Cross-Linked Human Serum Albumin Dimer Incorporating Sixteen (Tetraphenylporphinato)iron(II) Derivatives: Synthesis, Characterization, and O_2-Binding Property

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ABSTRACT: Recombinant human serum albumin (rHSA) was dimerized by bis(maleimido)hexane through the free thiol at Cys34. The molecular mass of the dimer [$(rHSA)_2$] was determined by native PAGE electrophoresis and MALDI−TOFMS spectrometry. As expected, the colloid osmotic pressure was only half that of the monomeric rHSA solution. Incorporation of (2-[8-{*N*-(2-methylimidazolyl)}-octanoyloxymethyl]-5,10,15,20-tetrakis(*o*-pivalamido)phenylporphinato)iron(II)s (FePs) into the hydrophobic cavities of the rHSA dimer provides a synthetic hemoprotein, [$(rHSA\text{-}FeP)_2$], which can reversibly bind and release dioxygen under physiological conditions (in aqueous media, pH 7.3, 37 °C) like hemoglobin and myoglobin. A maximum of *16* hemes (FePs) were incorporated into the $(rHSA)_2$ structure. On the basis of the isoelectric focusing measurement, the surface charge distributions of the $(rHSA)_2$ and $(rHSA\text{-}FeP)_2$ are identical to that of rHSA. The O_2-binding affinity ($P_{1/2}$: 30 Torr at 37 °C) and O_2-association and -dissociation rate constants of $(rHSA\text{-}FeP)_2$ (k_{on}, 2.4×10^7 M^{-1} s^{-1}; k_{off}, 4.7×10^2 s^{-1}) satisfy the requirements for a synthetic O_2 carrier as a red cell substitute.

Introduction

Human serum albumin (HSA) is the most abundant plasma protein, and it plays two major roles in our bloodstreams; (i) maintaining the colloid osmotic pressure (COP) and (ii) transporting many endogenous and exogenous compounds.[1,2] However, only 60% of the mass of the plasma proteins (5 g dL^{-1}), serum albumin is responsible for 80% of the COP, because of its lower molecular weight of 66.5 kDa relative to those of the globrins and its high negative net charges. We have found that a (tetraphenylporphinato)iron(II) derivative with a covalently linked axial imidazole, (2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamido)phenylporphinato)iron(II) (FeP), is incorporated into HSA and the obtained albumin−heme hybrid (HSA−FeP) can reversibly bind and release dioxygen under physiological conditions (in aqueous media, pH 7.3, 37 °C) in a fashion similar to hemoglobin and myoglobin.[3] A maximum of eight FeP molecules are included inside the HSA structure by hydrophobic interaction. The in vivo O_2-transporting efficacy of this artificial hemoprotein has also been evaluated by exchange transfusion into hemorrhagic shock rats.[3e] However, the HSA−FeP solution with a physiological HSA concentration (5 g dL^{-1}) can involve only 6 mM heme, which corresponds to 60% of the whole blood ([heme]: 9.2 mM). Of course, a highly condensed solution can dissolve more FeP, but the COP increases in proportion to the HSA content.

On the other hand, all mammalian albumins have a single thiol group resulting from an unpaired cysteine at position 34. The albumin dimers were first prepared using mercury(II) or oxidation with iodine through this special residue.[4] Unfortunately, these simple albumin dimers are unstable and dissociate to the monomer upon standing in the weakly alkaline solution.[5] On the basis of a recent X-ray diffraction analysis, the Cys34 thiol sits in a hydrophobic crevice with a depth of 9.5−10 Å;[6,7] therefore, direct dimerization may induce a distortion of the 10 Å pocket. Several polymerized HSA moieties, named *albumin microspheres* or *microbubbles*, were also prepared using gultaraldehyde or sonication.[8−11a] These spherical polymers more than 4 μm in diameter can act as agents of radiologic or ultrasonic imaging of particular areas of the circulatory system, and carriers of therapeutic drugs.[11b,c] However, the original high-ordered structure of albumin is all deformed in the polymer forms, and they segregate almost completely to the lung capillaries. To increase the heme concentration of the HSA−FeP solution while maintaining the COP constant, we designed a cross-linked HSA dimer for the host of FeP using a bifunctional bismaleimidohexane, which is very selective for thiol groups in neutral pH. At pH 7, the rate of reaction of maleimides with thiol is 1000-fold faster than with amines, and the produced thioether linkage cannot be cleaved under physiological conditions.[12]

We report herein for the first time, the synthesis, characterization, and O_2-binding properties of a new cross-linked rHSA hybrid incorporating *16* FeP molecules as O_2-binding sites. The $(rHSA\text{−}FeP)_2$ solution (10 g dL^{-1}) can dissolve the same amount of dioxygen relative to the whole blood at the physiological conditions expected in vivo.

