(O_2)-carrying albumin could be of extreme medical importance, however, it has also been known that the HSA-hemin hybrid is not able to reversibly coordinate O_2.

We have found that eight molecules of 2-[8-(2-methyl-1-imidazolyl)octanoyloxymethyl]-5,10,15,20-tetrakis[(α,α,α,α-*o*-pivaloylamino)phenyl]porphinatoiron(II) (FeP) are efficiently incorporated into recombinant HSA (rHSA),[3] which is now manufactured on a large scale by expression in *Pichia pastoris* as a host cell (Fig. 1).[4] The obtained synthetic hemoprotein (albumin-heme: rHSA-FeP) can bind and release O_2 under physiological conditions similar to hemoglobin and myoglobin, and showed a good compatibility with human whole blood. Hence, rHSA-FeP is recognized to be a synthetic O_2-carrying hemoprotein and one of the most promising materials not only as a blood replacement composition, but also as an O_2-delivering medicine.[5] In order to characterize the coordination structure of the active site in rHSA-FeP with O_2 and carbon monoxide (CO), we employed magnetic circular dichroism (MCD), resonance Raman (RR), and infrared (IR) spectroscopies. The electronic property and spin-state of the dioxygenated or carbonyl FeP in the rHSA host are described herein.

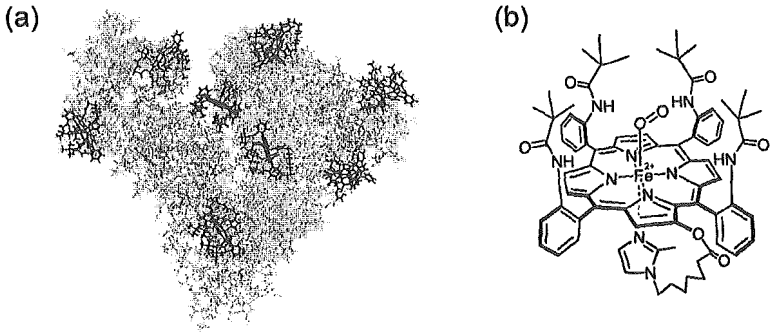

Figure 1. (a) The predicted three dimensional structure of rHSA included eight FeP molecules, (b) molecular structure of dioxygenated FeP.

Results and Discussion

a. MCD Spectroscopy

The application of MCD to natural hemoproteins and model hemes has been extensively studied as a fingerprint method for determining the oxidation state, spin state, and coordination structure

of the heme.[6] Under an N_2 atmosphere, the MCD spectrum of rHSA-FeP showed the formation of the five-coordinate ferrous high-spin complex of FeP with intramolecular coordinated proximal imidazole (Fig. 2). This result showed no ligation of the amino-acid residues of the protein, *ex.* histidine, tyrosine, cysteine, to the six-coordination site of the heme.[6a),c)] The X-ray analytical data of ferric hemin incorporated into the subdomain IB of HSA showed that the central Fe(III) ion is tightly bound to Tyr-161 to form a five-coordinate complex.[2] The bulky pivalamido-fences on the porphyrin plane could prevent access of the neighboring peptide residues to the six-coordination site of FeP. Upon exposure of this solution to O_2, the spectrum immediately changed and showed an S-shaped A-term MCD in the Soret region, which indicates a transformation to the ferrous low-spin complex.[6b)] The carbonyl rHSA-FeP also exhibited a similar A-term MCD band in the same region with a much stronger intensity. The wavelength where the value of $[\theta]_M$ is zero for the O_2- and CO-adduct complex coincided well with the absorption maxima in their corresponding UV-vis. spectra (Table 1). In all cases, the complicated shapes in the Q-band regions are similar to those of the previously reported studies (not shown).[6b)]

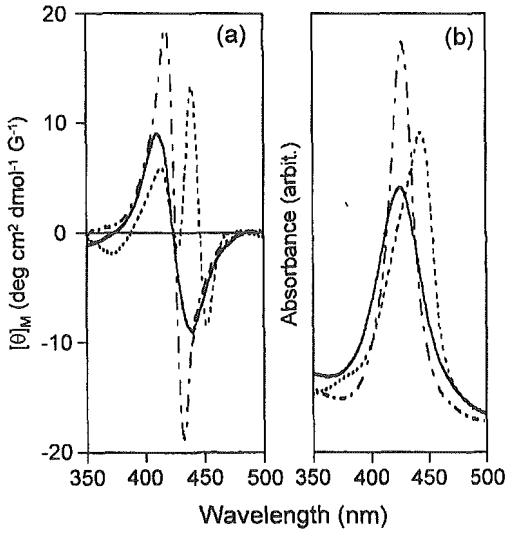

Figure 2. (a) MCD and (b) UV-vis. spectral changes of rHSA-FeP in phosphate buffer solution (pH 7.3) at 25°C: under N_2: - - - -, under O_2: ——, under CO: -·-·-.

Table 1. Absorption maximam wavelength (nm) of MCD ($[\theta]_M$) and UV-vis. spectra of rHSA-FeP in phosphate buffered solution (pH 7.3) at 25 °C.

	MCD	UV-vis.
N_2	371 (–4.6), 414 (+9.4), 428 (–1.1), 440 (+22), 452 (–13)	440
O_2	411 (+15), 435 (–8.6)	426
CO	418 (29), 433 (–7.1)	426

(+) positive, (–) negative.

b. RR and IR Spectroscopy

The RR spectrum of the rHSA-FeP solution under an N_2 atmosphere showed a medium but distinct band at 201 cm^{-1}, which corresponded to the characteristic Fe(II)-N(imidazole) stretching mode ($\nu_{Fe\text{-}N\varepsilon}$) of the five-coordinate ferrous high-spin porphyrin, and was quite identical to that of FeP in CH_2Cl_2 (Table 2).[3a),b),7,8] This means that unusual hindrance of the axial imidazole coordination of FeP is never accomplished in albumin. After exposure to O_2, the $\nu_{Fe\text{-}O2}$ vibration of dioxygenated FeP appeared at 564 cm^{-1}, suggesting an end-on type O_2-coordination.[7,8] The deformation modes of the porphyrin ring (ν_8 and ν_4) were also upshifted from 376 and 1356 cm^{-1} to 393 and 1371 cm^{-1}, respectively. These changes further supported the conversion from the five-coordinate high-spin to the six-coordinate low-spin spcies.

Table 2. The stretching frequencies (cm^{-1}) of coordinated O_2, CO and Fe(II)-N_ε, and Fe(II)-O_2 of rHSA-FeP at 25°C.

	solvent	ν^{16}_{O2}	ν^{18}_{O2}	ν_{CO}	$\nu_{Fe\text{-}N\varepsilon}$	$\nu_{Fe\text{-}O2}$
rHSA-FeP	p.b.[a] (pH 7.3)	1158	1079	1964	201	564
FeP[b]	CH_2Cl_2	1160	1079	1964	201	565
FepivP(DIm)[c]	Nujol/CH_2Cl_2	1159	1093	1969	200	564
Myoglobin[d]	p.b.[1] (pH 7.2)	1103	1065	1944	221	572
O_2 or CO (gas)[e]	–	1556	–	2143	–	–
O_2^- (metal-O_2^-)[e]	–	1100–1150	–	–	–	–

[a]p.b.: Phosphate buffer. b) Ref. [8]. c) FepivP: 5,10,15,20-Tetrakis[(α,α,α,α-*o*-pivaloylamino) phenyl]porphinatoiron(II), DIm: 1,2-Dimethylimidazole, Ref. [9]. d) Ref. [10], [11]. e) Ref. [12].

From the difference IR spectrum of the rHSA-FeP solution under $^{16}O_2$ and CO, we assigned the vibration stretching modes of the coordinated O_2 (ν^{16}_{O2}) and CO (ν_{CO}) to 1158 cm^{-1} and 1964 cm^{-1}, respectively (Table 2). Under $^{18}O_2$, the ν^{18}_{O2} appeared at 1079 cm^{-1}; the frequency shifting between the coordinated $^{16}O_2$ and $^{18}O_2$ is in good agreement with the calculated value from the harmonic oscilator prediction of the O-O stretching vibration. The ν_{O2} value was close to those of ionic superoxides and the end-on type O_2 bonding to the similar tetraphenylporphinatoiron(II) derivatives.[9] It is remarkable that the ν_{O2} and ν_{CO} values of rHSA-FeP are almost the same as those of FeP itself in CH_2Cl_2 solution. If the coordinated O_2 and CO have some weak interaction

with the surrounding amino-acid residue, they should be siginificantly shifted. Nevertheless, it has not been seen. Based on these findings, we can conclude that (i) the coordination geometry of O_2 to FeP in albumin is a bent/end-on configuration in the same manner as hemoglobin and myoglobin, and (ii) the micro-protein environment around FeP does not affect the coordinated O_2 and CO ligands.

