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Synthesis of protoheme IX derivatives with a covalently linked proximal base and their human serum albumin hybrids as artificial hemoprotein

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The simple one-pot reaction of protoporphyrin IX and ω-(*N*-imidazolyl)alkylamine or *O*-methyl-L-histidyl-glycine with benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate at room temperature produced a series of protoporphyrin IX species with a covalently linked proximal base at the propionate side-chain. The central iron was inserted by the general $FeCl_2$ method, converting the free-base porphyrins to the corresponding protoheme IX derivatives. Mesoporphyrin IX and diacetyldeuteroporphyrin IX analogues were also prepared by the same procedure. The Fe(II) complexes formed dioxygen (O_2) adducts in dimethylformamide at 25 °C. Some of them were incorporated into the hydrophobic domain of recombinant human serum albumin (rHSA), providing albumin–heme hybrids (rHSA–heme), which can bind and release O_2 in aqueous media (pH 7.3, 25 °C). The oxidation process of converting the dioxygenated heme in rHSA to the inactive Fe(III) state obeyed first-order kinetics, indicating that the μ-oxo dimer formation was prevented by the immobilization of heme in the albumin scaffold. The rHSA–heme, in which the histidylglycil tail coordinates to the Fe(II) center, showed the most stable O_2 adduct complexes.

Introduction

Numerous model compounds of hemoglobin (Hb) and myoglobin (Mb) have already been prepared and their O_2-binding equilibria and kinetics were extensively studied.[1] In particular, synthetic hemes having a sterically encumbered porphyrin platform can form stable O_2 adducts in organic solvent at room temperature. If we are to reproduce or mimic any biochemical reaction, the aqueous medium is particularly important. The dioxygenated complexes of highly-modified hemes are unfortunately oxidized to the ferric state in water. Human serum albumin (HSA) is the most abundant plasma protein in our circulatory system and solubilizes hydrophobic small molecules.[2] We have found that synthetic hemes are also spontaneously incorporated into HSA, which provides unique albumin–heme hybrids (HSA–hemes) and allows their Fe(II) states to remain stable in aqueous solution.[3] Actually, recombinant HSA[4] (rHSA) including tetrakis(α,α,α,α-*o*-pivalamidophenyl)porphinatoiron(II) with a covalently linked proximal base can reversibly bind and release O_2 under physiological conditions, and acts as an artificial O_2 transporter in the blood stream.[5] Our next target is to realize O_2 coordination to rHSA–heme involving protoheme IX in the same manner as natural Hb and Mb. The dioxygenation of protoheme IX has several advantages. (1) Synthetic procedures are rather simplified with respect to the highly modified tetraphenylporphyrin. (2) It has the same structure and thus the same spectra as do hemoproteins; this makes possible the study of subtle changes in the protein nanostructure. (3) Its metabolism process has been clarified,[6] which is an advantage for medical use as an artificial O_2 carrier.

We report herein the simple synthetic methodology of protoheme IX derivatives with a covalently-linked proximal imidazolyl arm and the O_2-binding properties of the obtained rHSA–hemes.

Results and discussion

Synthesis

The free-base porphyrins with a covalently linked proximal base (**1a**–**8a**, Scheme 1) were synthesized by the one-pot reaction of protoporphyrin IX, ω-(*N*-imidazolyl)alkylamine [R_2H; 3-(*N*-imidazolyl)propylamine, 4-(*N*-(2-methylimidazolyl))-butylamine or *O*-methyl-L-histidyl-glycine] for one propionic acid group, and a capping alcohol or amine on the other side (R_3H; MeOH, EtOH or $MeNH_2$) in the presence of benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) at 25 °C in pyridine [or dimethylformamide (DMF)] (Scheme 2). The carbonyl attachment was made through either an ester or an amide function. After the reaction, the mixture was poured into 10% NaCl solution, which led to the precipitation of the crude porphyrin. Centrifugation at 7000 g for 30 min gave a purple pellet. The pyridine (or DMF), BOP, R_2H and R_3H in the supernatant were all discarded at this point. The obtained precipitate was dissolved in $CHCl_3$ and showed several spots on a thin layer chromatograph. The anpolar band corresponds to the double R_3-substituted component (ex. protoporphyrin IX diethyl ester in the cases of **2**, **5**, **6**) and the second band is the desired porphyrin, which is purified by a silica gel chromatographic separation (yield: 20–30%). The iron was then inserted by the general $FeCl_2$ method with 2,6-lutidine in DMF solution, giving the corresponding hemins. Mesoporphyrin IX and diacetyldeuteroporphyrin IX also gave similar analogues (**7b** and **8b**). We obtained a mixture of two isomeric compounds that we were unable to separate.