Experimental Section

Materials. FeP was synthesized using our previously reported procedures.[13] A recombinant human serum albumin (rHSA, 25 wt %) was provided from Yoshitomi Pharmaceutical Ind., Ltd., and a natural plasma HSA was purchased from

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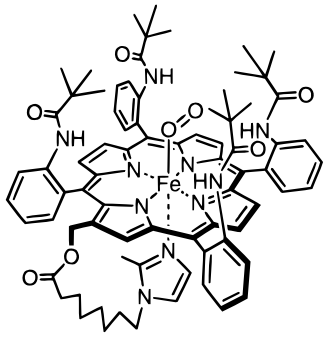
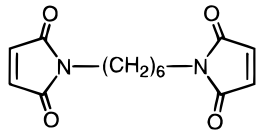


FeP



BMH

Bayer Co., Ltd. (Albumin Cutter, 5 wt %). Bismaleimidohexane (BMH) was purchased from Pierce Chemical Co.

Synthesis of rHSA Dimer. rHSA (25 wt %, 8.8 mL) and BMH (9.1 mg) were dissolved into 18.8 mL and 3 mL of phosphate buffered saline (PBS, pH 7.0, 10 mM), respectively. The BMH dispersion was sonicated for a few seconds. After nitrogen bubbling, each solution was slowly mixed and stirred for 12 h at 4 °C under a nitrogen atmosphere. The clear solution was then applied to gel permeation chromatography (Sephacryl S-100HR, $M_w = 5-250$ kDa, Pharmacia Co., Ltd.) with saline as the eluent at room temperature; the elution rate was approximately 0.5 mL min^{-1}. The fractionation was carefully monitored by a UV detector (AC-5100S, λ: 280 nm, ATTO) and the dimer band was collected. For further inspection, another gel permeation chromatographic run was performed on a Shimadzu LC-8A chromatograph equipped with a SPD-10A UV−vis detector using a Shodex GS-620HQ column at room temperature. The molecular weight of the rHSA dimer was measured by a Pharmacia Phastsystem using native PAGE in Phast Gel Gradient 8−25.[3c,d] The markers used are from an LMW Electrophoresis Calibration Kit. Finally, the pure rHSA dimer was concentrated by ultrafiltration (yield: 28%).

Molecular Mass of rHSA Dimer. The molecular mass was determined by a matrix-associated laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) system (Shimadzu Kompact MALDI 2). It was operated in the linear mode with an acceleration potential of 20 kV. The spectra were acquired in the positive ion mode and calibrated by bovine serum albumin. Sinapinic acid was used as the matrix.

Incorporation of FeP into rHSA Dimer. The inclusion of FeP into the rHSA dimer was carried out using the same method reported before,[3c] giving (rHSA−carbonyl FeP)$_2$ solution [FeP/(rHSA)$_2$ = 16 (mol/mol)]. During the preparation of the (rHSA−FeP−CO)$_2$ hybrid, the precipitated carbonyl−FeP was extracted with $CHCl_3$, and its concentration was assayed by the absorption spectrum. On the basis of the quantitative analysis of this free carbonyl−FeP molecule [ϵ_{max} (at 427 nm): 2.0×10^5 M^{-1} cm^{-1}], the binding numbers of FeP were determined.[3c,d] UV−vis absorption spectra were obtained on a Shimadzu V-570 spectrometer.

Physicochemical Properties. Isoelectric points were measured by a Pharmacia Phastsystem using isoelectric focusing (IEF) in pH 3−9 Phast Gel IEF 3−9.[3c,d] The temperature during the electrophoresis was maintained at 15 °C. The markers used are from an Isoelectric focusing calibration kit. The colloid osmotic pressure was measured by a Wescor 4420 colloidosmometer at 25 °C.

