# Human Serum Albumin Incorporating Synthetic Hemes as an $O_2$-Carrying Hemoprotein: Control of $O_2$-Binding Ability by Heme Structure

*Eishun Tsuchida,* Teruyuki Komatsu, Yasuko Mastukawa, Tomoyuki Okada*

Department of Polymer Chemistry, Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan

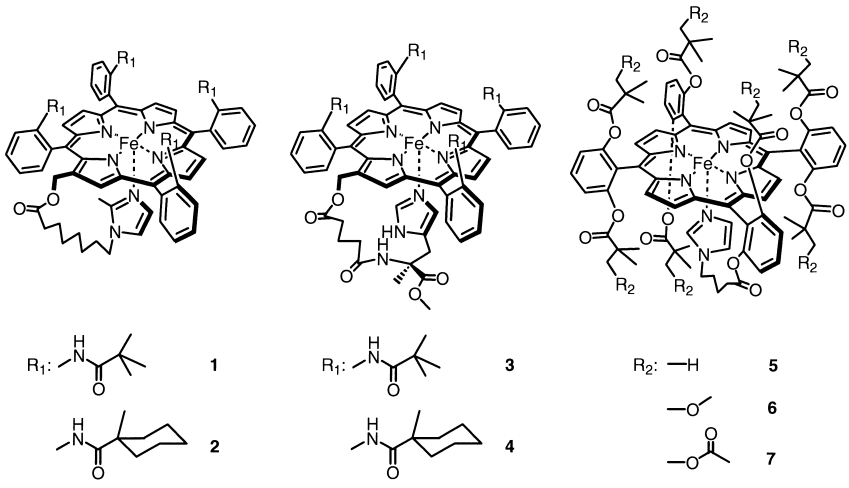
**Summary**: Incorporation of different structured synthetic hemes, 5,10,15,20-tetraphenylporphyrinatoiron(II) derivetives with a covalently linked proximal base [FeP(**1**) to FeP(**7**)], into human serum albumin (HSA), provides seven types of albumin-heme hybrids (HSA-FeP) with different $O_2$-binding abilities. An HSA host absorbs a maximum of eight FeP molecules in each case. The obtained all HSA-FePs can reversibly bind and release $O_2$ under physiological conditions (in aqueous media, pH 7.3, 37 °C) as similar as hemoglobin and myoglobin. The difference in the fence structures did not affect the $O_2$-binding parameters, however the axial histidine coordination significantly increased the $O_2$-binding affinity, which is ascribed to the low $O_2$-dissociation rate constants. The most remarkable effect of the heme structure appeared in the half-lifetime ($\tau_{1/2}$) of the $O_2$-adduct complex. The dioxygenated rHSA-FeP(**4**) showed an unusually long lifetime ($\tau_{1/2}$: 25 hr at 37 °C) which is ca. 13-fold longer than that of rHSA-FeP(**1**).

## Introduction

Human serum albumin (HSA) is the most abundant plasma protein, which binds and transports a great variety of metabolites and organic compounds in our blood stream.[1] This very common protein has suddenly become to attract much attention in many research fields, since its crystal structure was dissolved in 1989.[2] The 585 amino acids consist of a unique heart-shaped structure, that is made of three repeating domains I to III, and each one is constructed of two sub-domains. The majority of the ligand compounds are bound at one site within special hydrophobic cavities of the subdomains IIA and IIIA. We have also found that 5,10,15,20-tetrakis[α,α,α,α-*o*-(2,2-dimethylpropanamido)phenyl]-2-[8-(2-methyl-1-imidazolyl)octanoyloxymethyl]porphinatoiron(II) [FeP(**1**)] is incorporated into HSA, providing synthetic hemoprotein [HSA-FeP(**1**)] which can bind and release $O_2$ under physiological conditions like

