De Novo Design of Hemoprotein Model with Peroxidase Activity toward Lipophilic Peroxide

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A single-chained 49-mer-polypeptide tethering iron(III)-porphyrin folded into a certain conformation with bis-histidine coordination and consumed favorably linoleic acid hydroperoxide rather than H_2O_2.

There are increasing attractive studies in design and synthesis of polypeptide-metalloporphyrin complexes as hemoprotein models. A group of hemoprotein model systems are constructed by non-covalent complexes of exogenous iron-porphyrin (heme) derivatives toward four-α-helix bundle structure.[1] In another group, heme derivatives are covalently linked to the side chains of lysine residues in α-helix segments.[2] Porphyrin rings are also employed as the template to organize four parallel α-helices to form the bundle structure of polypeptides.[3] However, little is reported on the design of a single-chained hemoprotein model which folds into a predicted three-dimensional structure. We chose a βαβα-structure as a polypeptide scaffold, since the βα-unit is found in TIM barrel.[4] In order to place a porphyrin ring in the hydrophobic space in the folded structure, we designed a single-chained 49-peptide applying the amphiphilic α-helix and β-strand motifs (Figure 1).[5]

The Chou-Fasman parameters, $<P_\alpha>$ and $<P_\beta>$ of the α-helix segment, ELLKAHAELLK, are 1.27 and 0.90, respectively, while those parameters of the β-strand segment, AVEVKVA, are 1.24 and 1.12, respectively.[6] Though the β-strand segment is more likely to form α-helical conformation, the alternating hydrophobic and hydrophilic amino acid residues in the sequence were expected to separate the side chains to form β-structure in the amphiphilic manner. The iron(III)-5-(4-carboxyphenyl)-10,15,20-tritolylporphyrin was chosen to be connected on the side chain of ornithine residue at the hydrophobic side of the β1-segment (Figure 1). Two histidine residues were also placed at the hydrophobic faces of two α-helix segments to coordinate to the iron(III). Peptide synthesis was carried out by solid-phase synthesis and segment-condensation method in Boc chemistry.[7] The desired 49-peptide tethering iron(III)-porphyrin, **2** (Figure 1) was obtained as fluffy brown powder after lyophilization.

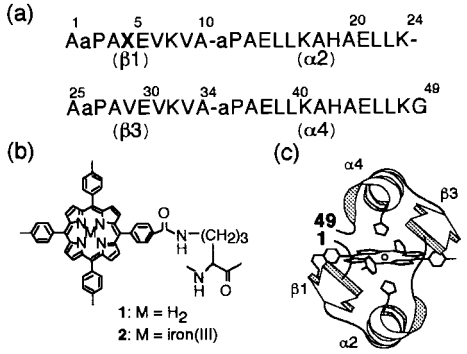

Figure 1. Structure of 49-peptide tethering iron(III)-porphyrin. (a) Amino acid sequence of 49-peptide. One letter symbols of amino acids are A, Ala; a, D-Ala; E, Glu; G, Gly; H, His; K, Lys; L, Leu; P, Pro; V, Val; X, L-Orn(Por(M)). (b) Structure of L-Orn(Por(M)). (c) Illustration of the folded βαβα-structure of **2** with bis-histidine coordination to iron(III).

Far-UV CD profile of **2** in 20 mM Tris HCl buffer (pH 7.2) showed a shoulder at the vicinity of 210–220 nm and a negative band at 204 nm (Figure 2(a)). The ellipticity at 222 nm of **2** in aqueous solution was -13000 deg cm^2 dmol^{-1} (33% α-helix content).[8] Since the presumed βαβα-structure should contain 45% α-helix for 49-peptide sequence in design, this result suggests that **2** may form a conformation with perturbation in α-helix by a bulky porphyrin ring. We could not find out the evidence of β-structure in the CD spectra. The β-strands in design might be extended through the hydrophobic interactions with α-helix segments. In methanol, **2** exhibited 80% α-helix content (θ_{222} = -31800 deg cm^2 dmol^{-1}), indicating the transition of extended conformation in aqueous solution to α-helix structure by the addition of methanol.