Conclusion

The coordination structure and spin-state of the active site in the synthetic O_2-carrying hemoprotein, *albumin-heme*, has been elucidated by MCD, RR, and IR spectroscopy. Under an N_2 atmosphere, all the FePs produce a five-coordinate high-spin complex with an intramolecularly coordinated axial imidazole similar to FeP itself in a non-ligated solvent, such as toluene and CH_2Cl_2. In the hydrophobic cavity of rHSA, the amino-acid residues may bind to the six-coordination site of the intercalated FeP, however, the four rigid pivaloyl-fences on the porphyrin ring protect the access of the peptide residue as a base. This is in contrast to the fact that planar protoporphirinatoiron(III) tightly coordinates to the Tyl-161 in the subdomain IB of HSA.[2] The coordination structure and electronic state of the dioxygenated and carbonyl complex of FeP in rHSA also showed a similarity to those in the organic solvents. Although the association kinetics of these gaseous ligands to rHSA-FeP are largely affected by the protein environment around the indivisual FeP,[3c)] the coordinated O_2 and CO are efficiently shielded by the four pivalamido-substituents which are presented as the nearest neighbor, and not influenced by the surrounding peptide residues.

This work was partially supported by the Health Science Research Grants (Artificial Blood Project) of the Ministry of Health, Labor, and Welfare, Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports, Japan.

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Microcalorimetry Investigation of Synthetic Hemoprotein (Albumin-heme)

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ABSTRACT

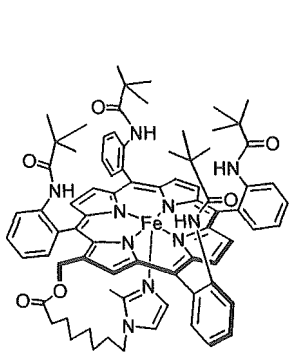
Recombinant human serum albumin (rHSA) incorporating the iron(II) complex of the tetraphenylporphyrin derivative (FepivP or FecycP) is a synthetic O_2-carrying hemoprotein [albumin-heme (rHSA-FepivP or rHSA-FecycP)], which acts as a red blood cell substitute. The association and dissociation behavior of FepivP and FecycP with rHSA has been initially investigated by isothermal titration calorimetry. A strong heat release appeared after the injection of albumin-heme into a large molar excess of rHSA. This exothermic enthalpy change was due to the transference of hemes to the other free albumins. The difference in the heme binding affinity to rHSA can be manifested in the enthalpy term. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: synthetic hemoprotein; albumin-heme; calorimetry; biomaterials; heat capacity

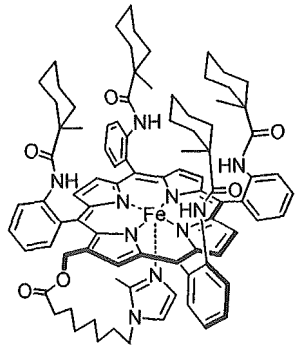
INTRODUCTION

Recombinant human serum albumin (rHSA) including the synthetic heme, 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivaloylamino)phenylporphinatoiron(II) (FepivP) or 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis{α,α,α,α-*o*-(1-methylcyclohexanoyl)amino] phenylporphinatoiron(II) (FecycP) (albumin-heme; rHSA-FepivP or rHSA-FecycP) is an artificial hemoprotein, which can reversibly bind and release O_2 in aqueous solution (pH 7.3) [1–4].

The recent results on the physiological responses and O_2-transporting efficacy of rHSA-hemes definitely proved that it satisfies the initial clinical requirements for a red blood cell substitute [5, 6]. It has also been found that a maximum of eight heme molecules are accommodated into certain domains of one albumin molecule by a hydrophobic interaction [2]; which is twice the heme molecules in hemoglobin. Nevertheless, the incorporated heme derivatives may dissociate from rHSA and transfer to the other components in the blood stream, for instance, free plasma albumin, if the non-covalent binding force is not strong enough. Unfortunately, it is not very easy to elucidate the transference of hemes from the rHSA-heme to



FepivP



FecycP

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other albumins in the circulatory system. Therefore it is reported for the first time the association and dissociation behavior of FepivP and FecycP to rHSA by microcalorimetry analysis using inductive titration calorimetry (ITC). In addition, their transference rate constants were also determined.

EXPERIMENTAL

Preparation of rHSA-heme Solution

The FepivP and FecycP were synthesized as described in the literature [3]. The rHSA (25 wt%) was provided by the NIPRO Corporation. The rHSA-heme solution ([heme]/[rHSA]: 1–8 (molar ratio), [rHSA]: 5 wt%, in phosphate buffered saline solution (pH 7.3)) was prepared according to our previously reported procedure with some modifications [7]. Finally, the red-colored solution was filtered using a DISMIC 25CS045AS.

Dialysis of rHSA-heme

rHSA-FepivP_8 was diluted using phosphate buffer solution (pH 7.3) to make a final concentration of 20 μM for FepivP ([rHSA]: 0.015 wt%). Of this solution 5 ml was sealed in a cellulose acetate membrane tube (cut off size, 14 kDa, Viskase Companies, Inc.) and dialyzed against 0.015 wt% albumin in phosphate buffer (100 ml, pH 7.3) for 24 hr at room temperature. The UV-vis absorption spectra were recorded using a JASCO V-570 spectrophotometer, and all the measurements were normally carried out at 25 °C.

Microcalorimetry Analysis

The microcalorimetry measurements were carried out using a Microcal MCS ITC CELL (Fig. 1). The rHSA solution (2 ml, 0.23 mM) was first injected into the sample titration cell and kept at 37 °C by heating the cell. When the baseline was constant, the rHSA-heme solution ([rHSA]: 0.23 mM) was stepwise injected into the sample titration cell by the injection controller (each time 10 μl, time between injections is 300 sec) at 37 °C. The albumin solution (0.23 mM) was also injected into the albumin solution in the sample titration cell for determining the control value.

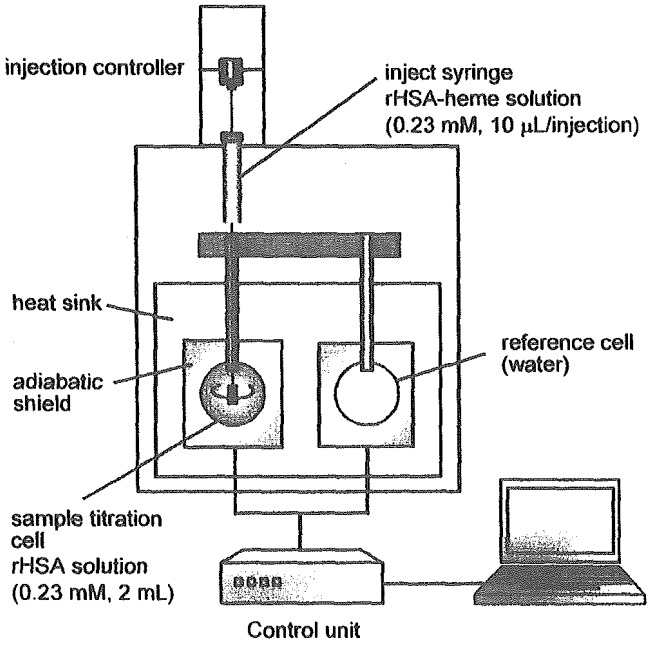

FIGURE 1. Isothermal titration calorimetry (ITC) apparatus.