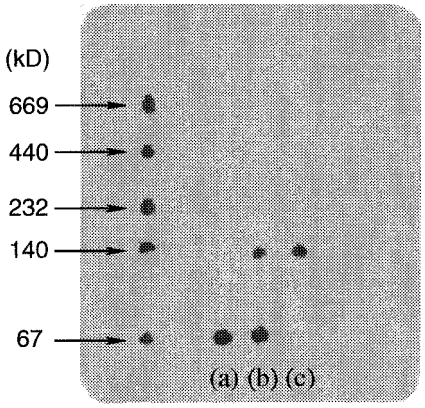


Figure 1. Native PAGE electrophoretic patterns of (a) rHSA, (b) rHSA after reaction with BMH for 12 h at 4 °C, and (c) isolated rHSA dimer.

O_2-Binding Equilibrium and Kinetics. O_2-binding to the FePs in (rHSA-FeP)$_2$ was expressed by

$$\mathrm{FeP} + \mathrm{O_2} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{FeP{-}O_2} \qquad (K = k_{on}/k_{off}) \tag{1}$$

The O_2-binding affinity (gaseous pressure at half O_2-binding for FeP, $P_{1/2} = 1/K$) was determined by UV−vis absorption spectral changes during O_2 titration.[3d,14−16] FeP concentrations of 10−20 μM were normally used for the absorption spectroscopy. The spectra were recorded within the range of 350−700 nm. The thermodynamic parameters for O_2 binding (ΔH, ΔS) were determined by the van't Hoff plots of K. The O_2-association and -dissociation rate constants (k_{on}, k_{off}) were determined using a Unisoku TSP-600 laser flash photolysis apparatus.[14−16]

Results and Discussion

Synthesis and Characterization of rHSA Dimer. The Cys34−Cys34 disulfide-linked HSA dimer has several disadvantages: (i) a side reaction of another residue during preparation, (ii) low stability, (iii) disordered structure of the albumin units, which induces decreased binding numbers of the ligands,[17] etc. Bifunctional bismaleimides are of interest as cross-linking agents for selective coupling between cystein residues. We used a hydrophobic and flexible bis(maleimide), namely bis(maleimido)hexane (BMH), because the thiol group of Cys34 of HSA is located in the hydrophobic pocket and its spacer length of 16.1 Å is suitable for the coupling. At the beginning of the reaction, BMH was not dissolved in PBS, but slowly disappeared during the reaction. The dimer formation was confirmed by the native PAGE measurement—the appearance of about a 140 kDa band (Figure 1)—and the GPC measurements, which displayed two significant peaks for the rHSA and rHSA dimer (Figure 2). After 12 h, the dimer's content became constant. The relatively low yield is probably due to the fact that part of the rHSA forms a mixed disulfide with cystine or glutatione. The pure rHSA dimer was isolated by Sephacryl-100 chromatography (Figure 2); the GPC profile represented a single peak, and the native PAGE showed a monospot of 140 kDa (Figure 1).

The molecular mass of the rHSA dimer (133 179.6 Da) was measured by matrix-associated laser desorption/ionization time-of-flight mass spectrometry (MALDI−TOFMS) (Figure 3). The rHSA dimers showed a sharp signal for a molecular mass of m/z 132 494 Da, which

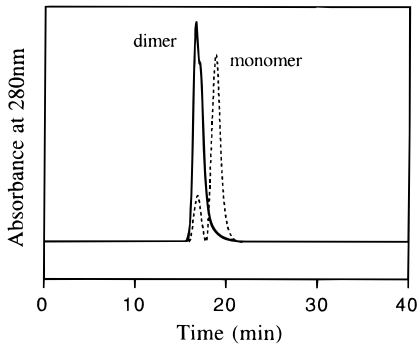


Figure 2. GPC profiles of rHSA dimer with bis(maleimido)-hexane: solid line, pure dimer fraction after the separation; dotted line, mixture after reaction with BMH for 12 h at 4 °C.

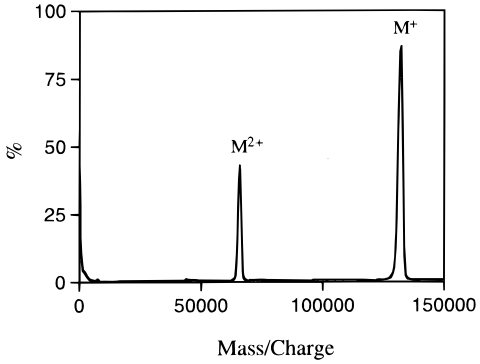


Figure 3. MALDI−TOFMS of rHSA dimer.

