O_2-Transport Albumin: A New Hybrid-Haemoprotein Incorporating Tetraphenylporphinatoiron(II) Derivative

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2-[8-{N-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamido)phenylporphinatoiron(II) is incorporated into the human serum albumin, which reversibly forms a stable O_2-adduct under physiological conditions (in aqueous medium, pH 7.4, 37 °C) similar to haemoglobin.

We report for the first time the reversible O_2-binding to human serum albumin (HSA) incorporating the tetraphenylporphinatoiron(II) derivative bearing a covalently bound axial base under physiological conditions (in aqueous medium, pH 7.4, 37 °C). This is similar to the action of haemoglobin (hb).

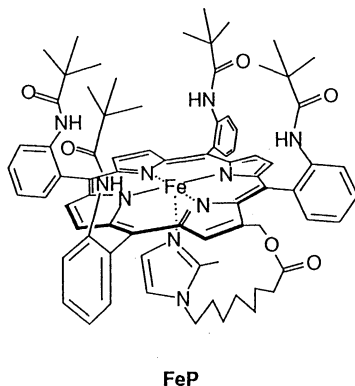
Serum albumin is the second most abundant constituent in blood plasma. Besides contributing to the colloid osmotic pressure of blood, albumin serves as a transport protein for numerous endogeneous and exogeneous compounds. For example, haemin liberated from hb is trapped with HSA in the blood stream and transported to the liver during the plasma haem degradation process. Several studies have reported the attempt to characterize the interaction between HSA and porphyrin derivatives; HSA contains one high affinity binding site of haemin as well as additional sites of much low affinity.[1-5] However, less attention has been paid to the function of the HSA-porphyrin complex. Marden et al. demonstrated that the reduced deoxy HSA-haem complex bound CO in aqueous medium, but the dioxygenated species could not be detected.[6] Bonaventura et al. preliminary reported the reversible spectral change of HSA incorporating tetrakis(*o*-pivalamido)phenylporphinatoiron(II) in the presence of a large excess of molar axial imidazole upon exposure to dioxygen,[7] but its structure and the O_2-binding formation have not been clarified.

We have recently found that a tetraphenylporphinatoiron(II) derivative bearing a covalently bound axial base, 2-[8-{N-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamido)phenylporphinatoiron(II) (FeP), was efficiently incorporated into HSA and this HSA-FeP complex reversibly transported dioxygen under physiological conditions. This paper describes the O_2-, CO-binding performances of HSA-FeP as a new O_2-transport haemoprotein.

FeP was synthesized according to our previous paper.[8] The HSA-FeP was prepared as follows. The dimethyl sulfoxide (DMSO) solution of Fe(III)P was added to the phosphate buffer solution (pH 7.4, 33 mmol dm^{-3}) of HSA, which is fatty acid free. The mixture was ultrafiltrated and diluted several times using phosphate buffer. A small excess of $Na_2S_2O_4$ was then added to the solution which resulted in reduction of the central Fe(III) ion of the porphyrin.

The UV-vis. absorption spectrum of HSA-FeP was identical with that of the monomeric FeP in DMSO solution. The interaction between FeP and HSA was examined using its fluorescence emission spectrum. As FeP is a fluorescence quencher, the intensity at 340 nm for HSA, originating from a triptophane residue (Trp^{214}) in domain II, decreased with the [FeP]/[HSA] ratio; FeP strongly quenched the emission of the Trp^{214}. This suggested that the FeP binding site is located in the middle part of HSA, in the proximity of Trp^{214} in domain II. From fluorescence titrations with FeP in the range of HSA concentration, a Scatchard plot was made.[1,9] This plot showed that there is a single FeP binding site to HSA (intercept of the *x*-axis: 1.3) with a binding constant of 2.6 x 10^6 mol dm^{-3}. The isoelectric point of HSA-FeP was determined to be 4.5 from the isoelectric focusing measurement, which agreed with that of HSA itself.

After adding CO gas on the solution of HSA-FeP and shaking, the CO- adduct was immediately obtained. The FeP-CO was then efficiently transformed to a five-N-coordinated (deoxy) species by irradiation with a 500 W halogen lamp under a nitrogen atmosphere in the ice-water bath. The visible absorp-



FeP

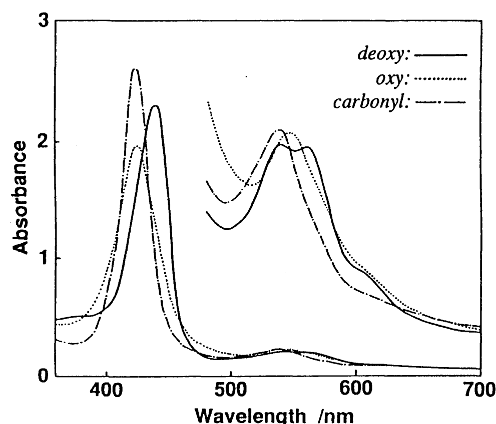


Figure 1. Visible absorption spectral change of O_2- and CO-binding to HSA-FeP in phosphate buffer (pH 7.4, 33 mmol dm^{-3}) at 25 °C.