The CD spectra at the Soret band showed the split CD (a positive and a negative bands from longer wavelength) in 0-40% methanol, a negative Cotton effect in 50% methanol, and no induced CD in 60–100% methanol (Figure 2(b)). The split CD at the Soret band suggests that the iron(III)-porphyrin is sandwiched by two histidine imidazoles in α-helix segments to afford chiral exciton coupling. Similar chirality in porphyrin-peptide conjugate has been also reported in the literatures.[2]

The details of the situation of the iron(III)-porphyrin in the folded conformation of **2** were further studied by UV/vis spectra. Dominant features in the UV/vis spectrum of **2** in 20 mM Tris HCl buffer (pH 7.2) were low-spin β (Q_v) and α (Q_o) bands at 551 nm and 575 nm, respectively, and the Soret band at 420 nm (Figure 2(c)).[9] These bands indicate that the iron(III) is predom-

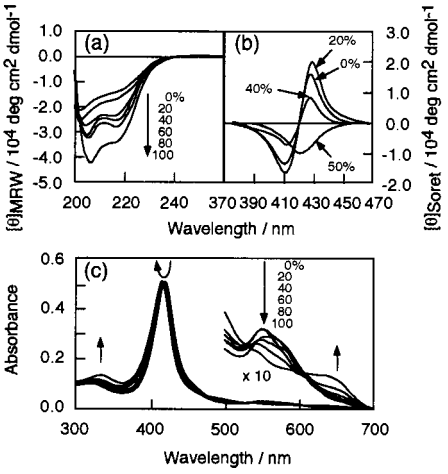

Figure 2. (a) Far-UV CD and (b) CD at the Soret band of **2** ([**2**] = 10 μM), and (c) UV/vis spectra ([**2**] = 5.0 μM) of **2**. All spectra were recorded in 20 mM Tris HCl buffer (pH 7.2) containing various methanol contents at 25 °C.

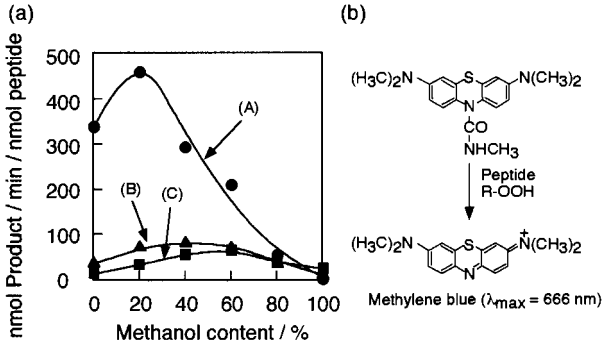

Figure 3. (a) The initial rates of MCDP oxidation catalyzed by **2** in the presence of LPO ([**2**] = 50 nM and [LPO] = 100 μM) (A), CHP ([**2**] = 0.5 μM and [CHP] = 20 mM) (B), and H_2O_2 ([**2**] = 0.5 μM and [H_2O_2] = 20 mM) (C) in 20 mM Tris HCl buffer (pH 7.2) containing various methanol contents at 25 °C. [MCDP] = 200 μM. (b) Oxidation scheme of MCDP to methylene blue in the presence of peptide and peroxide.

inantly in the low-spin state. The absorbance at 420 nm was in proportion to peptide concentrations between 0.6 and 15 μM. These facts also suggest that the iron(III) in **2** in aqueous solution is coordinated intramolecularly with two imidazoles at the histidine side chain on α-helix segments as the 5th and 6th axial ligands.

In the UV/vis spectrum in methanol, high-spin β-band (Q_v) and porphyrin π-iron charge transfer band were observed at 495 nm and 640 nm, respectively, with the disappearance of low-spin β (Q_v) and α (Q_o) bands.[9] The Soret band was shifted to 416 nm in methanol from 420 nm (in aqueous solution). Probably, the transition of extended conformation to α-helix structure in **2** by methanol forced to dissociate the imidazoles from iron(III). The change in UV/vis spectra in 40–80% methanol content was attributed to the dissociation of one of two imidazoles coordinated to the iron(III) (Figure 2(c)). It is interesting that the iron(III) coordination with imidazoles was thus controllable by the content of the helix-stabilizing solvent such as methanol.