RESULTS AND DISCUSSION

There are two possibilities for the transference of heme from rHSA-heme to the other free rHSA: the first is through the bulk aqueous solution, and the second is direct transfer when the both molecules encounter and contact each other. The first route was studied by dialysis of the rHSA-heme solution against the aqueous albumin solution. The cutoff size of the dialysis membrane (M_w 14 kDa) used is much smaller than the molecular weight of rHSA, but is large enough for the hemes to pass through. Thus, rHSA-heme has no chance to meet rHSA, and it provides the only possibility for a dissociated heme to cross the membrane and rebind to the free rHSA molecules. This would increase the heme absorption in the UV-vis spectrum ($\lambda_{max} = 425$ nm) 425 nm) of the outer rHSA solution. Our dialysis results indicated that the heme concentration in the inner phase remained unaltered, and absolutely no heme absorbance was observed in the outer phase (Fig. 2). These dialysis experiments clearly showed that it is impossible for hemes to transfer through bulk aqueous solution, probably due to the extremely low solubility of FepivP and FecycP in water.

This result was again confirmed by microcalorimetry measurements using ITC while rHSA-FepivP_8 was added to a phosphate buffer solution (pH 7.4, 37 °C). In general, the mixing of two solutions led to very little thermal change via solvation, dissociation (exothermic change), association

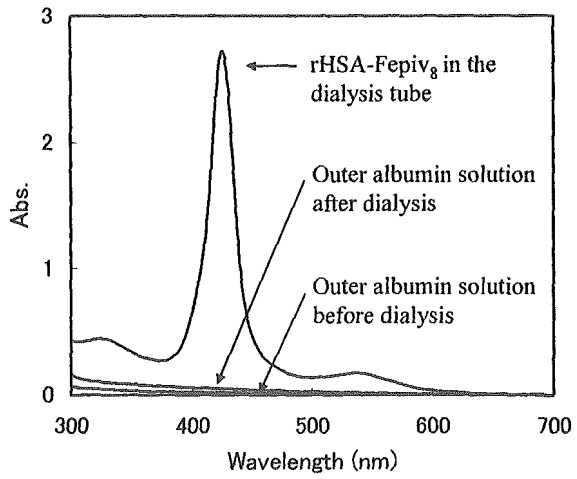

FIGURE 2. UV-vis absorption spectra for the dialysis experiment of rHSA-FepivP_8 in the rHSA solution after 24 hr at 4 °C.

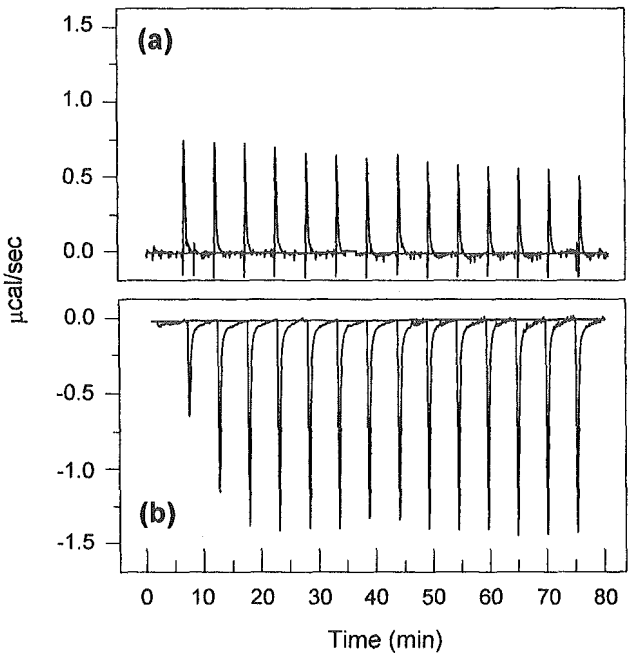

FIGURE 3. Microcalorimetry changes in the ITC measurements: (a) rHSA injection into phosphate buffer solution; (b) rHSA-FepivP$_8$ injection into a large molar excess of rHSA solution.

(endothermic change), or other physical effects. The microcalorimetry measurement is useful to detect such minute thermodynamic differences and help to analyze the weak interaction between the molecular components among the mixed system (Fig. 1) [8]. The injection of rHSA into the phosphate buffer solution showed a small endothermic change (0.5 μcal/sec), which is probably due to the solvation effect of albumin in water (Fig. 3a). Regarding rHSA-FepivP$_8$, if the dissociation of FepivP takes place, the observed endothermic peak would increase compared to the case of rHSA alone. However, the additions of rHSA-FepivP$_8$ showed identical endothermic peaks, which implies that rHSA-FepivP$_8$ is stable in the aqueous buffer solution without heme dissociation.

The ITC measurements were further used to analyze the interaction between the rHSA-heme and free rHSA molecules. In our experimental setup, the large excess moles of rHSA (0.23 mM, 2 ml) was maintained in the sample titration cell at 37 °C, providing enough acceptors for the dissociated heme ([heme]/[rHSA in the cell] = 1/50) in the chamber. Under these conditions, the transference of heme from the rHSA-heme to the free rHSA molecules will contribute all of the heat energy change that occurs after the mixing. Indeed, repeated titrations of rHSA-FepivP$_8$ into the rHSA solution demonstrated significant negative ITC peaks, which means that exothermic reactions occurred with each injection (Fig. 3b). This observation implied that the transference of FepivPs to the other free albumin occurs and makes a more stable form than that of rHSA-FepivP$_8$, resulting in a decrease in the total energy for the entire system.

An FepivP analogue, FecycP, contains 1-methylcyclohexanoyl groups, which gives the porphyrin platform a greater hydrophobic property. The rHSA-FecycP hybrid showed the same O_2-equilibria and O_2-kinetics parameters as those of rHSA-FepivP, but its half-life time ($\tau_{1/2}$) of the dioxygenated species is five-fold longer than that of rHSA-FepivP [3]. The difference in the fence-structure on the porphyrin plane significantly affects to the stability of the O_2-adduct complex. Upon injection of rHSA-FecycP$_5$ into the excess moles of albumin, a strong exothermic change was also observed like that seen in rHSA-FepivP$_8$. The more hydrophobic FecycP showed larger enthalpy changes during the transference compared to those of FepivP. For instance, the exothermic change of rHSA-FecycP$_4$ is four-fold higher than that of rHSA-FepivP$_4$. This coincided with the fact that FecycP has a large binding constant to rHSA (e.g. K_1 for FecycP = 6.0×10^6 M^{-1}; K_1 for FepivP = 1.6×10^6 M^{-1}).

In order to carefully evaluate the thermodynamic properties of the FepivP and FecycP bindings to rHSA, several rHSA-FepivP and rHSA-FecycP hybrids with different [heme]/[rHSA] molar ratio were prepared. The ITC results showed that the released heat energy gradually decreased and was accompanied by a decrease in the binding numbers of the heme (Fig. 4). In principle, all the negative peaks due to the heme transference should include an endothermic component due to heme dissociation from the rHSA-heme. Although the maximum binding number of heme in one rHSA molecule was eight, the binding constant of the eighth heme for rHSA ($K_8 = 1.3 \times 10^4$ M^{-1}) is *ca.*

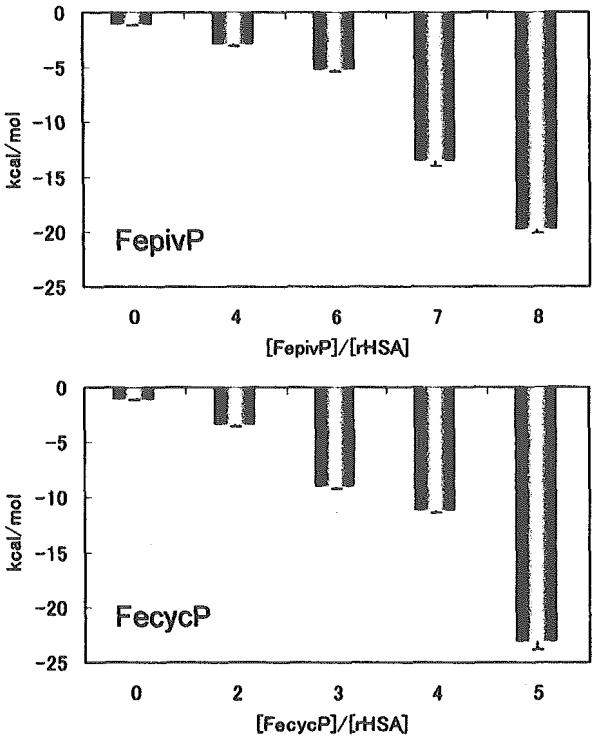

FIGURE 4. The exothermic enthalpy changes observed after the injections of rHSA-FepivP and rHSA-FecycP into a large molar excess of rHSA solution.