Traylor and co-workers reported many pioneering studies on "chelated hemes".[7] They synthesized compound **1b**, for instance, using an acid anhydride procedure directly from protohemin chloride.[7e] First, the protohemin dimethyl ester was partially hydrolyzed and, after purification, the mono acid was coupled to a 3-(*N*-imidazolyl)propylamine by the pivaloyl chloride method. Nevertheless, reaction mixtures involving the diacid and monoacid are normally insoluble in common organic solvents, therefore, the yield of this reaction largely depends on the separation techniques. In contrast, our simple procedure makes it possible to synthesize a series of new protoporphyrins with a wide variety of proximal bases and end-capping groups of the other propionic acid.

Dioxygenation of heme in DMF solution

The obtained hemin complexes **1b**–**8b** in DMF solution were reduced to the corresponding Fe(II) complexes using a solution

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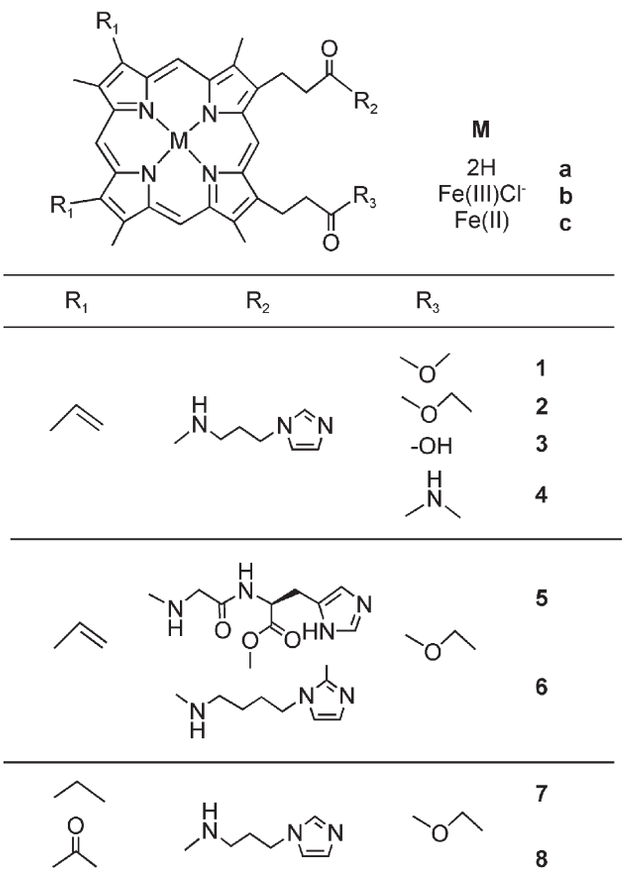


Scheme 1

of the crown ether-dithionite as reducing agent.[8] The UV-vis absorption spectrum of **2c** [Fe(II) complex] under a nitrogen (N_2) atmosphere showed a single broad band in the α,β region around 520–580 nm. This indicates the formation of a typical five-*N*-coordinate high-spin complex,[7] in which the proximal imidazole group intramolecularly coordinates to the central Fe(II) ion in the non-coordinating solvent (DMF) (Fig. 1). Because 2-methyl-imidazole significantly inhibits a sixth ligand binding to the *trans*-position, **6c** also demonstrated a similar 5-coordinated spectrum in DMF solution. Upon bubbling of the O_2 gas through the solution of **2c**, the spectral pattern immediately changed to that of the O_2 adduct complex. After adding carbon monoxide (CO) gas, the heme changed to a very stable carbonyl complex. Similar absorption changes were observed for all the heme derivatives, **1c**–**8c**. The absorption maxima (λ_{max}) of compounds **1c**–**8c** in DMF solution under N_2, O_2 and CO atmospheres are summarized in Table 1.

The positions and the relative intensities of all peaks were independent of the temperature changes from 5 to 25 °C. In general, the electron density of the porphyrin ring systematically changes the λ_{max} of the B-band and Q-band.[9] The replacement of the vinyl groups at the 3,8-positions of protoheme IX with ethyl groups (from **2c** to **7c**) produced a hypsochromic shift. In contrast, changing the vinyl groups to electron withdrawing acetyl groups (from **2c** to **8c**) produced a bathochromic shift.

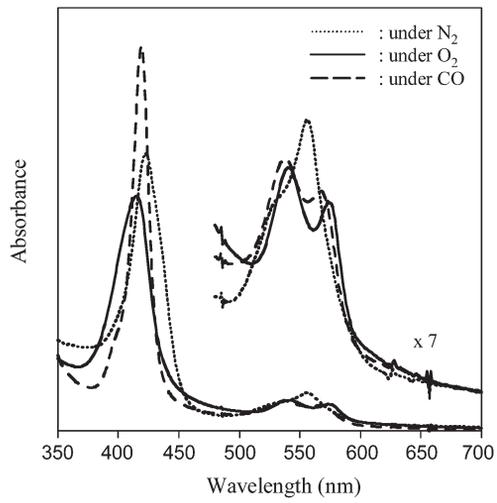


Fig. 1 UV-vis spectra of **2c** in DMF at 25 °C.