To utilize the hydrophobic core in the folded conformation of **2** as a lipid peroxide-specific reactive site, here, we demonstrated peroxidase-like oxidation reaction catalyzed by **2** in the presence of oxidants such as H_2O_2, cumene hydroperoxide (CHP), and more hydrophobic linoleic acid hydroperoxide (LPO). A peroxidase sensitive dye, 10-(*N*-methylcarbamoyl)-3,7-bis(dimethylamino)-10*H*-phenothiazine (MCDP, Kyowa Medex Co. Ltd.) was employed as a reductant to detect the rates of the consumption of peroxides by **2**.[10] The rate of MCDP oxidation was obtained by monitoring the increase in absorbance at 666 nm corresponding to the absorption of the generated methylene blue molecule ($\varepsilon_{666} = 9.6 \times 10^4$ M^{-1} cm^{-1}). With increasing concentrations of oxidants, peroxidase activities of **2** were measured in aqueous solution (pH 7.2) to determine the Michaelis-Menten parameters, the results being summarized as follows: K_M(LPO) = 1.3×10^{-5}; K_M(CHP) = 2.5×10^{-2}; and $K_M(H_2O_2)$ = 2.0×10^{-2} (M); and k_{cat}/K_M(LPO) = 6.4×10^5; k_{cat}/K_M(CHP) = 6.2×10^1; and $k_{cat}/K_M(H_2O_2)$ = 2.7×10^1 (M^{-1} s^{-1}). These results suggest that the lipid peroxide such as LPO was more favorably incorporated into the hydrophobic core (K_M(LPO) << K_M(CHP) ≈ $K_M(H_2O_2)$), where the iron(III)-porphyrin sits, than CHP and H_2O_2 in aqueous solution. As a peroxidase model, **2** reacted specifically with LPO by 2.4×10^4 times more sensitive than H_2O_2. The initial rate of MCDP oxidation catalyzed by **2** in the presence of three different oxidants showed the bell-shaped kinetics depending on methanol content (Figure 3). In the presence of 100 μM LPO, the maximal initial rate was given at 20% methanol. The addition of methanol (20%) may allow **2** to take the optimal conformation for effective accommodation of hydrophobic LPO. Interestingly, in the presence of 20 mM CHP and 20 mM H_2O_2, the maximal initial rates were obtained at 40% and 60% methanol, respectively. The oxidants with higher hydrophobicity required lower volume percent of methanol in the reaction mixture to give the maximal rate enhancements. Namely, the tight conformation with bis-histidine coordination seems to disturb the easy access of less hydrophobic substrate to the iron(III) in the hydrophobic interior of **2**. These changes in activities by solution conditions are surprisingly well consistent to both solvent depending low- and high-spin states of iron(III) and folding of **2**.

In summary, we designed and synthesized an iron(III)-porphyrin-bound βαβα-polypeptide with bis-histidine coordination to the iron(III) which is predominantly in low-spin state in aqueous solution (pH 7.2). The spin state of iron(III) in **2** was controlled by volume percent of an α-helix-stabilizing solvent such as methanol. Peroxidase-like oxidation activity of **2** occurred specifically in the presence of LPO rather than H_2O_2 at low methanol content, reflecting that the hydrophobic inside was used as a binding site of the oxidant.

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7 The peptide was synthesized according to the literature (H. Mihara, Y. Tanaka, T. Fujimoto, and N. Nishino, *J. Chem. Soc., Perkin Trans. 2*, **1995**, 1915). The protected intermediates Boc-(25-49)-OBzl, Boc-(11-49)-OBzl, and Boc-[Orn(Por)^5]-(1-49)-OBzl were successfully purified by gel filtration chromatography (Sephadex LH-20 and LH-60, DMF, 2.0 x 90 cm). The protecting groups of 49-peptide were removed by HF-anisole (9:1) for 60 min at 0 °C. The crude **1** was purified by HPLC equipped with a Superdex Peptide HR 10/30 column (Pharmacia Biotech, eluted with 20% acetonitrile/0.1% TFA) and characterized by MALDI TOF-MS, *m/z* found 5618.9, calcd. 5616.7. The iron was inserted into **1** with iron(II) acetate in acetic acid for 1 day at 30 °C under N_2 atmosphere to yield **2**, and then the crude **2** was purified by gel filtration chromatography (Sephadex G-50, 40% acetic acid, 2.0 x 90 cm). The purity was confirmed by RP-HPLC (>95%).
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