TABLE 1. Averaged Enthalpy Changes Per Mole of Heme (ΔH_{heme}) and Apparent Transference Rate Constants of Hemes (k_{app}) in the Titration of rHSA-heme into the rHSA Solution at 37 °C

Heme/rHSA	rHSA-FepivP				rHSA-FecycP			
	4	6	7	8	2	3	4	5
$-\Delta H_{heme}$ (kcal mol^{-1})	0.6	0.8	2.1	2.7	2.4	4.0	3.4	5.5
k_{app} (10^2 M^{-1} sec^{-1})	15.5	26.1	35.5	39.1	1.7	4.3	5.9	9.3

1/90 lower than the initial constant K_1 of 1.2×10^6 M^{-1}[2]. Since the concentration of the free albumin in the chamber was high enough, the weakly bound hemes would immediately jump into the other albumins to form a more stable complex. In fact, rHSA-FecycP$_2$ still showed a slight exothermic enthalpy change, representing the fact that even the second FecycP in rHSA moves to the other free rHSA. We then calculated the averaged enthalpy change per mole of heme (ΔH_{heme}) in all cases (Table 1). The ΔH_{heme} values increased by increasing the heme numbers. If the assumption is true, the injection of rHSA-heme$_n$ into the large excess of rHSA leads to dissociation of the $(n-1)$ hemes from the hybrid, and produces the $(n-1)$ molecules of rHSA-heme$_1$. In this case, the exothermic enthalpy change for the each association process of the heme to the highest affinity site in the other free rHSA is constant. Nevertheless, the overall ΔH_{heme} values increased by increasing the heme numbers. This means that the endothermic enthalpy component due to the heme dissociation decreased in the high heme/rHSA region. The hemes bound at the low binding affinity sites of the rHSA-heme are easily transferred to the other rHSA. The difference in the heme binding affinity to rHSA can be manifested in the enthalpy term.

The apparent heme transference rate constants were also calculated from the ITC kinetics (Table 1). The slower transference of FecycP implied the stronger binding properties with rHSA than that of FepivP.

CONCLUSIONS

The ITC measurement provides a new method to investigate the association and dissociation behavior of the heme derivatives with the rHSA molecule. The heme transference from rHSA-heme to the other free rHSA did not occur through the bulk aqueous solution, but was observed when the rHSA-heme was mixed with the large molar excess of albumin. More stable structures of the rHSA-heme could be formed by the immediate distribution of the hemes molecules. rHSA-FecycP is presumably a more stable structure with less heme transference than rHSA-FepivP in the circulatory system.

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原著

テトラ(シクロヘキサノイル基)を有するヘムを包接したヒト血清アルブミン-ヘム複合体の酸素結合反応

Oxygen-Binding Reaction of Human Serum Albumin Hybrid Including Heme with Tetra(cyclohexanoyl)-Groups

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Teruyuki Komatsu, Seiji Ishihara, Eishun Tuchida

和文抄録

組換えヒト血清アルブミン（rHSA）に4つのピバロイル基を有するテトラフェニルポルフィリン鉄（ヘム）錯体（FepivP）を包接させて得たアルブミン-ヘム複合体は，生理条件下で酸素を可逆的に吸脱着できる人工酸素運搬体（赤血球代替物）である．本報では，アルブミン-ヘム酸素錯体のさらなる安定度向上を目指し，ヘム中心鉄近傍に疎水性の高いシクロヘキサノイル基を4つ導入した新しいヘム誘導体（FecycP）を合成，それを包接させたrHSA複合体の構造，酸素及び一酸化炭素結合能，血液適合性を明らかにしたので報告する．rHSA 1分子に対するFecycPの最大包接数は8．rHSA-FecycPとrHSAの等電点，CDスペクトルパターンが一致したことから，ヘム包接後もアルブミンの二次構造に変化はないと考えられる．酸素親和性（$P_{1/2}^{O_2}$）は34Torr（37℃），酸素結合に伴う熱力学パラメーター（⊿H，⊿S）は包接ヘム数に依存せず-59 kJ mol^{-1}，-108 JK mol^{-1}であった．レーザーフラッシュホトリシス法を用いて，酸素及び一酸化炭素の結合解離速度定数（k_{on}，k_{off}）を決定．気体分子の再結合過程は多相性を示し，包接サイト近傍分子環境の影響を受けているものと推察された．酸素錯体半減期（9 h，37℃）は，従来系（rHSA-FepivP）に比べて長く，この値はミオグロビンに匹敵する値であった．得られた赤色のrHSA-FecycP水溶液（[rHSA]: 5 g dL^{-1}, FecycP/rHSA=4, 8 (mol/mol)）は室温での長期保存も可能．rHSA-FecycPと全血液を1:1（v/v）の割合で混合した場合も，凝集・沈殿を認めず，血液適合性も高い．

Abstract

Recombinant human serum albumin (rHSA) incorporating tetraphenylporphinatoirn(II) (heme) derivative with four pivaloyl-groups (FepivP), albumin-heme, is an artificial oxygen-carrier (red cell substitute), which can reversibly bind and release oxygen under physiological conditions. This paper describes for the first time the structure, oxygen- and carbon monoxide-binding abilities, and blood compatibility of an rHSA hybrid including new heme derivative having more hydrophobic cyclohexanoyl-groups around the central iron (FecycP), rHSA-FecycP. The maximum binding numbers of heme to one albumin molecule were determined to be eight. Since the isoelectric point and CD spectral pattern of rHSA-FecycP were identical to those of rHSA itself, the two-dimensional structure of albumin could be unchanged after the incorporation of heme. The oxygen-binding affinity ($P_{1/2}^{O_2}$) of rHSA-FecycP was 34 Torr, and its thermodynamic parameters (⊿H,⊿S) were -59 kJ mol^{-1} and -108 J $K^{-1}mol^{-1}$, respectively. The oxygen- and carbon monoxide-association and dissociation rate constants (k_{on}, k_{off}) were also measured by laser flash photolysis. The rebinding kinetics of these gaseous ligands consisted of multiple-exponentials; therefore we supposed that oxygen- and carbon monoxide-binding reactions to FecycP are affected by the molecular environment around the each heme-sites. The half-lifetime of the oxygenated rHSA-FecycP (9 h, 37℃) was significantly longer than that of rHSA-FepivP and closed to the myoglobin's. The obtained red-colored solution was stable and showed a long shelf life at room temperature. The equivalent mixture of rHSA-FecycP and whole blood showed no coagulation and precipitation, which suggested the high blood compatibility of rHSA-FecycP.

Keywords

oxygen-binding reaction, human serum albumin, heme, albumin-heme, synthetic oxygen-carrier, red cell substitute

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1．緒言

現在，我国におけるアルブミン製剤の年間使用量は約190万L（2002年度，原料血漿換算）であり，低蛋白血症による病態，人工心肺を使用する心臓手術のほか，その多くは出血性ショックに対する循環血漿量の補充液として用いられている[1]．もし，この血清アルブミンにの酸素輸送能を付与することができれば，人工酸素運搬体（赤血球代替物）としての臨床利用価値はきわめて高く，例えば救急医療現場における救命措置への適用を考えただけでも，その絶大な効果が容易に推測される．

興味あることに，血漿蛋白質の中にはヘムを取り込んでヘム蛋白質を構成するものもいる．Hbから血流中へ遊離したプロトポルフィリン鉄（III）錯体（ヘミン）は，細胞毒性や活性酸素を発生するため，ヘモペキシンと呼ばれる糖蛋白質により速やかに捕捉され肝臓へと運ばれる[2]．ヘモペキシン-ヘミン複合体のX線結晶構造解析の結果から，ヘミン分子は2つのヒスチジン残基との軸配位，プロピオン酸側鎖とアミノ酸残基間の多重水素結合により，非常に強く固定されている様子が明らかにされている（$K>10^{12}M^{-1}$）[3]．しかし，ヘモペキシンの血中濃度は $<17\mu M$と低いため，これが飽和した場合には，血清アルブミンがその機能代替を果たす．アルブミンは血漿中で最も多量に存在する蛋白質であり，ヘミンとの結合定数も約10^8M^{-1}と高い[4]．ごく最近，著者らのグループは，アルブミン-ヘミン-ミリスチン酸複合体の結晶構造解析に初めて成功した（分解能：3.2Å）[5]．ヘミンはアルブミンのサブドメインIBに取り込まれ，Tyr-161との軸配位，3つの塩基性アミノ酸残基との水素結合により固定されている．窒素雰囲気下でこの水溶液に亜二チオン酸を添加し，ヘミンを鉄（II）錯体（ヘム）へ還元すると，可視吸収スペクトルはTyr-161が配位した鉄（II）5配位高スピン錯体を形成するが，中心鉄（II）は酸素との接触により瞬時に酸化され，安定な酸素錯体を得ることはなかった[6]．