Table 1. O_2- and CO-binding parameters of the HSA incorporating FeP in aqueous medium at 25 °C

	Solvent	O_2			CO		
		$P_{1/2}$/ Torr	10^{-8} k_{on}/ $dm^3mol^{-1}s^{-1}$	10^{-3} k_{off}/ s^{-1}	10^2 $P_{1/2}$/ Torr	10^{-6} k_{on}/ $dm^3mol^{-1}s^{-1}$	10^2 k_{off}/ s^{-1}
HSA-FeP	p.b. (pH 7.4)[a]	8.0	2.4	3.2	1.4	4.4	8
FeP[b]	toluene	38	1.6	46	0.6	2.9	17
Red blood cell	p.b. (pH 7.4)[c]	8.8	0.00011	0.00016	57	0.014	1
Hb-α (R-state)	p.b. (pH 7.0)[d]	0.22	0.33	0.013	0.14	4.6	0.9
Mb	p.b. (pH 7.0-7.4)[c]	0.82	0.2	0.03	0.28	0.3	0.15

[a]p.b.: Phosphate buffer, 33 mmol dm^{-3}. [b]From Ref. 8. [c]From Ref. 13. [d]From Ref. 10.

tion spectrum of the Fe(II) deoxy state of HSA-FeP (λ_{max}: 439, 542, and 563 nm) changed to that of its O_2-adduct upon exposure to dioxygen (λ_{max}: 424 and 548 nm) (Figure 1). The spectrum immediately and reversibly changed in response to the O_2-pressures. The O_2-adduct changed to the corresponding CO-adduct again after adding CO gas to the solution (λ_{max}: 424 and 540 nm). The O_2-binding affinity [$P_{1/2}(O_2)$: the O_2-partial pressure at half O_2-binding for the porphinatoiron(II)] of HSA-FeP was estimated to be 17 Torr at 37 °C. The half-lifetime ($\tau_{1/2}$) of the O_2-adduct with respect to the Fe(III)porphyrins was 16 h in aqueous solution at 25 °C. On the other hand, FeP homogenized with a surfactant (Triton X-100) in aqueous medium, *i.e.* micelle solution, was rapidly and irreversibly oxidized upon exposure to dioxygen. That is, a micro-environment around FeP in HSA acts as a pseudo-haempocket which enables the formation of an O_2-adduct that is stable against irreversible oxidation. The thermodynamic parameters for the O_2-binding, enthalpy changes (ΔH) and entropy changes (ΔS) for HSA-FeP was estimated to be -69 kJ mol^{-1} and -190 J K^{-1} mol^{-1}, respectively, which are comparable to those of hb. These results showed that HSA-FeP is a new type of O_2-carrier replacing hb.

The kinetic parameters of O_2- and CO-binding to HSA-FeP were explored by laser flash photolysis (Table 1).[8,10-12] The higher O_2-binding affinity of HSA-FeP compared with that in toluene [$P_{1/2}(O_2)$ = 38 Torr] mainly arises from the decrease in the O_2-dissociation rate constant. On the other hand, CO-binding parameters in HSA were almost the same as those in toluene. It is assumed that a high polar amide-environment surrounds the FeP moiety constructed by HSA polypeptide causes an increase in the stability of the dioxygenated species, leading to a reduced $k_{off}(O_2)$. This agrees quite well with the result of the high O_2-binding affinity of FeP in amide solution (*e.g.* $P_{1/2}(O_2)$ in DMF: 0.8 Torr). Furthermore, experiments on the CO-binding kinetics of HSA incorporating FeP on a nanosecond time scale exhibited rapid geminate recombination reactions, which are often observed in hb and also in the HSA-haem-CO complex.[6]

In conclusion, FeP was equivalently incorporated into the microdomain nearby Trp^{214} of HSA. The HSA incorporating FeP reversibly transports dioxygen under physiological conditions and its O_2- and CO-binding capacity make it suitable as a red blood cell substitute; this hybrid-haemoprotein acts as a new type of O_2-carrying molecule.

Recently, recombinant HSA (r-HSA) has been manufactured by gene cloning and expression in *saccharomyces cerevisiae* or *eschrichia coli, etc.* and its utility is a topic of current interest in blood substitute chemistry. Further study on the preparation and characterization of the r-HSA incorporating FeP as a totally synthetic O_2-carrier is now in progress by the authors.

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