Table 1 Absorption maxima (λ_{max}) of heme derivatives in DMF under various conditions

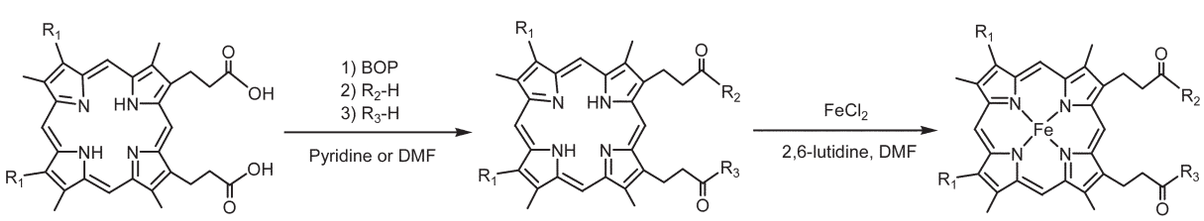
	λ_{max}/nm		
Compound	Under N_2	Under O_2	Under CO
1c (15 °C)	427, 530, 558	414, 543, 575	420, 540, 569
1c (25 °C)	424, 532, 559	412, 542, 575	420, 539, 567
2c (5 °C)	422, 531, 556	415, 541, 574	419, 537, 567
2c (25 °C)	421, 533, 557	409, 539, 571	418, 537, 565
3c (25 °C)	426, 537, 559	415, 543, 575	420, 539, 567
4c (5 °C)	421, 527, 555	413, 540, 572	417, 536, 564
5c (5 °C)	419, 529, 551	406, 537, 569	412, 534, 562
5c (25 °C)	423, 533, 557	408, 539, 573	419, 538, 567
6c (5 °C)	430, 555	413, 547, 576	418, 538, 561
7c (25 °C)	414, 523, 548	407, 531, 563	409, 529, 556
8c (5 °C)	440, 541, 571	432, 552, 579	434, 549, 576
8c (25 °C)	439, 545, 569	431, 552, 580	433, 548, 577

We could not find any significant difference in the absorption maxima of **1c**–**6c**, because modification of the propionic acids did not affect the electron density of the porphyrin macrocycle.

Preparation of rHSA–heme

Aqueous solutions of rHSA–heme were prepared by injecting an ethanol solution of the carbonylated heme into an aqueous solution of rHSA. The inclusion of heme into rHSA was confirmed by the following results: (1) Sepharose gel column chromatography showed the elution peaks of heme and rHSA coincided at the same position, (2) during dialysis of the rHSA–heme solution against phosphate buffer, the outer aqueous phase did not contain the heme component. The UV-vis absorption spectra of the obtained solution showed that the heme was retained as a CO adduct complex.

The binding number of heme in one rHSA was determined to be 0.9–1.1 (mol/mol) by assaying the iron and rHSA concentrations. The binding constant of **1b** for rHSA was estimated to be *ca.* 4×10^6 M^{-1}, which is approximately 1/25 of that for protohemin IX itself to albumin (*ca.* 1×10^8 M^{-1}).[10] Polar heme derivatives **3c** with monopropionic acid and **4c** with a methyl-



BOP: benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate

Scheme 2 Synthesis of protoheme IX derivatives.

Table 2 Absorption maxima (λ_{max}) of rHSA–hemes in phosphate buffer solution (pH 7.3) at 25 °C

	λ_{max}/nm		
Compounds	Under N_2	Under O_2	Under CO
rHSA–**1c**	420, 536, 561	414, 540, 567	419, 541, 566
rHSA–**2c**	420, 538, 561	416, 540, 567	421, 543, 567
rHSA–**5c**	422, 539, 561	418, 540, 571	422, 541, 569
rHSA–**8c**	444, 549, 571	432, 551, 580	440, 555, 578

amide capping group at the porphyrin periphery were partially oxidized to the Fe(III) state during the inclusion process. Since the binding force of the heme derivative to rHSA is a hydrophobic interaction,[11] relatively polar porphyrins may not be incorporated into a certain domain of rHSA and easily oxidized compared to more apolar ones.

The circular dichroism spectra of the rHSA–hemes (rHSA–**1c**, –**2c**, –**5c**, –**7c** and –**8c**) are almost identical to that of rHSA itself (not shown). This suggests that the secondary structure of the albumin host molecule did not change after incorporation of the hemes. Furthermore, the isoelectric points of these rHSA–hemes were all 4.8, which is the original value of rHSA. The surface net charges of rHSA remained unaltered after heme incorporation.

Dioxygenation of rHSA–heme in aqueous solution

Light irradiation of the CO adduct complex of rHSA–heme (rHSA–**1c**, –**2c**, –**5c**, –**6c**, –**7c** and –**8c**) under an N_2 atmosphere led to CO dissociation and demonstrated new spectral patterns with well-defined α and β bands. For example, the typical absorption spectral changes of rHSA–**2c** are shown in Fig. 2.

