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Dominant structural factors for complexation and denaturation of proteins using carboxylic acid receptors

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ABSTRACT

Complexation accompanied by denaturation of protein with synthetic carboxylic acid receptors was investigated, to evaluate the key factors for recognition of proteins. The synthetic receptors used were tetraphenylporphyrin (TPP) derivatives and receptors bearing multiple (2–8) carboxylic acid groups. The complexation behavior was quantified from the absorption in the far UV CD spectrum attributed to the secondary structure of the protein. TPP derivatives bearing multiple carboxylic acid groups in the side chains exhibited higher affinity than other receptors that were smaller and had fewer carboxylic acid groups. As the degree of complexation was influenced by the pH and ionic strength in aqueous solution, electrostatic interaction was one of the most important factors for the recognition of proteins. Complexation was also estimated by observation of fluorescence quenching of the TPP derivatives. The stoichiometry of the complexes between lysozyme and the porphyrins was investigated by quantitative analysis of the denaturation using CD spectra. From the results of Job plots and slope analysis for the amount of denatured protein, formation of 1:1 complexes was confirmed. The equilibrium association constants (K_{ass}) for lysozyme and the TPP receptors ranged from 0.6×10^6 to $1.1 \times 10^6\ M^{-1}$. The lytic activity of lysozyme was partially lost in the presence of anionic TPP derivatives, due to complexation and denaturation.

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1. Introduction

In recent years, synthetic molecules that recognize amino acid residues located on the exterior surfaces of proteins have been developed for various biological uses. Such synthetic receptors can be used for the detection of a targeted protein in medical use, modulation of protein functionalities, and development of a novel protein separation system [1–5].

The concept for the design of protein surface receptors is different to that of conventional receptors such as inhibitors and antagonists, which bind to well-defined internal cavities in the targeted protein. The protein surface receptors can be roughly divided into two types. One type involves formation of an *n*:1 supramolecule between receptor and protein, by recognition of targeted residues. Lysine residue can be guests for macrocyclic receptors. 18-Crown-6 and calix[6]arene derivatives, for example, form stable complexes with the amino group of the lysine residue by incorporating it into their cavities [6–13]. Complexation with the macrocyclic receptors enables protein to dissolve in organic solvents [7–14] and ionic liquids [15,16]. Dissolution of enzymes via complexation with the macrocyclic receptors enhances the enzymatic activities in organic solvents [17–21]. Such *n*:1 complexation is available for protein modification, but discrimination of the distribution of residues on the protein surface over a large surface area is impossible.

By contrast, synthetic receptors that form a 1:1 supramolecule with protein via multiple interactions can recognize the structure of a whole protein, and control the functionality. For recognition of multiple functional groups that are widely distributed in a whole protein, the receptor should have a large central domain and multiple functional groups at the periphery. A synthetic receptor linked to four constrained peptide loops using calix[4]arene as the platform was found to act as a receptor for various proteins [22–25]. Mallik and co-workers developed multidentate ligands bearing multiple transition metal ions for recognition of histidine residues distributed on protein surfaces [26]. Rottelo and co-workers reported recognition of proteins using gold nanoparticles functionalized with anionic groups [27–29]. Thus, the development of artificial receptors or materials for protein recognition with large surface area has attracted considerable attention.

Hamilton and co-workers developed various tetraphenylporphyrin (TPP) derivatives for recognition of various proteins, by introducing multiple functional groups and amino acid residues at the periphery [30–38]. The TPP derivatives were found to be attractive materials for preparing protein receptors that showed strong affinity for proteins. In addition, TPP derivatives are highly

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fluorescent and show emission intensity changes on binding to a targeted protein. A TPP derivative bearing eight carboxyl groups exhibited quite strong affinity (K_d = 20 nM) for the cationic protein cytochrome *c* (Cyt *c*) [30]. Moreover, a series of TPP derivatives whose fluorescence emission changes on binding to protein surfaces has been developed as a protein "fingerprinting" array [31]. The library of TPP derivatives bearing different amino acids or amino acid derivatives at the periphery was prepared using a mixed condensation strategy. The protein-detecting array based on the TPP derivatives showed a unique pattern of fluorescence change on interaction with targeted proteins. Unambiguous identification of a variety of proteins was achieved using an eight-porphyrin array, after processing the data matrix by principal component analysis (PCA) clustering [32]. Furthermore, complexation with TPP derivatives promotes disruption of the tertiary and secondary structure of proteins at low concentration. A TPP derivative bearing four anionic Tyr-Asp residues binds tightly to Cyt *c* and lowers the melting temperature of the protein to room temperature [33]. Complexation with copper TPP derivatives accelerates denaturation and proteolytic degradation of heme proteins with trypsin [34–36]. Moreover, tetrabiphenylporphyrin-based receptors show sub-nanomolar affinity for complexation with protein and enhance protein unfolding [37]. Thus TPP derivatives show high affinity for proteins, and the complexation can be used for protein identification and denaturation, and inhibition of protein–protein interaction [38–41].