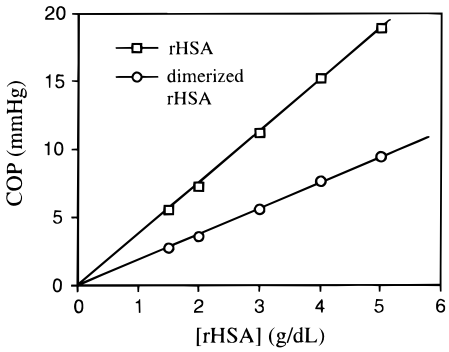


Figure 4. Colloid osmotic pressure of rHSA dimer solution and monomeric rHSA solution.

can be assigned to the molecular ion peak. The peak at *m*/*z* 66353.2 Da is probably the charge state 2+ of the rHSA dimer, but the intensity was somewhat sharp and strong for the charged state. The rHSA dimer might be partially dissociated to the monomeric form during the sample preparation with sinapinic acid and/or the laser ionization.

As expected, the colloid osmotic pressure (COP) of the $(rHSA)_2$ solution (5 g dL^{-1}) showed 9 mmHg, which is only half that of rHSA (Figure 4). Since the surface charge distribution of the dimer was not changed (vide infra), the essential biological roles as serum albumin, i.e., control of COP, plasma expansion, and ligand binding capability, are probably sustained after the dimerization.

The maximum molecular number of FeP incorporated into certain domains of monomeric HSA was eight with binding constants from 10^6-10^4 M^{-1}.[3b,c] If the three-dimensional structure of the rHSA unit in the cross-linked dimer is not changed, 16 FePs can be bound into $(rHSA)_2$. Sollenne and co-workers reported that the

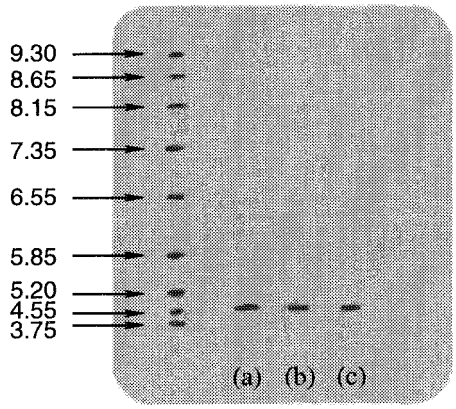


Figure 5. IEF PAGE electrophoretic patterns of (a) rHSA, (b) isolated rHSA dimer, and (c) $(rHSA-FeP)_2$.

L-tryptophane could not bind to the disulfide HSA dimer, but strongly binds to the monomeric HSA.[13] The absence of the L-tryptophane binding site in the dimer presumably reflects its inclusion within the dimer interface. On the basis of the quantitative analyses of the free carbonyl−FeP in the $(rHSA-FeP)_2$ solution using a previously reported procedure (see Experimental Section),[3c,d] the binding ratios were calculated to be 99% for $FeP/(rHSA)_2 = 16$ (mol/mol). Therefore, the maximum binding numbers of FeP to an rHSA were determined to be *16*.

The isoelectric points (pI) of the $(rHSA)_2$ and $(rHSA-FeP)_2$ were all 4.8, which was exactly the same as that of the monomeric rHSA (Figure 5). The FeP molecule without any ionic side chains nonspecifically interacts with a hydrophobic cavity of the rHSA dimer, so its surface net charges are always identical. We thus concluded that hydrophobic interaction is the major force of the FeP binding to $(rHSA)_2$ and that its incorporation does not induce any changes in the surface charge distribution of the host rHSA dimer.