 CCC 1022-1360/00/$ 17.50+.50/0

hemoglobin.[3-5] The obtained HSA-FeP(**1**) solution has a good compatibility with human whole blood and can quantitatively transport $O_2$ in vivo.[6] Furthermore, recombinant HSA (rHSA) is now manufactured on a large scale by expression in the *Pichia pastoris* as a host cell, therefore rHSA-FeP becomes an entirely synthetic $O_2$-carrying hemoprotein.[7] These albumin-heme hybrids are recognized to be one of the most promising materials not only as a blood replacement composition, but also as an $O_2$-carrying medicine which will be adopted in several clinical applications, ex., myocardial infraction, anemia, and tracheal blockade, *etc.* However, to use this synthetic $O_2$-carrier for such diseases, its $O_2$-transporting abilities should be adjusted to the required conditions. We believe that the control of the $O_2$-binding property can be realized by the optimizing of the chemical structure of the incorporated heme. This paper describes the $O_2$-binding abilities of the human serum albumin incorporating seven types of synthetic hemes, 5,10,15,20-tetraphenylporphyrinatoiron(II) derivatives with a covalently linked proximal base [FeP(**1**) to FeP(**7**), Formula 1].[8,9]




Formula 1. Synthetic hemes FeP(**1**) to FeP(**7**)

## Heme incorporation into human serum albumin

A maximum of eight FeP(**1**) molecules were incorporated into certain domains of human serum albumin with binding constants from $10^6 - 10^4$ $M^{-1}$.[4] Other FePs were

also expected to bind to HSA or rHSA in a similar fashion, and the binding sites would also be identical. For instance, from the quantitative analyses of the absorption intensity for the Soret band of aqueous rHSA-FeP(**4**), the maximum binding numbers of FeP(**4**) to an rHSA were determined to be eight. The other FePs [FeP(**2**) – FeP(**7**)] also showed the same results. The binding numbers are always eight and independent of the substituents and proximal bases. The predicted 3D structure of the rHSA hybrid incorporating eight FeP(**1**) molecules into the hydrophobic cavities are demonstrated in Figure 1.

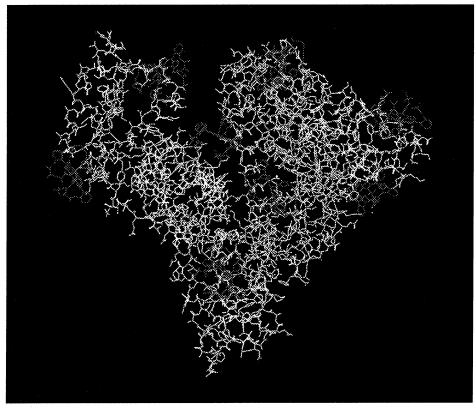


Figure 1. Predicted structure of rHSA hybrid incorporating eight FeP(**1**) molecules by molecular simulation using Insight II.

## $O_2$-binding parameters of human serum albumin–hemes

The UV-vis. absorption spectrum of the aqueous rHSA hybrid including carbonyl FeP(**3**) showed the formation of the typical CO-coordinated low-spin tetraphenyl-porphinatoiron(II) derivative ($\lambda_{max}$: 433, 548 nm). Light irradiation of this solution under an $O_2$ atmosphere led to CO dissociation, affording the $O_2$-adduct complex ($\lambda_{max}$: 433, 553 nm). Upon exposure of the dioxygenated rHSA-FeP(**3**) to $N_2$, the UV-vis. absorption spectrum changed to that of the five-N-coordinated high-spin iron(II) complex with an intramolecularly coordinated axial histidine ($\lambda_{max}$: 441, 548, 568 nm). This $O_2$-binidng was reversibly observed and kinetically stable under physiological

conditions (pH 7.3, 37 °C). Similar dioxygenations were also observed in the human serum albumin hybrids with FeP(**2**) – FeP(**7**) (Table 1).