他方，我々は4つのピバロイル基を有するテトラフェニルポルフィリン鉄錯体（FepivP）でもアルブミンに効率よく包接され，得られたアルブミン-ヘム複合体が生理条件下（生理塩水中，pH7.3，37℃）でヘモグロビン（Hb）やミオグロビン（Mb）と同じように酸素を吸脱着できることを明らかにしている[7-11]．赤外吸収，共鳴ラマン，磁気円偏光二色性スペクトルなどから，酸素配位錯体の電子状態を詳細に解析．生体内でも"酸素運搬のできる血清ヘム蛋白質"として機能することを動物実験から定量的に検証している[12]．また現在，人工赤血球として開発が先行しているHb製剤の欠点である血圧亢進（副作用）も全く観測されていない[13]．アルブミンは Hb に比べ等電点が低く，内皮細胞を取り囲む基底膜との間に負電荷どうしの静電反発を生じるため，その血管内皮透過性が低く，血管内皮弛緩因子である一酸化窒素を消去しないので，急激な血圧変動は見られないのである．

我々はアルブミン-ヘム酸素錯体のさらなる安定度向上を目指し，ヘム中心鉄近傍に疎水性の高いシクロヘキサノイル基を4つ導入したヘム誘導体（FecycP，Fig.1）を合成[14]，それを包接させたrHSA複合体の酸素錯体寿命が従来系（rHSA-FepivP）に比べ4.5倍長くなることを見出した．本報ではrHSA-FecycPの構造，酸素及び一酸化炭素結合能，血液相溶性について報告する．

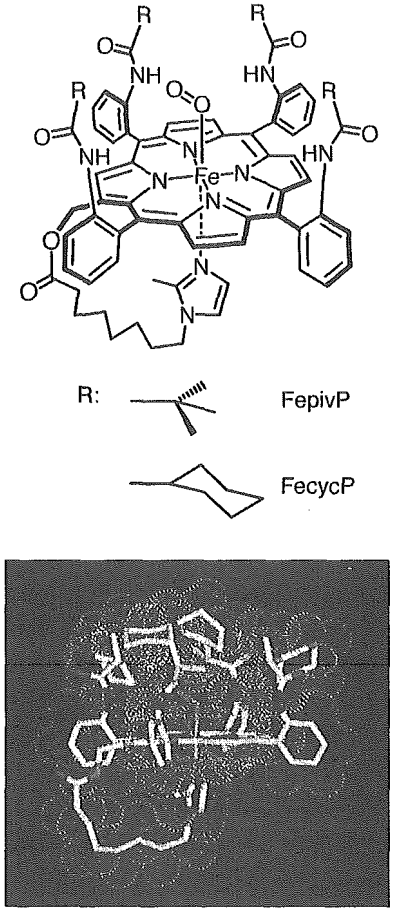

Figure 1. Structures of FecycP and FepivP, and the simulated structure of oxygenated FecycP. The esff forcefield simulation was performed using an Insight II system (Molecular Simulations Inc.) . The structure was generated by alternative minimizations and annealing dynamic calculations from 1,000 K to 100 K. Dielectric constant was fixed at 2.38 D, corresponding to toluene solution. The dotted surface represents the van der Waals radius.

2．実験方法

2.1 試薬，試料，装置

FecycP(2-[8-{N-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-o-methylcyclohexanoylamino) phenylporphinatoiron(FecycP))は既報に従い合成した[14]．組換えヒト血清アルブミン（rHSA）25gdL^{-1}（Albrec®）はニプロ（株）より提供頂いた．従来法により，アルブミン-ヘム

([FecycP]/[rHSA] = 4, mol/mol, rHSA-FecycP (4), または8, mol/mol, rHSA-FecycP (8)) 水溶液を調製[14]. 紫外可視吸収 (UV-vis.) スペクトルは 日本分光V-570を用い350－700 nmの範囲で測定. 円偏光二色性 (CD) スペクトルは日本分光J-725を用いて測定した (25℃). 等電点電気泳動は, Pharmacia Phastgel Systemを用いて測定. アルブミン濃度の定量はアルブミンテストワコー (和光純薬工業) を用い, ヘム (鉄イオン) 濃度の定量は誘導結合高周波プラズマ分光分析 (セイコー電子工業SPS7000A) により行った. FecycP酸素錯体の分子シミュレーションはInsight II (Molecular Simulations Inc.) を用いて構築, 分子力学計算と分子動力学計算を1000 Kから100 Kまで降温させながら交互に実施 (誘電率は2.38 D (トルエン溶液中) に設定) した.

2.2 酸素及び一酸化炭素結合パラメーター

得られたrHSA-FecycP水溶液 ([FecycP]=20 μM, pH7.3) へ酸素及び一酸化炭素を通気し, UV-vis.スペクトル変化を測定, スペクトルパターンから酸素錯体及び一酸化炭素錯体の形成を判定した. また, 酸素錯体吸収 (λ_{max}: 555 nm) の吸光度変化より酸素錯体半減期 ($\tau_{1/2}$) を算出. 異なる酸素及び一酸化炭素分圧に対するUV-vis.スペクトルの連続変化から, Drago式またはHill式を用いて酸素親和性 [$P_{1/2}^{O_2}=(K^{O_2})^{-1}$] 及び一酸化炭素親和性 ($P_{1/2}^{CO}$) を決定した[9,14]. また, 異なる温度におけるK^{O_2}測定から, van't Hoff プロットを作成, 酸素結合に伴う熱力学パラメーター (ΔH, ΔS) を算出した[9].

さらに, レーザーフラッシュホトリシス装置 (ユニソクTSP-600, Nd:YAGレーザー第二高調波 (532 nm)) を用いて, 酸素及び一酸化炭素結合解離速度定数 (k_{on}, k_{off}) を決定[9,14]. 気体分子の再結合反応が多相系からなる場合は, 吸光度の減衰曲線をmultiple-exponential curveでフィティングし, 速い成分と遅い成分に分離, 各々の速度定数を算出した[9].

また, キャピラリー粘度計 (Anton Paar DCR) を用いた溶液粘度測定 (37℃) からrHSA-FecycPと血液適合性を評価した.

3. 結果および考察

3.1 アルブミン-ヘムの構造

得られたrHSA-FecycP溶液のrHSA濃度及びFecycP (Fe) 濃度を定量し, [FecycP]/[rHSA] が4及び8 (mol/mol) であることを確認した. [FecycP]/[rHSA] を10 (mol/mol) 以上で調製した場合でも, 得られたアルブミン-ヘム溶液中の[FecycP]/[rHSA] は8～9 (mol/mol) であったことから, rHSA 1分子に対するFecycPの最大結合数を8分子と決定した. rHSA-FecycPのCDスペクトルパターンはFecycPの包接数によらずrHSAと同等であり, ヘムが結合した後でもアルブミンの二次構造に変化はない (α-ヘリックス含量：67%) (Fig. 2). また, rHSA-FecycP (4, 8) の等電点 (pI) も包接ヘム数によらず4.8と一定で, この値はFecycPを結合していないrHSAの値と一致した. FepivPと同様, FecycPはrHSAの疎水ドメインへ疎水性相互作用により取り込まれていると考えられる.

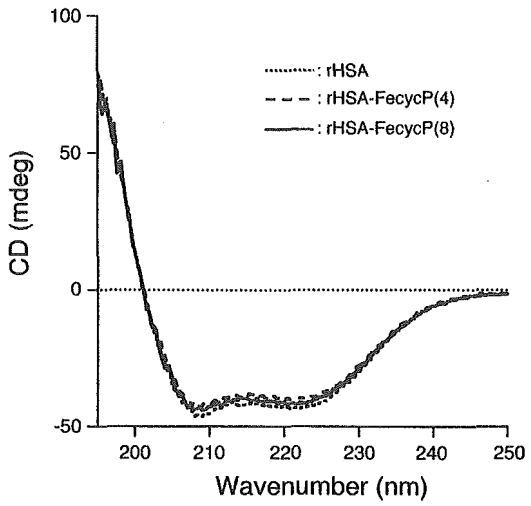

Figure 2. CD spectra of rHSA and rHSA-FecycP in water at 25℃.