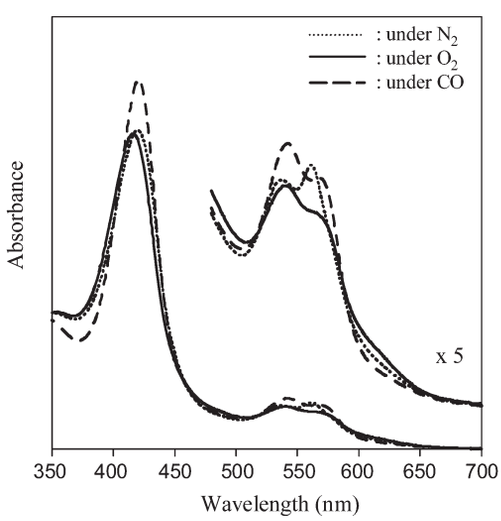


Fig. 2 UV-vis spectra of rHSA–**2c** in phosphate buffer solution (pH 7.3) at 25 °C.

From the nature of these spectra, we concluded that the obtained Fe(II) complexes are a mixture of Fe(II) 5-coordinated (high-spin) and 6-coordinated (low spin) species. It implies that the sixth coordinate position of the heme might be partially occupied by some amino acid residue of the protein scaffold. Upon exposure of O_2 to the Fe(II) complex of rHSA–**1c**, the spectrum changed to that of the O_2 adduct species. Although the aqueous micelle solution of **1c** with 5% surfactant (cetyltrimethylammonium bromide) forms a CO adduct complex, dioxygenation was not stable enough to measure the spectrum at 25 °C.[7e] In contrast, rHSA–**1c**, –**2c**, –**5c**, and –**8c** formed O_2 adduct complexes at 25 °C (pH 7.3) except for rHSA–**6c** and –**7c** (Table 2). The introduction of a methyl group to the 2-position of the imidazole ring is widely recognized to reduce the O_2 and CO binding affinities.[1] In this case, the strength of the imidazole coordination to the Fe(II) center is too weak to produce a stable O_2 adduct complex.

Table 3 Half-life ($\tau_{1/2}$) and O_2 binding affinity ($P_{1/2}$) of rHSA–hemes in phosphate buffer solution (pH 7.3) at 25 °C

Compounds	$\tau_{1/2}$/min	$P_{1/2}$/Torr
rHSA–**1c**	20	0.1
rHSA–**2c**	50	0.1
rHSA–**5c**	90	0.1
rHSA–**8c**	50	0.4

The oxidation process of dioxygenated rHSA–heme to the inactive Fe(III) state obeyed first-order kinetics. This indicates that the μ-oxo dimer formation was prevented by the immobilization of heme in the albumin structure. The half-life of the O_2 adduct complexes ($\tau_{1/2}$) and the O_2 binding affinities ($P_{1/2}$) of rHSA–hemes are summarized in Table 3. The histidylglycyl tail coordinated protoheme (**5c**) in rHSA showed the most stable O_2 adduct complex ($\tau_{1/2}$: 90 min) with respect to the imidazole bound ones. The more hydrophobic ethylpropionate (**2c**) also contributed to prolong the stability of the O_2 adduct complex relative to the methylpropionate protoheme (**1c**).

The $P_{1/2}$ values of rHSA–**1c**, –**2c** and –**5c** are 0.1 Torr at 25 °C. On the other hand, rHSA–**8c** showed a higher $P_{1/2}$ value (low O_2-binding affinity) compared to the others. The acetyl groups at the 3,8-positions of **8c** decrease the electron density of the porphyrin macrocycle, therefore $P_{1/2}$ could be significantly reduced. Traylor and co-workers found that the O_2 binding affinity of the chelated heme was sensitive to the electron density at Fe(II) and thus to the substituents at the heme periphery. The O_2 binding constant decreased by 1/6 upon changing the substituent from a vinyl to an acetyl group.[12] Our experimental data of hemes in rHSA are quite consistent with their observations.

Conclusion

A convenient one-pot synthesis of protoporphyrin IX derivatives with a covalently linked proximal base has been described. rHSA successfully incorporates the protoheme derivatives, providing an artificial hemoprotein, which can form an O_2 adduct complex at 25 °C. The rHSA–heme, in which the histidylglycil tail intramolecularly coordinates to the Fe(II) center, showed the most stable O_2 adduct complex with the relatively high O_2 binding affinity of 0.1 Torr.

Experimental

Materials and apparatus

All reagents were used as supplied commercially unless otherwise noted. All solvents were normally purified by distillation before use. DMF was distilled under reduced pressure in N_2. Pyridine was refluxed over and distilled from P_2O_5. The water was deionized using an ADVANTEC GS-200 system. The rHSA (Albrec®, 25 wt%) was obtained from NIPRO Corp. (Osaka).