Table 1. Absorption maxima ($\lambda_{max}$) of human serum albumin-heme hybrids in phosphate buffer solution (pH 7.3) under $N_2$, $O_2$ and CO.

| | $N_2$ | $O_2$ | CO |
|---|---|---|---|
| rHSA-FeP(**1**) | 443,542,567 | 426,552 | 427,539 |
| rHSA-FeP(**2**) | 445,543,567 | 428,555 | 429,545 |
| rHSA-FeP(**3**) | 441,548,568 | 433,553 | 433,548 |
| rHSA-FeP(**4**) | 442,548,568 | 429,553 | 431,548 |
| HSA-FeP(**5**) | 438,539,558 | 425,549 | 427,545 |
| HSA-FeP(**6**) | 438,540,560 | 426,549 | 427,546 |
| HSA-FeP(**7**) | 438,542,563 | 426,549 | 427,546 |

The $O_2$-coordinations to FePs in human serum albumin are expressed by equation 1.

$$\mathrm{FeP} + \mathrm{O_2} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{FeP\text{-}O_2} \qquad \cdots (1)$$

$$[K = (P_{1/2})^{-1} = k_{on}/k_{off}]$$

The $O_2$-association and dissociation rate constants ($k_{on}$, $k_{off}$) were explored by laser flash photolysis.[5] The detailed kinetic evaluation of human serum albumin-heme hybrids gave the following three results.

(i) The absorption decays accompanying the $O_2$ recombination were composed of three-phases of the first-order kinetics; the curves were fit by a triple-exponential equation.[5] The minor (<10%) and the fastest component was independent of the $O_2$ concentrations. It should be correlated with a base elimination.[10]

**(ii)** From the careful inspections of the two slower phases, the association rate constants for the fast and slow rebindings [$k_{on}$*(fast)* and $k_{on}$*(slow)*] of $O_2$ were calculated (Table 2). The $k_{on}$*(fast)* values are 4 – 7-times larger than $k_{on}$*(slow)*.

**(iii)** The concentration ratios of the fast and slow reactions were 2 – 3.

Based on these findings, we can conclude that the $O_2$ association to the FePs in the hydrophobic domains of the serum albumin is influenced by the molecular microenvironments around each $O_2$-coordination site, e.g., steric hindrance of the amino acid residue and difference in polarity.

Table 2. $O_2$-Binding parameters of human serum albumin-heme hybrids in phosphate buffer solution (pH 7.3) at 25°C.

| | $10^{-6}$ $k_{on}$ ($M^{-1}s^{-1}$) | | $10^{-1}$ $k_{off}$ ($s^{-1}$) | | $P_{1/2}$ | $\tau_{1/2}$ |
|---|---|---|---|---|---|---|
| | *fast* | *slow* | *fast* | *slow* | (Torr) | (hr) |
| rHSA-FeP(**1**) | 34 | 9.5 | 75 | 20 | 13 | 2 |
| rHSA-FeP(**2**) | 46 | 7.3 | 98 | 16 | 13 | 9 |
| rHSA-FeP(**3**) | 36 | 6.1 | 5.9 | 1.0 | 1 | 5 |
| rHSA-FeP(**4**) | 54 | 8.8 | 8.9 | 1.4 | 1 | 25 |
| HSA-FeP(**5**) | 11 | 1.5 | 50 | 6.9 | 28 | 5 |
| HSA-FeP(**6**) | 11 | 2.0 | 41 | 7.6 | 23 | 2 |
| HSA-FeP(**7**) | 8.9 | 2.3 | 34 | 8.8 | 23 | 2 |

The $P_{1/2}$ of the human serum albumin-heme hybrids were determined on the basis of the UV-vis. spectral changes during the $O_2$ titration (Table 2).[5] According to the kinetic experiments, the $P_{1/2}$ values were divided into two components using our previously reported equation.[5] Nevertheless, the calculated $P_{1/2}$ for the fast and slow phases were all identical in each case. The rHSA-FeP(**1**) and rHSA-FeP(**2**) showed the same $O_2$-binding affinities (13 Torr), indicating that the fence structure on the porphyrin plane causes only small changes in their $O_2$-equilibria and -kinetics. The double-sided series, FeP(**5**), FeP(**6**), and FeP(**7**) in albumin, showed very similar $O_2$-binding parameters, also supporting the above assumption. In contrast, the axial histidine coordination provided a 13-fold larger $O_2$-binding affinity compared to those of the 2-methylimidazole linked analogues [FeP(**1**) or FeP(**2**)], which is ascribed to the low $O_2$-dissociation rate constants.