O_2−Binding Property. The UV−vis absorption spectrum of the aqueous rHSA dimer including the carbonyl FeP complex $(HSA\text{-}FeP-CO)_2$ showed a typical low-spin (tetraphenylporphinato)iron(II) derivative (λ_{max}: 427, 539 nm). Light irradiation with a lamp on this solution under flowing O_2 led to CO dissociation yielding the O_2-coordinated species (λ_{max}: 426, 552 nm). Upon exposure of $(rHSA\text{-}FeP-O_2)_2$ to argon, the UV−vis absorption spectrum changed to that of a five-N-coordinated species (λ_{max}: 442, 543, 569 nm). This dioxygenation was reversible over the range of Po_2 expected in vivo, e.g., 40−110 Torr, and stable under physiological conditions; the half-life ($\tau_{1/2}$) of the O_2-coordinated species was 8 h at 25 °C and 2 h at 37 °C. The O_2-binding affinities [$P_{1/2}$: O_2 pressure at half O_2 binding for the (porphyrinato)-iron(II)] and its thermodynamic parameters (ΔH, ΔS) were determined (Table 1).[3d,14−16] The $P_{1/2}$ value of $(rHSA\text{-}FeP)_2$ (30 Torr at 37 °C) was almost identical to that of red blood cells (27 Torr),[18] and its O_2-binding equilibrium curve showed Langmuir type absorption; the Hill coefficient was 1.0 (Figure 6). It is known that cooperativity observed in the O_2 binding to hemoglobin has implications for physiological use. Nevertheless, it is also the fact that 78% of $(rHSA\text{-}FeP)_2$ is dioxygenated at the O_2-pressure in the lungs (P_{O_2}: ca. 110 Torr) and only 56% is dioxygenated in muscle tissue (P_{O_2}: ca. 40 Torr). Consequently, the O_2-transporting efficiency (OTE) of $(rHSA-FeP)_2$ between the lungs and muscle tissue

Table 1. O_2-Binding Affinities, Thermodynamic Parameters, and Kinetic Parameters of (rHSA-FeP)$_2$ in Phosphate Buffer (pH 7.3) at 25 °C

	$P_{1/2}$/Torr	ΔH/kJ mol^{-1}	ΔS/J K^{-1} mol^{-1}	$10^{-7}k_{on}$/ M^{-1} s^{-1}	$10^{-2}k_{off}$/ s^{-1}
(rHSA-FeP)$_2$	12 (30)	−61	−116	2.4	4.7
rHSA-FeP	13 (33)	−59	−109	2.7	6.0
Hb (T-state)α[b]	40	−57 to −65[c]	−116 to −133[c]	0.29	1.8
Mb[d]	0.37−1	−63 to −88[e]	−159 to −234[e]	1−2	0.1−0.3
red blood cells	9 (27)				

[a] At 37 °C in parentheses. [b] pH 7, 20 °C, ref 18. [c] pH 7.4, ref 19. [d] pH 7−7.4, 20 °C, ref 14. [e] Reference 20.

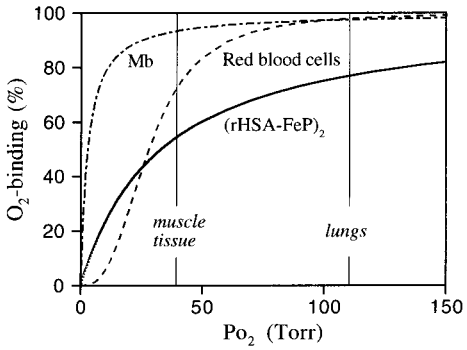


Figure 6. O_2-binding equilibrium curve of (rHSA−FeP)$_2$ at 37 °C.

becomes 22%, which is nearly the same (23%) as that of the red blood cells.

On the basis of the laser flash experiments, the O_2-association and -dissociation rate constants (k_{on}, k_{off}) of (rHSA−FeP)$_2$ were determined (Table 1).[3d,14−16] It has also recently been found that the O_2 recombination of rHSA−FeP obeyed three-phase kinetics.[3d] In the case of (rHSA−FeP)$_2$, the assumption of multiphase rebinding of dioxygen is too complicated, so we employed the monophasic first-order reaction of O_2 association. It is remarkable that these apparent k_{on} and k_{off} values were almost same as those for the monomeric rHSA−FeP.

In conclusion, recombinant human serum albumin was dimerized by a hydrophobic cross-linker, bis-(maleimido)hexane, providing a stable rHSA dimer which can absorb (tetraphenylporphinato)iron(II) derivatives as O_2-binding sites. The (rHSA−FeP)$_2$ can act as a totally synthetic O_2-carrying hemoprotein instead of hemoglobin. The major molecular force of the FeP binding is a hydrophobic interaction like monomeric rHSA−FeP, which did not change the physicochemical properties of the rHSA units. The O_2-binding ability ($P_{1/2}$ = 30 Torr; OTE = 22%, etc.) should satisfy the clinical requirements for an artificial O_2 carrier as a red blood cell substitute. Moreover, the (HSA−FeP)$_2$ solution (10 g dL^{-1}), which shows physiological COP value, can dissolve the same amount of dioxygen as the whole blood at the range of P_{O_2} expected in vivo.

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