In these precedents, anionic TPP derivatives bearing multiple carboxylic acid groups showed remarkably high affinity for various proteins. The affinity of the carboxylic acid compounds for proteins is considered to be based on electrostatic interaction between the carboxylic acid groups and cationic residues on the targeted protein, as well as hydrophobic interaction at the central porphyrin core and the hydrophobic domain of the protein. In the present study, the complexation and denaturation behavior of proteins was systematically studied using a series of synthetic carboxylic acid receptors. The receptors had different numbers (2–8) of carboxylic acid groups, and the sizes and structures of the core domains were also different. Denaturation of proteins, arising from their secondary structure, by complexation with the carboxylic acid receptors, was observed using circular dichroism (CD) spectroscopy in the far UV region. Lysozyme was used as a model protein for complexation with the carboxylic acid receptors, because of its relatively high isoelectric point (p*I* = 11.1) and moderate molecular size (M.W. = 14,300 g mol^{-1}). The affinities of receptors for lysozyme monitored by the changes of CD spectra were compared, to identify the key structural factors for complexation. The effects of the pH and ionic strength of the aqueous solution used for denaturation of lysozyme were also studied, to identify the dominant interactions for protein recognition. Furthermore, loss of the enzyme activity of the lysozyme by complexation with the receptors was also studied.

2. Experimental

2.1. Materials

Fig. 1 shows the molecular structures of the commercial TPP derivatives that were used. The following analytical grade reagents were purchased as receptors for proteins and employed without further purification: tetrakis(4-carboxyphenyl) porphyrin (TCPP) and tetrakis(4-sulfonatophenyl) porphyrin (TPPS) (Tokyo Kasei Co. Ltd., Tokyo, Japan); and tetrakis(4-*N*,*N*,*N*-trimethylamiophenyl) porphyrin (TTMAPP) (Sigma–Aldrich Co., St. Louis, MO). Fig. 2 shows the molecular structures of the carboxylic acid receptors that were used. An octacarboxylic acid TPP derivative (Asp-TPP) and a hexacarboxylic acid compound (Asp-TRI) were prepared according

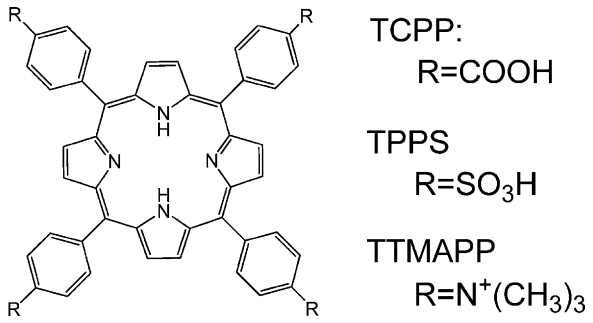

Fig. 1. Molecular structures of commercial TPP derivatives used in this study.

to the procedures described in Sections 2.2 and 2.3. Analytical grade protoporphyrin disodium salt (PP) and 1,4-phenylenediacetic acid (DI) (Wako Pure Chemical Industries, Ltd., Osaka, Japan); and 1,3,5-tricarboxybenzene (trimesinic acid; TRI) (Tokyo Kasei Co. Ltd., Tokyo, Japan) were used as received. The following protein reagents were used as received: cytochrome *c* from horse heart and lysozyme from egg white (Sigma–Aldrich Co., St. Louis, MO); albumin from bovine serum, and hemoglobin from bovine blood (Wako Pure Chemical Industries, Ltd., Osaka, Japan). L-Aspartic acid di-t-butyl ester hydrochloride (H-Asp(OtBu)-OtBu), (Merck, Hohenbrunn, Germany) for synthesis of receptors was used as received. All other reagents were reagent grade and were used as received.

2.2. Synthesis of a tetraphenylporphyrin asparaginic acid derivative (Asp-TPP)

The tetraphenylporphyrin asparaginic acid derivative Asp-TPP was synthesized according to the synthetic route shown in Scheme 1, as follows. To a solution of TCPP (474 mg, 0.60 mmol) in N,N-dimethylformamide (DMF) was added H-Asp(OtBu)-OtBu (845 mg, 3.0 mmol) and *N*-hydroxybenzotriazole (HOBt, 405 mg, 3.0 mmol), and the mixture was stirred in an ice bath for 0.5 h. A solution containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl, 575 mg, 3.0 mmol) and triethylamine (304 mg, 3.0 mmol) was then added dropwise, and the mixture was stirred in an ice bath. After 16 h, the solvent was removed *in vacuo* and the residue dissolved in chloroform. The solution was washed with aqueous hydrochloric acid, aqueous sodium hydrogen carbonate and distilled water. After drying with anhydrous sodium sulfate, the solvent was evaporated *in vacuo*, and the residue recrystallized from chloroform and hexane. The tetra asparaginic acid di-tert-butyl ester TPP derivative Asp(OtBu)-OtBu-TPP was obtained as a purple solid, then hydrolyzed using TFA/H_2O (29:1). After 2.5 h the solvent was removed *in vacuo*, TFA in the residue was completely removed by azeotropic distillation with ethanol, and the residue was recrystallized from ethanol and hexane. The following properties of the product Asp-TPP were observed: green powder; ^{1}H NMR (400 MHz, pyridine, 25 °C) −1.01 (2H, s, C-NH-C), 5.22–5.37 (8H, m, C-CH_2-CO), 7.54–7.59 (4H, m, m, N-CH), 9.85 (8H, d, Ar-H), 10.17 (8H, d, Ar-H), 10.53 (8H, s, Py-H), 11.34 (4H, d, CO-NH-C). ^{13}C NMR (400 MHz, DMSO, 25 °C) δ = 35.85, 49.60, 119.42, 126.05, 133.43, 134.17, 144.16, 166.10, 171.88, 172.64.

2.3. Synthesis of a tripodal asparaginic acid compound (Asp-TRI)

A tripodal asparaginic acid compound (Asp-TRI) was synthesized according to the synthetic route shown in Scheme 2, as follows. To a solution of TRI (168 mg, 0.80 mmol) in N,N-dimethylformamide (DMF) was added H-Asp(OtBu)-OtBu (1.13 g,

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