Thin-layer chromatography was carried out on 0.2 mm precoated plates of silica gel 60 F254 (Merck). Purification was performed by silica gel 60 (Merck) column chromatography. The infrared spectra were measured with a JASCO FT/IR-410 spectrometer. The UV-vis absorption spectra were recorded by a JASCO V-570 spectrophotometer. The ^{1}H-NMR spectra were recorded using a JEOL Lambda 500 spectrometer. Chemical shifts were expressed in parts per million downfield from Me_4Si as the internal standard. The FAB-MS spectra were obtained using a JEOL JMS-SX102A spectrometer.

Synthesis of porphyrin derivatives

O-Methyl-L-histidyl-glycine[13] and 4-(*N*-(2-methylimidazolyl))-butylamine[14] were synthesized according to the reported procedures.

3,18-Divinyl-8-(3-methoxycarbonyl)ethyl-12-(3-(*N*-imidazolyl)propylamido)ethyl-2,7,13,17-tetramethylporphyrin (1a). A pyridine (7 mL) solution of 3-(*N*-imidazolyl)propylamine (35 μL, 0.29 mmol) was added dropwise to protoporphyrin IX (200 mg, 0.36 mmol) and benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate (411 mg, 0.93 mmol) in pyridine (20 mL) and stirred for 30 min at room temperature. The mixture was reacted for 4 h at 40 °C. After the addition of methanol (10 mL), the solution was stirred for another 12 h at 40 °C. The mixture was then poured into a 10% NaCl solution (1 L, 4 °C) and the suspension was centrifuged for 30 min at 7000*g*. The supernatant was discarded and the precipitate was collected and dried *in vacuo.* The residue was chromatographed on a silica gel column using $CHCl_3/CH_3OH$ = 8/1 (v/v) as the eluent. The main band was collected and dried at room temperature for several hours *in vacuo*, giving compound **1a** as a purple solid (75 mg, 20%). R_f = 0.3 ($CHCl_3/CH_3OH$ = 8/1 (v/v)); IR (NaCl) ν = 1731 (C=O, ester), 1646 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 408, 506, 542, 575, 630 nm; ^{1}H-NMR ($CDCl_3$) δ: −4.0 (s, 2H, inner), 1.8–2.4 (m, 4H, –(C*H*$_2$)$_2$–Im), 2.7 (m, 4H, –C*H*$_2$–COO–, NH–C*H*$_2$–), 3.2 (t, 2H, –CONH–C*H*$_2$–), 3.3–3.7 (m, 18H, por–CH_3, –CH_2–CO–, –$COOCH_3$), 4.2 (d, 4H, por–CH_2–), 5.4 (s, 1H, Im), 6.0–6.3 (m, 4H, =CH_2 (vinyl)), 6.4 (d, 1H, Im), 6.6 (d, 1H, Im), 8.0–8.4 (m, 2H, –CH= (vinyl)), 9.7 (m, 4H, *meso*); MS *m*/*z*: 681.67.

Fe(III) complex of 1a (1b). Iron(II) chloride tetrahydrate (106 mg, 0.53 mmol) was added to a dry DMF (10 mL) solution of **1a** (36 mg, 53 μmol) and 2,6-lutidine (30 μL, 0.27 mmol) under an N_2 atmosphere. The reaction mixture was stirred at 70 °C for 3 h. After confirming the disappearance of the porphyrin's fluorescence (600–800 nm, ex. 400 nm), the solution was cooled to room temperature and poured into 10% NaCl solution (1 L, 4 °C). The suspension was centrifuged for 30 min at 7000*g* and the supernatant was discarded. The precipitate was dried *in vacuo* and chromatographed on a silica gel column using $CHCl_3/CH_3OH$ = 8/1 (v/v) as the eluent. The main band was collected and dried at room temperature for several hours *in vacuo* to give compound **1b** as a brown solid (27 mg, 68%). R_f = 0.3 ($CHCl_3/CH_3OH$ = 8/1); IR (NaCl) ν = 1728 (C=O, ester), 1646 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 389, 513, 641 nm; HR-MS *m*/*z*: calcd for $C_{41}H_{43}O_3N_7Fe$: 737.2777, found: 737.2778 [M^+].