## Stability of $O_2$-adduct complex of human serum albumin–hemes

Accompanying the autooxidation of the central iron(II), the absorption band ($\lambda_{max}$: 549–555 nm) slowly disappeared at 37 °C, leading to the formation of the inactive iron(III) porphinate.[11] The effect of the heme structure on the half-lifetime ($\tau_{1/2}$) of the $O_2$-adduct complex was rather remarkable. (i) The cyclohexanoyl fences gave ca. 5-fold longer $\tau_{1/2}$ values compared to those of the pivaloyl derivatives, and (ii) the proximal histidine coordination increased $\tau_{1/2}$ by a factor of 3. As a result, (iii) the dioxygenated rHSA-FeP(**4**) showed an unusually long lifetime ($\tau_{1/2}$: 25 hr at 37 °C)

which is ca. 13-fold longer than that of rHSA-FeP(**1**). In double-sided series, the introduction of the polar-substituents on the fence groups led to reduce the stability of the $O_2$-adduct species.

## Conclusions

Human serum albumin incorporating FePs having an intramolecularly coordinated histidine residue formed stable $O_2$-adduct complexes under physiological conditions. In particular, dioxygenated rHSA-FeP(**4**) showed a high stability, and its half-lifetime reached a value similar to that of the native Hb A ($\tau_{1/2}$: 36 hr at 37 °C).[12] Although the $O_2$-binding affinity of rHSA-FeP(**4**) is too high to use as a blood replacement composition, it can be utilized as an $O_2$-carrying medicine for dioxygenation of the hypoxia region in cancer tissue, *etc.* As a red blood cell substitute, rHSA-FeP(**2**), which has a similar $P_{1/2}$ value to red blood cells, is promising. In the blood stream, the apparent lifetime of the $O_2$-coordinated albumin-heme is prolonged by a factor of 4,[6] so that the rHSA-FeP(**2**) is expected to transport $O_2$ in the circulatory system for more than 1.5 days.

This work was partially supported by the Core Research for Evolutional Science and Technology, JST, 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.

[1] T. Peters Jr., "All about Albumin, Biochemistry, Genetics, and Medical Applications", Academis Press Inc., San Diego, 1996.
[2] D. C. Carter, X.-M. He, S. H. Munson, P. D. Twigg, K. M. Gernert, M. B. Broom, T. R. Miller, *Science* **1989**, *244,* 1195.
[3] T. Komatsu, K. Ando, N. Kawai, H. Nishide, E. Tsuchida, *Chem. Lett.* **1995**, 813.
[4] T. Komatsu, K. Hamamatsu, J. Wu, E. Tsuchida, *Bioconjugate Chem.* **1999**, *10,* 82.
[5] T. Komatsu, Y. Matsukawa, E. Tsuchida, *Bioconjugate Chem.* **2000**, *11,* 772.
[6] E. Tsuchida, T. Komatsu, K. Hamamatsu, Y. Matsukawa, A. Tajima, A. Yoshizu, Y. Izumi, K. Kobayashi, *Bioconjugate Chem.* **2000**, *11,* 46.
[7] E. Tsuchida, T. Komatsu, Y. Matsukawa, K. Hamamatsu, J. Wu, *Bioconjugate Chem.* **1999**, *10,* 797.
[8] T. Komatsu, Y. Matsukawa, E. Tsuchida, *Bioconjugate Chem.* **2001**, *12,* submitted.
[9] T. Komatsu, T. Okada, M. Moritake, E. Tsuchida, *Bull. Chem. Soc. Jpn.* **2001**, *74,* in press.
[10] J. Geibel, J. Cannon, D. Campbell, T. G. Traylor, *J. Am. Chem. Soc.* **1978**, *100*, 3575.
[11] M. Momentau, C. A. Reed, *Chem. Rev.* **1994**, *94,* 659.
[12] A. Monsouri, K. H. Winterhalter, *Biochemistry* **1973**, *12,* 4946.