3.2 酸素及び一酸化炭素結合能

rHSA-FecycP (4, 8) のUV-vis.スペクトルは, 窒素雰囲気下でλ_{max}：445, 543, 567nmを示し, ヘム鉄 (II) が5配位高スピン錯体 (deoxy体) であることを示唆している[7]. そこへ酸素を通気すると, スペクトルパターンは酸素分圧の増大に伴い等吸収点を持ちながら連続的に変化し, 酸素錯体型 (λ_{max}：428, 555nm) (oxy体) へ移行した (Fig. 3). この酸素結合解離は酸素／窒素の吹き込みに伴い可逆的に繰り返し観測された. また, 一酸化炭素を吹き込むと, きわめて安定なcarbonyl体 (λ_{max}：429, 545 nm) が得られた. 異なる酸素分圧に対するUV-vis.スペクトル変化から算出した酸素親和性 ($P_{1/2}^{O_2}$) は34～35 Torr (37℃) でヒト赤血球 (27 Torr) やHb (T-state) α

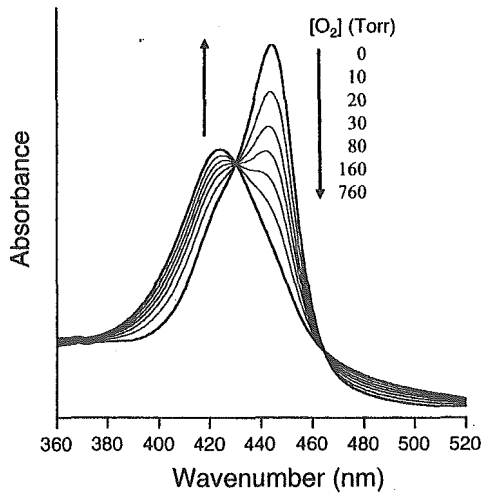

Figure 3. UV-vis. absorption spectral changes of rHSA-FecycP (4) dependent of the oxygen partial pressure in phosphate buffer solution (pH 7.3) at 37℃.

Table 1. O_2-Binding equilibrium parameters and half-lifetime of rHSA-FecycP in phosphate buffer solution (pH 7.3).

	$P_{1/2}$ [Torr][a]	ΔH [kJ mol^{-1}]	ΔS [J K^{-1}mol^{-1}]	$\tau_{1/2}$ [hr][a]
rHSA-FecycP(4)	34	-59	-108	9
rHSA-FecycP(8)	35	-59	-107	9
rHSA-FepivP(4)[b]	36	-60	-114	2
rHSA-FepivP(8)[b]	33	-60	-112	2
Red cell[c]	27			
Hbα	40[d]	-57 to -65[c]	-116 to -133[c]	35[f]
Mb[d]	40[d]	-57 to -65[c]	-116 to -133[c]	12[g]

[a]At 37°C. [b]Ref. 7. [c]pH 7.4, ref. 15. [d]T-state, pH 7, 20°C, ref. 16. [e]pH 7.4, ref 17. [f]At 37°C, pH 7.2, ref. 18. [g]At 35°C, pH 7.0, ref. 19.

(40 Torr) に近い (Table 1)[15,16]. Hbの酸素結合過程に見られる協同現象 (アロステリック効果) は観測されないが (Hill係数：1.0), 例えば肺 (P^{O_2}: 110 Torr) ～末梢組織 (P^{O_2}: 40 Torr) 間におけるrHSA-FecycPの酸素運搬効率 (22%) は赤血球の値 (22%) に匹敵することから, 人工酸素運搬体としての十分な酸素輸送能力を有していると言ってよい.

van't Hoff プロット (Fig. 4) から決定した酸素結合反応のエンタルピー変化 ΔH (-59 kJ mol^{-1}) 及びエントロピー変化 ΔS (-107 J K^{-1}mol^{-1}) は, 包接ヘム数によらず一定で, rHSA-FepivPや赤血球の値と同等であった (Table 1)[7,17]. 4つのピバロイル基をシクロヘキサノイル基に変換しても, 酸素親和性やその熱力学パラメーターに変化はないことが明らかとなった.

また, 酸素錯体半減期 ($\tau_{1/2}$) は9 h (37℃) と長く, この値はMbの $\tau_{1/2}$：12hにも迫る[18,19]. 酸素配位座近傍に疎水性の

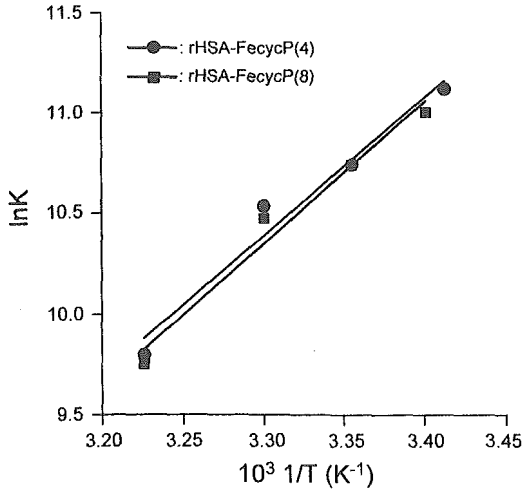

Figure 4. van't Hoff plots of oxygen-binding affinity of rHSA-FecycP in phosphate buffer solution (pH 7.3).

Table 2. O_2-Association and dissociation rate constant of rHSA-FecycP in phosphate buffer solution (pH 7.3) at 25℃.

	k_{on} [M^{-1}s^{-1}]		k_{off} [s^{-1}]	
	fast	*slow*	*fast*	*slow*
rHSA-FecycP(8)	4.6×10^7	7.3×10^6	9.8×10^2	1.6×10^2
rHSA-FepivP(8)[a]	3.4×10^7	9.5×10^6	7.5×10^2	2.0×10^2
Hb (T-state)α[b]	2.9×10^6		1.8×10^2	

[a]Ref. 9. [b]pH 7, 20°C, ref. 16.

Table 3. CO-Binding parameters of rHSA-FecycP in phosphate buffer solution (pH 7.3) at 25℃.

	$P_{1/2}{}^{CO}$ [Torr]	k_{on} [M^{-1}s^{-1}]	
		fast	*slow*
rHSA-FecycP(8)	0.04	5.9×10^6	8.9×10^5
rHSA-FepivP(8)	0.1	4.9×10^6	6.7×10^5
Hb (T-state)α[a]	0.3	2.2×10^5	

[a]Auqeous, pH 7-7.4, 20°C, ref. 21, 22.

高いシクロヘキサノイル基を4つ導入したことにより, 配位酸素へのプロトン付加が誘起する一分子酸化過程が抑制できたためと考えられる. FecycP酸素錯体の分子シミュレーション結果からも, 4つのシクロヘキサン環が, 配位酸素分子を取り囲むように位置している様子がわかる (Fig. 1).

アルブミン-ヘム水溶液にレーザーフラッシュを照射すると, 配位酸素や一酸化炭素の解離と再結合が観測される. この瞬時に起こる非平衡状態から平衡状態へのUV-vis.スペクトル変化から, 配位気体分子の結合解離速度定数 (k_{on}, k_{off}) が算出できる[9,13]. rHSA-FecycPへの一酸化炭素再結合過程 (deoxy体からcarbonyl体へ戻る際の吸光度の時間変化) はtriple-exponential curvesのフィッティングが最適であった. 最も速い成分は一酸化炭素分圧に依存せず, 軸塩基配位子であるイミダゾリル基の脱離を経由した反応と考えられる[9,20]. 一方, 残りの2成分は一酸化炭素濃度に比例し, これらがdeoxy体への一酸化炭素再結合反応に相当する. アルブミン内部に取り込まれたヘムへの一酸化炭素結合は, ヘム近傍の微小な分子環境の相違に影響を受けるため, 厳密には各サイトでそれぞれ異なる速度定数を示すものと推測されるが, 大別して速い反応と遅い反応の二つの過程として解析することができた[9,13].