3,18-Divinyl-8-(3-ethoxycarbonyl)ethyl-12-(3-(*N*-imidazolyl)-propylamido)ethyl-2,7,13,17-tetramethylporphyrin (2a). The synthetic procedure of compound **2a** was the same as that used for **1a** except for using ethanol instead of methanol. Yield 30%; R_f = 0.4 ($CHCl_3/CH_3OH$ = 10/1); IR (NaCl) ν = 1650 (C=O, amide), 1732 (C=O, ester) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 409, 544, 580, 633 nm; ^{1}H-NMR ($CDCl_3$) δ: −4.1 (s, 2H, inner-NH), 0.8–0.9 (t, 3H, –COO–CH_2–C*H*$_3$), 1.3–1.5 (t, 2H, –CONH–CH_2–C*H*$_2$–), 3.0–3.1 (t, 2H, –CH_2–Im), 3.1–3.3 (m, 4H, –CH_2–COO), 3.5–3.7 (m, 12H, por–CH_3), 3.8–3.9 (m, 2H, –COO–C*H*$_2$–CH_3), 4.2–4.4 (d, 4H, por–CH_2–), 6.1 (s, 1H, Im), 6.1–6.4 (q, 5H, =CH_2 (vinyl), Im), 6.6–6.7 (d, 1H, Im), 6.9–7.0 (d, 1H, Im), 8.1–8.3 (m, 2H, –CH= (vinyl)), 9.8–10.2 (m, 4H, *meso*); MS *m*/*z*: 695.29.

Fe(III) complex of 2a (2b). Iron insertion to **2a** was carried out by the same procedure as in the **1b** preparation. Yield 80%; R_f = 0.3 ($CHCl_3/CH_3OH$ = 8/1); IR (NaCl) ν = 1651 (C=O, amide), 1725 (C=O, ester) cm^{-1}, UV-vis ($CHCl_3$) λ_{max} = 406, 520, 578 nm; HR-MS *m*/*z*: calcd. for $C_{42}H_{45}O_3N_7Fe$: 751.2933, found: 751.2953 [M^+].

3,18-Divinyl-8-(3-carboxy)ethyl-12-(3-(*N*-imidazolyl)propyl-amido)ethyl-2,7,13,17-tetramethylporphyrin (3a). Sodium hydroxide (2 N, 4.5 mL) was added to the methanol (10 mL) solution of **2a** (266 mg, 0.38 mmol) and the mixture was stirred for 12 h at room temperature. It was brought to dryness *in vacuo.* Methanol was added to the residue and the mixture was added dropwise to 10% NaCl solution (pH 2, 4 °C). It was centrifuged for 30 min at 7000*g* and the precipitate was collected and dried *in vacuo*, affording compound **3a** as a brown solid (187 mg, 78%), IR (KBr) ν = 1652 (C=O, amide), 1707 (C=O, –COOH) cm^{-1}; UV-vis (DMSO) λ_{max} = 409, 508, 543, 578, 631 nm; ^{1}H-NMR (d_6-DMSO) δ: −3.5 (s, 2H, inner-NH), 1.6–1.7 (t, 2H, –CONH–CH_2–C*H*$_2$–), 2.8–2.9 (t, 2H, –CH_2–Im), 3.1–3.3 (m, 2H, –CONH–C*H*$_2$–), 3.5–3.9 (m, 12H, por–CH_3), 4.2–4.4 (d, 4H, por–CH_2–), 6.1 (s, 1H, Im), 6.1–6.4 (q, 5H, =CH_2 (vinyl), Im), 6.6–6.7 (d, 1H, Im), 6.9–7.0 (d, 1H, Im), 8.5–8.6 (m, 2H, –CH= (vinyl)), 10.2–10.4 (m, 4H, *meso*); MS *m*/*z*: 670.41.

Fe(III) complex of 3a (3b). Iron insertion to **3a** was carried out by the same procedure as in the **1b** preparation. Yield 80%; IR (KBr) ν = 1646 (C=O, amide), 1707 (C=O, –COOH) cm^{-1}; UV-vis (DMSO) λ_{max} = 403, 508, 631 nm; HR-MS *m*/*z*: calcd. for $C_{40}H_{41}O_3N_7Fe$: 723.2620, found: 724.2668 [M + H^+].

3,18-Divinyl-8-(3-methylamido)ethyl-12-(3-(*N*-imidazolyl)-propylamido)ethyl-2,7,13,17-tetramethylporphyrin (4a). Compound **4a** was synthesized according to the same procedure as for **1a** except for using methyl amine instead of methanol. Yield 20%; R_f = 0.5 ($CHCl_3/CH_3OH$ = 3/1); IR (NaCl) ν = 1631 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 409, 509, 543, 579, 632 nm; ^{1}H-NMR (CD_3OD, $CDCl_3$) δ: −4.0 (s, 2H, inner), 1.8–2.4 (m, 4H, –(C*H*$_2$)$_2$–Im), 2.5 (t, 3H, –CONH–C*H*$_3$), 2.9 (m, 2H, –CONH–C*H*$_2$–), 3.3 (m, 4H, –C*H*$_2$–CONH–), 3.4–3.6 (m,12H, por–CH_3), 5.5 (s, 1H, Im), 6.0 (s, 1H, Im), 6.1–6.4 (m, 4H, =CH_2 (vinyl)), 6.8 (m, 1H, Im), 8.1–8.3 (m, 2H, –CH= (vinyl)), 9.7–9.9 (q, 4H, *meso*); MS *m*/*z*: 680.69.

