 T. Sasaki and E. T. Kaiser, *J. Am. Chem. Soc.*, **111**, 380 (1989); K. S. Åkerfeldt, R. M. Kim., D. Camac, J. T. Groves, J. D. Lear, and W. F. DeGrado, *J. Am. Chem. Soc.*, **114**, 9656 (1992); E. Tsuchida and T. Komatsu, *Methods Enzymol.*, **231**, 167 (1994).

5 J. Takeda, S. Ohta, and M. Hirobe, *J. Am. Chem. Soc.*, **109**, 7677 (1987); I. Tabushi and M. Kodera, *J. Am. Chem. Soc.*, **108**, 1101 (1986).

6 λmax(TFE) 470, 570, 620 nm (Mn(III)-TAPP) and 290, 430 nm (flavin). Fluorescence emission of the flavin was observed at 500 nm excited at 430 nm. Gel-electrophoresis of the protein with sodium dodecylsulfate afforded a satisfactory result on the molecular weight as 11000 (calcd. 10600).

7 N. J. Gibson and J. Y. Cassim, *Biochemistry*, **28**, 2134 (1989).