一方, rHSA-FecycP (4, 8) への酸素結合過程は2成分から構成された. レーザーフラッシュ照射後の酸素再結合過程には, 軸配位子であるイミダゾールの解離を経由した反応は見られないことが知られており[20], 酸素の再結合も一酸化炭素の場合同様, 2成分系として解析した. rHSA-FecycP (4, 8) の酸素及

び一酸化炭素結合パラメーターをTable 2, 3にまとめる[16,21,22].

また，rHSA-FecycP（4, 8）の一酸化炭素親和性（$P_{1/2}^{CO}$）は0.04Torrで，rHSA-FepivPの$P_{1/2}^{O_2}$：0.1Torrに比べて小さい（一酸化炭素親和性は高い）．これはFecycPの酸素配位座近傍における極性が，FepivPに比べて低いためと考えている．両者のCO結合速度定数（k_{on}^{CO}）が，それぞれ5.9×10^6，$4.9\times10^6 M^{-1}s^{-1}$と同等であったことから，FecycPの高い一酸化炭素親和性は小さな解離速度定数に起因するものと推察される．この結果はHbやMbの低い一酸化炭素親和性が遠位ヒスチジンの立体障害ではなく，ヘムポケットの高い極性によるという最近の解釈を支持している[23,24].

3.3 アルブミン-ヘムの血液適合性

rHSA-FecycP溶液の粘度曲線をFig. 5に示す．rHSA-FecycP（4, 8）は共にニュートン粘性を示し，ずり速度$230s^{-1}$における粘度は1.2cPで，rHSAとほぼ同等であった．rHSA-FecycP溶液を血液と1:1（v/v）で混合した場合，沈殿・凝集は全く認められず，安定な混合溶液として存在した（2.0cP（$230s^{-1}$））．rHSA-FecycP溶液の高い血液適合性が実証された．

4．結論

組換えヒト血清アルブミンに4つのシクロヘキサノイル基を有するヘム誘導体を包接させたアルブミン-ヘム複合体は，従来系に比べて安定な酸素錯体を形成できる．酸素配位座近傍に疎水性のシクロヘキサン環を4つ導入したことにより，配位酸素へのプロトン（水）付加が誘起する一分子酸化過程が抑制されたためと考えられる．さらに我々は，分子内配位している軸塩基（2-メチルイミダゾリルオクタノイル基）をHbと同じヒスチジル基に変換すると，酸素錯体半減期が25h（37℃）まで延命することも見出している[12]（この場合，酸素親和性は1.0Torrと高い）．rHSA-FecycPの酸素親和性（34Torr）はrHSA-FepivPの値と変わることなく，半減期のみが9 hに延長され，その安定度はMb（$\tau_{1/2}$：12h）に迫った．得られたrHSA-FecycP溶液は，室温2年間の保存が可能．また，酸素及び一酸化炭素の結合解離過程には，速い相と遅い相の二つの反応が存在した．包接サイトの構造の違い（例えばアミノ酸残基が酸素結合部位周辺に立体障害基として存在するなど）により，各ヘムへの酸素及び一酸化炭素結合速度に差が生じたものと考えられる．rHSA-FecycPと全血液を同容量で混合した場合も凝集・沈殿の惹起を認めず，血液適合性も高い．最近，このrHSA-FecycPを用いた動物実験から，本製剤が生体内でも安全性高く酸素運搬できることを明らかにしている．現在，赤血球代替物としてのさらなる機能評価を進めているので，その詳細は次報で紹介したい．

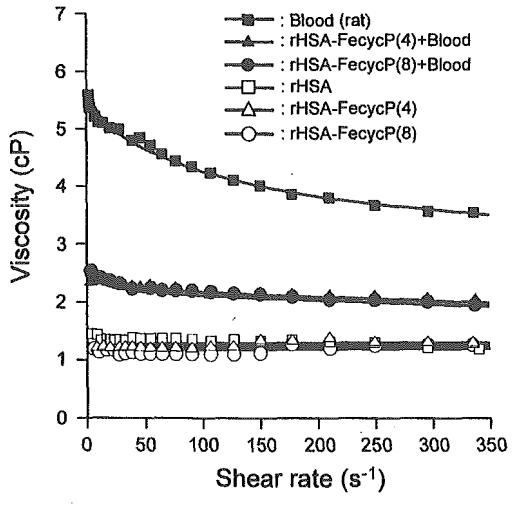

Figure 5. Viscosity of rHSA-FecycP solution with whole blood at 37℃.

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Oxygen-Carrying Plasma Hemoprotein "Albumin-Heme": Nitric Oxide Binding and Physiological Responses after Administration in vivo

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Summary: Recombinant human serum albumin complexed with tetraphenylporphinatoiron(II) derivative, "albumin-heme (rHSA-FeP)", is a synthetic oxygen (O_2)-carrying plasma hemoprotein, which becomes a new class of red blood cell substitute. The UV-vis. absorption and ESR spectroscopy revealed that rHSA-FeP formed six-coordinate nitrosyl complex after exposure of nitric oxide (NO) gas. Although the NO-binding affinity of rHSA-FeP ($P_{1/2}^{NO}$: 1.7×10^{-6} Torr, pH 7.3, 25 °C) is 9-fold higher compared to that of hemoglobin (Hb), the administration of this artificial hemoprotein solution into anesthetized rat does not induce an acute increase in blood pressure (hypertension), which is often observed in Hb-based O_2-carriers due to the depletion of NO (endothelial derived relaxing factor).

Keywords: albumin-heme; hemoprotein; hypertension; nitric oxide; oxygen carrier

Introduction

Recently, one of the hemoglobin(Hb)-based oxygen(O_2)-carriers, which consists of polymerized bovine Hb, was approved for human use in South Africa as the first artificial red cell substitute.[1,2] It shows some superior characteristics compared to stored human blood, for instance, compatibility with all blood type and two-year room temperature stability. However, the administration of such Hb-solutions often elicit an acute increase in blood pressure, because the Hb molecules extravasuate through the vascular endothelium and depletes nitric oxide (NO; endotherial-derived relaxing factor), thus inducing vasoconstriction. Although the precise mechanism of this bradycardia is still controversial, the unfavorable hemodynamic alterations may limit the use of these modified-Hb solutions as blood replacement compositions.

We have shown that the recombinant human serum albumin (rHSA) incorporating a tetraphenylporphinatoiron(II) derivative with a covalently linked proximal base, 2-[8-{N-(2-

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methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamido)phenylporphiphinatoiron(II) (FeP) [rHSA-FeP, Figure 1], can reversibly bind and release O_2 under physiological conditions (in aqueous media, pH 7.3, 37 °C) in the same manner as Hb and myoglobin (Mb).[3] The exchange transfusion tests with hemorrhagic rats demonstrated that rHSA-FeP satisfies the initial clinical requirements for an O_2-carrying resuscitative fluid.[4] The only source of our present concern was that the small rHSA-FeP molecule (8 × 3 nm) injected into the blood vessels would be eliminated from the circulations, and contributes to the significant consumption of NO in the interstitial space between the endothelium and vascular smooth muscle.

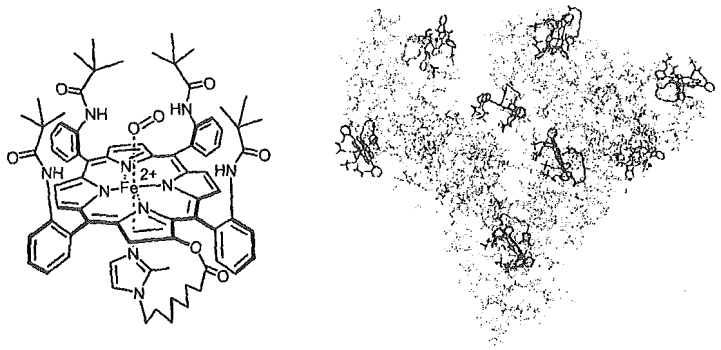

Fig. 1. Oxygenated FeP and predicted structure of rHSA complexed with eight molecules of FeP. The rHSA molecule is colored gray and FePs are shown in dark gray. The figure was made with insight II (Molecular Simulations).