Fe(III) complex of 4a (4b). Iron insertion to **4a** was carried out by the same procedure as in the **1b** preparation. Yield 67%; R_f = 0.3 ($CHCl_3/CH_3OH$ = 5/1); IR (NaCl) ν = 1646 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 408, 521, 565 nm; HR-MS *m*/*z*: calcd. for $C_{41}H_{44}O_3N_7Fe$: 736.2937, found: 736.2938 [M^+].

3,18-Divinyl-8-(3-ethoxycarbonyl)ethyl-12-(((3-*N*-glycyl-L-histidinyl)-9-oxymethyl)carbonyl)ethyl-2,7,13,17-tetramethyl-porphyrin (5a). The synthetic procedure of compound **5a** was same as that used for **1a**. DMF was used instead of pyridine, because it dissolves *O*-methyl-L-histidyl-glycine. Yield 15%; R_f = 0.4 ($CHCl_3/CH_3OH$ = 15/1); IR (NaCl) ν = 1635 (C=O, amide), 1725 (C=O, ester) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 405, 505, 541, 577, 627 nm; ^{1}H-NMR ($CDCl_3$) δ: −4.6 (s, 2H, inner-NH), 2.7–2.9 (m, 2H, Im–CH_2–), 3.0–3.5 (m, 18H, por–CH_3, –CH_2–C*H*$_2$–CO–NH–, –CH_2–C*H*$_2$–COO–C*H*$_2$–CH_3), 3.6 (s, 2H, –CONH–C*H*$_2$–CONH–), 3.8 (s, 3H,–OCH_3), 4.0–4.3 (d, 4H, por–CH_2–), 4.3–4.5 (m, 1H, α-CH), 6.0–6.4 (m, 4H, =CH_2 (vinyl)), 7.4 (s, 1H, Im–H), 8.0–8.3 (m, 5H, –CH= (vinyl), Im–H), 9.8–10.0 (m, 4H, *meso*-H); MS *m*/*z*: 782.68.

Fe(III) complex of 5a (5b). Iron insertion to **5a** was carried out by the same procedure as in the **1b** preparation. Yield 75%; R_f = 0.5 ($CHCl_3/CH_3OH$ = 8/1); IR (NaCl) ν = 1660 (C=O, amide), 1734 (C=O, ester) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 388, 508, 637 nm; HR-MS *m*/*z*: calcd. for $C_{44}H_{46}O_6N_8Fe$: 838.2890, found: 839.2929 [M + H^+].

3,18-Divinyl-8-(3-ethoxycarbonyl)ethyl-12-(4-(*N*-(2-methyl-imidazolyl))butylamido)ethyl-2,7,13,17-tetramethylporphyrin (6a). Compound **6a** was synthesized by the same procedure as for **1a** except for using 4-(*N*-(2-methylimidazolyl))butylamine instead of 3-imidazolylpropylamine. Yield 20%; R_f: 0.1 ($CHCl_3$/CH_3OH = 8/1); IR (NaCl) ν = 1732 (C=O, ester), 1651 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 408, 506, 542, 576, 630 nm; ^{1}H-NMR ($CDCl_3$) δ: −4.2 (s, 2H, inner-H), 0.4–0.6 (m, 4H, CONH–CH_2–(C*H*$_2$)$_2$–), 1.4–1.5 (d, 3H, Im–CH_3), 2.2–2.4 (m,

2H, –CONH–CH_2–), 2.8–3.1 (m, 4H, por–CH_2–CH_2–), 3.2–3.3 (t, 2H, –CH_2–Im), 3.4 (s, 3H, –COO–CH_3), 3.5–3.8 (m, 12H, por–CH_3), 4.2–4.4 (t, 4H, por–CH_2–), 5.6–5.7 (d, 1H, Im), 5.8 (m, 1H, Im), 6.1–6.4 (q, 4H, =CH_2 (vinyl)), 8.1–8.2 (m, 2H, –CH= (vinyl)), 9.8–10.1 (m, 4H, *meso*); MS *m*/*z*: 709.72.

Fe(III) complex of 6a (6b). Iron insertion to **6a** was carried out by the same procedure as in the **1b** preparation. Yield 64%; R_f = 0.2 ($CHCl_3$/CH_3OH = 8/1); IR (NaCl) ν = 1732 (C=O, ester), 1652 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 401, 580, 630 nm; HR-MS *m*/*z*: calcd. for $C_{43}H_{47}O_3N_7Fe$: 765.3090, found: 766.3184 [M + H^+].