Nitrosyl Complex

We have shown that covalently attaching the imidazolyl group directly to the porphyrin periphery inhibits the proximal base elimination, which is triggered by the NO-binding to the trans side.[5] The UV-vis. absorption spectrum of the rHSA-FeP solution showed maxima at 425 and 546 nm after the exposure of NO gas (1% in N_2) [Figure 2 (A)]. The carbonyl rHSA-FeP (λ_{max}: 428, 541 nm) also moved slowly to the same species after flowing the NO. Further addition of the 100% CO gas to these solutions led to the spectral changes in the carbonyl complexes. These results indicated that (i) the nitrosylation of FeP is reversible and (ii) the central iron was not oxidized during the reaction. ESR spectroscopy was also employed to

confirm the coordination structure of this nitrosyl rHSA-FeP. The absorption curve of the frozen solution at 77 K showed the characteristic shape of rhombic symmetry around the paramagnetic center and very similar to those of the nitrosyl Hb and Mb [Figure 2 (B)].[6] The obtained g values (g_1, g_{II-1}, g_3) are also in good agreement with those of the six-coordinate nitrosyl Hb, Mb, as well as FeP in toluene.[5a,6,7] If the imidazolyl moiety is dissociated from the central iron(II) of the FeP, the spectrum of the five-coordinate nitrosyl complexes become a sharp intense triplet associated with g_3; this was not observed. Based on these findings, it can be concluded that FeP forms the six-coordinate nitrosyl complex with an intramolecularly bound 2-methylimidazolyl arm in the albumin structure.

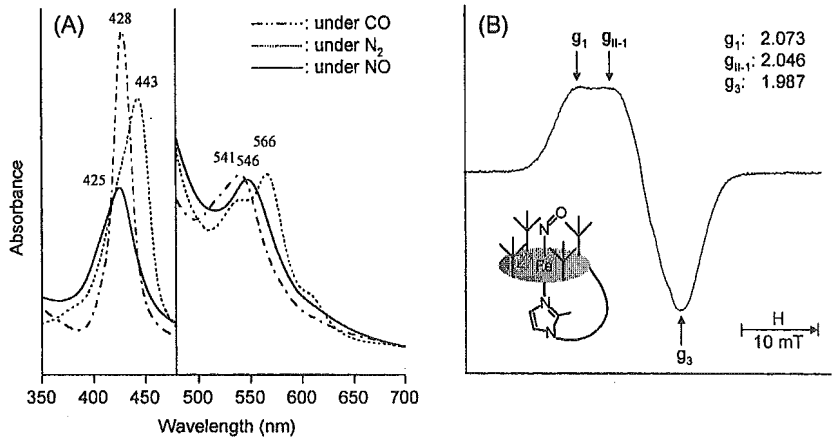

Fig. 2. (A) UV-vis. Absorption spectral changes of rHSA-FeP in phosphate buffer solution (pH 7.3) at 298 K and (B) ESR spectrum of nitrosyl rHSA-FeP in frozen phosphate buffer (pH 7.3) at 77K.

NO-Binding Parameters

The NO-association and -dissociation rate constants (k_{on}^{NO}, k_{off}^{NO}), and NO-binding affinity ($P_{1/2}^{NO}$) of rHSA-FeP are summarized in Table 1.[5b] Kinetically, the low NO-binding affinity (high $P_{1/2}^{NO}$ value) of rHSA-FeP compared to that of FeP in toluene arises from the decreased association rate constant. It has been widely recognized that the NO-binding to Fe(II)porphyrins and natural hemoproteins are diffusion controlled.[8-10] Actually, Hb and Mb have identical

k_{on}^{NO} values (1.7–1.8 × 10^7 $M^{-1}s^{-1}$) which are 10-fold lower than that of the naked Fe(II)protoporphyrin (1-methylimidazole) complex.[9] Interestingly, a similar decrease is observed in our artificial hemoprotein, *i.e.*, incorporation of FeP into the albumin matrix restricts the NO access to the central iron which led to a 60-fold reduction in its NO-association rate.

Table 1. NO-Binding parameters of rHSA-FeP in phosphate buffer solution (pH 7.3) at 25 °C.

	Solvent	10^{-8} k_{on} ($M^{-1}s^{-1}$)	10^4 k_{off} (s^{-1})	10^6 $P_{1/2}$ (Torr)
rHSA-FeP	pb[a]	0.15	0.67	1.7
FeP	toluene	8.9	2.3[b]	0.018
Hb(T-state)[c]	ab[d]	0.18	40	15
Mb[e]	pb[f]	0.17	1.2	2.7
FePP(1-MeIm)[g]	water[h]	1.8	2.9	0.57

[a]pb: Phosphate buffer (30 mM, pH 7.3).
[b]The dissociation rate of NO was determined by k_{on}^{NO}/K^{NO}.
[c]From ref. 9.
[d]ab: Aqueous buffer (pH 8.0).
[e]At 20 °C. From ref. 11.
[f]pb: Phosphate buffer (50 mM, pH 7.0).
[g]FePP(1-MeIm): Fe(II)protoporphyrin(1-methylimidazole) complex, at 22 °C. From ref. 9.
[h]20% N-Methylimidazole solution (pH 9.0).

Change of Blood Pressure after Administration

The rHSA-FeP strongly binds NO; the NO-binding affinity is still 9-fold higher compared to that of Hb and enough high to react 1 μM NO in the wall of the vasculator, therefore one can anticipate that the injection of this O_2-carrying plasama hemoprotein into the blood vessels may induce unfavorable hypertension. In order to clarify the hemodynamic behavior after the administration of rHSA-FeP, we tested a top-load dose of this solution in anesthetized rats.[12] Contrary to our expectations, only a negligibly small change in the mean arterial pressure (MAP) was observed after the administration (Figure 3). If anything, the difference from the baseline (ΔMAP) slowly decreased to –6.8 ± 3.4 Torr within 20 min and remained constant during the monitoring period. The response is quite similar observed following infusion with an equivalent volume of rHSA (5 g/dL) in this experimental setup. In contrast, the administration

of extracellular Hb solution elicited an acute increase in blood pressure (ΔMAP: 16±1.9 mmHg), followed a graduated decrease throughout the 60 min period of observation. Why does rHSA-FeP not induce the hypertension? The molecular size is almost the same as Hb, and its association rate constant for NO binding (k_{on}^{NO}: 1.5×10^7 $M^{-1}s^{-1}$) is high for the rapid scavenging of NO. The answer probably lies in the negatively charged molecular surface of the albumin vehicle. One of the unique characteristics of serum albumin is its low permeability through the muscle capillary pore, which is less than 1/100 that for Hb due to the electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells.[13] The isoelectric point of rHSA-FeP (pI = 4.8) is exactly the same as that of rHSA itself, because the FeP molecule without any ionic residue interacts non-specifically with the hydrophobic cavity of rHSA.[3a,b]

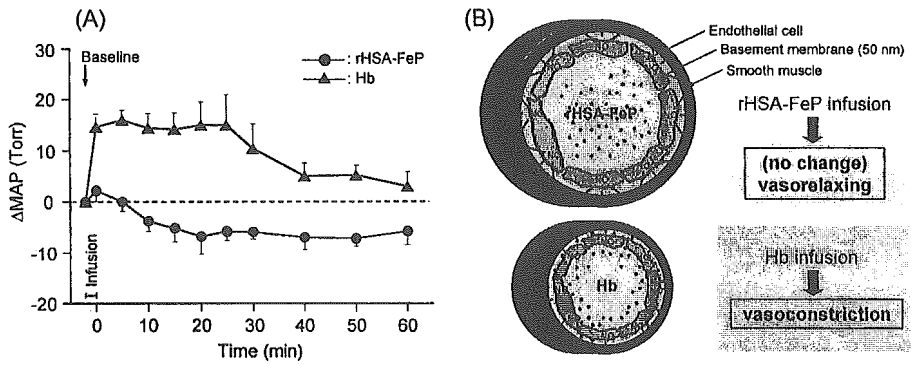

Fig. 3. (A) Changes of mean arterial pressure (MAP) before and after the infusion of rHSA-FeP solution ([rHSA]: 5 g/dL, FeP/rHSA: 4 (mol/mol), 300 mg/kg) and extracellular Hb solution (5 g/dL, 300 mg/kg) in the anesthetized rats (significant difference from baseline: $p < 0.05$, n=5). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± standard error. Basal value is 90.1 ± 3.0 Torr. (B) Schematic illustration of section of aorta and changes of diameter of the vessels after administration of rHSA-FeP and Hb solutions.