3,18-Diethyl-8-(3-carboxy)ethyl-12-(3-(*N*-imidazolyl)propyl-amido)ethyl-2,7,13,17-tetramethylporphyrin (7a). Compound **7a** was synthesized by the same procedure as for **1a** except for using mesoporphyrin IX instead of protoporphyrin IX. Yield 10%; R_f: 0.4 ($CHCl_3$/CH_3OH = 20/1); IR (NaCl) ν = 1732 (C=O, ester), 1651 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 408, 506, 542, 576, 630 nm; ^{1}H-NMR ($CDCl_3$) δ: 0.8 (m, 3H, CH_3–CH_2–O–), 1.6 (m, 2H,–CH_2–CH_2–Im), 1.8 (t, 6H, CH_3–CH_2–Por), 2.9 (m, 2H, CH_3–CH_2–O–), 3.1 (m, 4H, –CH_2–COO–), 3.2 (m, 2H, –NH–CH_2–), 3.6 (m, 12H, CH_3–Por), 3.8 (m, 2H,–CH_2–Im), 4.1 (m, 4H,CH_3–CH_2–Por), 4.4 (m, 4H, Por–CH_2–), 6.6 (s, 1H, –NHCO–), 6.0–6.8 (d, 3H, Im), 10.0 (m, 4H, *meso*); MS *m*/*z*: 699.32.

Fe(III) complex of 7a (7b). Iron insertion to **7a** was carried out by the same procedure as in the **1b** preparation. Yield 62%; R_f: 0.2 ($CHCl_3$/CH_3OH = 6/1); IR (NaCl) ν = 1732 (C=O, ester), 1668 (C=O, amide) cm^{-1}; UV-vis (DMF) λ_{max} = 394, 566, 591 nm; MS *m*/*z*: calcd for $C_{42}H_{49}O_3N_7Fe$: 755.3292, found 755.3246 [M^+].

3,18-Diacetyl-8-(3-carboxy)ethyl-12-(3-(*N*-imidazolyl)propyl-amido)ethyl-2,7,13,17-tetramethylporphyrin (8a). Compound **8a** was synthesized by the same procedure as for **1a** except for using diacetyldeuteroporphyrin IX instead of protoporphyrin IX. Yield 27%; R_f: 0.1 ($CHCl_3$/CH_3OH = 6/1); IR (NaCl) ν = 1735 (C=O, ester), 1651 (C=O, amide, ketone) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 423, 516, 551, 586, 640 nm; ^{1}H-NMR ($CDCl_3$) δ: 1.5 (m, 2H, –CH_2–CH_2–Im), 2.9–3.1 (M, 4H, –CH_2–Im, –NH–CH_2–), 3.2–3.3 (m, 16H, –CH_2–COO, CH_3–Por), 3.4 (m, 6H, CH_3–CO–), 3.6 (m, 3H, CH_3–OCO–), 4.1 (m, 4H, Por–CH_2–), 6.0 (d, 1H, Im), 6.6 (m, 1H, Im), 6.9 (m, 1H, Im), 10 (m, 4H, *meso*); MS *m*/*z*: 712.

Fe(III) complex of 8a (8b). Iron insertion to **8a** was carried out by the same procedure as in the **1b** preparation. Yield 64%; R_f: 0.1 ($CHCl_3$/CH_3OH = 6/1); IR (NaCl) ν = 1735 (C=O, ester), 1651 (C=O, amide, ketone) cm^{-1}; UV-vis (DMF) λ_{max} = 418, 550, 578 nm; HR-MS *m*/*z*: calcd. for $C_{41}H_{43}O_5N_7Fe$: 769.2675, found 769.2697 [M^+].

Preparation of ferrous complex in DMF solution

The central Fe(III) ion of the porphyrin derivatives were reduced to the Fe(II) state using the complex of 18-crown-6 ether with $Na_2S_2O_4$ in DMF under aerobic conditions as previously reported.[8]

Preparation of rHSA–heme

Aqueous ascorbic acid (0.2 M, 10 μL) was added to an ethanol solution of the hemin derivative (2 mM, 1 mL) under a CO atmosphere. After complete reduction of the central Fe(III) ion, the ethanol solution (2 mM, 25 μL) was injected into the phosphate buffer solution (1 mM, pH 7.3, 2.5 mL) of rHSA (20 μM) under an Ar atmosphere. The formation of carbonyl rHSA–heme was confirmed by its UV-vis spectrum. The binding ratio of heme to rHSA was estimated by each concentration. The heme concentration was measured by the assay of iron ion using inductively coupled plasma spectrometry (Seiko, SPS7000A). The rHSA concentration was determined by bromocresol green along with the Albumin Test Wako kit (Wako Pure Chemical Industries).

Measurement of O_2 binding ability

The half-life of the O_2 adduct complex was determined by the time course of spectral changes, and the O_2 binding affinity ($P_{1/2}$) was determined by spectral changes at various partial pressures of O_2 according to previous reports.[2,15] rHSA–heme concentrations of 20 μM were normally used for UV-vis absorption spectroscopy. The spectra were recorded within the range of 350–700 nm.

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