= Electronic Supporting Information =

Haemoglobin wrapped covalently by human serum albumin mutants containing Mn(III) protoporphyrin IX: an O_2 complex stable in H_2O_2 solution

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Experimental

Materials and apparatus

Human serum albumin [wild-type, HSA(wt)] was purchased from Japan Blood Products Organization. Recombinant HSA mutant [HSA(Y161H)] was prepared according to our previously reported site-directed mutagenesis expression techniques.[20,21] Pure bovine haemoglobin (Hb) was purified from fresh bovine blood purchased from Tokyo Shibaura Zouki Co., Ltd.[14] Mn(III) protoporphyrin IX (MnPP) was purchased from Frontier Scientific, Inc. Other chemicals of special grades were used without further purification. The water was deionized (18.2 MΩcm) using water purification systems (Elix UV and Milli Q Reference; Millipore Corp.). UV-Vis absorption spectra were recorded using a UV-visible spectrophotometer (8543; Agilent Technologies Ltd.) equipped with a temperature control unit 89090A. Isoelectric focusing (IEF) was performed using an electrophoresis power supply (EPS 601; GE Healthcare UK Ltd.) with an IEF gel (Novex pH 3–10; Invitrogen Corp.). The protein marker used was an IEF calibration kit Broad pI (pH 3–10; GE Healthcare UK Ltd.).

Preparation of Hb–HSA(Y161H)$_3$ cluster

The Hb–HSA(Y161H)$_3$ cluster was prepared according to our previously reported procedure.[14] The DMSO solution of crosslinker, *N*-succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC; Tokyo Chemical Industry Co., Ltd.) (20 mM, 0.2 mL) was added dropwise into phosphate buffered saline (PBS) solution (pH 7.4) of carbonyl Hb (0.1 mM, 2 mL). After stirring for 2 h at 25 °C under the darkness, the reaction mixture was applied to gel filtration chromatography (GFC) with a Sephadex G25 (superfine) column to remove unreacted crosslinker. The obtained SMCC-bound Hb (maleimide activated Hb) was concentrated to 2 mL ([Hb] = 0.1 mM) using a centrifugal concentrator (Vivaspin 20 ultrafilter, 10 kDa MWCO; GE Healthcare UK Ltd.). Then this solution (1 mL) was added gradually into HSA (1 mM) in PBS (pH 7.4, 1 mL) with subsequent stirring under dark conditions for 5 h at 4 °C. A part of reaction mixture was applied to size-exclusion chromatography (SEC) on an HPLC system (LaChrome Elite; Hitachi High-Technologies Corp.) with a Shodex Protein KW-803 column (Showa Denko K.K.) using phosphate buffer (PB, pH 7.4, 50 mM)

as the mobile phase. The elution curve demonstrated new multiple peaks at the high molecular weight region. The three major components were identified as Hb–HSA(Y161H)$_4$ heteropentamer (minor), Hb–HSA(Y161H)$_3$ heterotetramer and Hb–HSA(Y161H)$_2$ heterotrimer. Then the resultant solution was subjected to GFC with a Superdex 200pg column (GE Healthcare UK Ltd.) using PBS (pH 7.4) as the running buffer. We collected main fractions before the HSA peak (Fig. S1). The unreacted free HSA was excluded completely. By Hb and total protein assays, the average HSA/Hb ratio of the harvested Hb–HSA(Y161H)$_m$ cluster was determined to be 3.0, which is shown as Hb–HSA(Y161H)$_3$. The protein concentration was assayed by Pierce 660-nm Protein Assay Kit (Thermo Fisher Scientific K.K.).

Preparation of HSA–MnPP complexes and Hb–[HSA(Y161H)–MnPP]$_3$ cluster

The HSA(wt)–MnPP and HSA(Y161H)–MnPP complexes (MnPP/HSA = 1.0) were prepared as described in the literature.[21] On the other hand, the ethanolic MnPP solution (2.0 mM, 30 μL, PBS) was slowly added to the Hb–HSA(Y161H)$_3$ solution (10 μM, 2 mL, PBS) and the obtained mixture was incubated for 1 h with gentle stirring in the dark at 25 °C, affording Hb–[HSA(Y161H)–MnPP]$_3$ cluster [MnPP/Hb-HSA(Y161H)$_3$ = 3.0].

H_2O_2 dismutation activity

H_2O_2 dismutation activity (catalase activity) of the HSA(Y161H)–MnPP complex was evaluated by measuring the concentration of generated O_2 using an O_2-monitoring system (Oxygraph; Hansatech Instruments Ltd.). The Oxygraph system consists of a highly sensitive Clark type polarographic O_2-electrode disc mounted within an electrode chamber and connected to the electrode control unit. First, the PBS solution of HSA(Y161H)–MnPP (10 μM, 0.98 mL) was poured into the electrode chamber and the dissolved O_2 was measured with gentle stirring under aerobic condition at 25 °C. After confirming the [O_2] level constant for 30 s, the 50 mM H_2O_2 in PBS (20 μL) was added to make the [H_2O_2] in the medium to 1 mM. Immediately after the addition of H_2O_2, an increase in the dissolved O_2 was observed. The same experiments were performed at various H_2O_2 concentrations (0–16 mM). From the relationship between the initial rate constant (v_0) and [H_2O_2], we determined the

Michaelis constant (K_m) and catalytic constant (k_{cat}) of HSA(Y161H)−MnPP complex using Lineweaver–Burk plots. The same experiments were also conducted for HSA(wt)−MnPP complex.

O_2-binding ability

The visible absorption spectra of deoxy (under N_2), oxy (under O_2), and carbonyl (under CO) forms of the Hb–HSA(Y161H)$_3$ and Hb–[HSA(Y161H)–MnPP]$_3$ clusters ([Hb]: 10 μM, PBS, pH 7.4) were obtained in accordance with our previously reported methods.[14] The O_2 affinity (P_{50}: O_2-partial pressure where Hb is half-saturated with O_2) and Hill coefficient (n) were determined using an automatic recording system for O_2-equilibrium curve (Hemox Analyzer; TCS Scientific Corp.) using PBS (pH 7.4) at 37 °C. The sample was oxygenated by an increasing O_2-partial pressure and deoxygenated by flushing with N_2.

O_2-complex stability in H_2O_2 solution

The O_2-complex stability of the Hb–[HSA(Y161H)–MnPP]$_3$ cluster in aqueous H_2O_2 was evaluated by the time course of metHb formation level. The PBS solution (pH 7.4) of oxy Hb–[HSA(Y161H)–MnPP]$_3$ cluster ([Hb] = 10 μM, 2 mL) was put into a 10-mm-path length optical quartz cuvette. Aqueous H_2O_2 was injected into the solution and the top of the cuvette was sealed with a gas permeation film (AeraSeal Film MAF710; Gel Co.), which allows air exchange and which prevents water evaporation. The absorption intensity (A_t) at 630 nm based on metHb formation was monitored under aerobic conditions with gentle stirring for 120 min at 25 °C. After the measurement, a slightly excess $K_3[Fe(CN)_3]$ was added to determine the absorption intensity of the entirely oxidized metHb form (A_{100}). From the absorbance increase, the metHb level [$(A_t - A_0)/(A_{100} - A_0) \times 100$ (%)] (A_0 is the absorption intensity at 630 nm before H_2O_2 injection) was ascertained. The same experiments were conducted for native Hb, Hb–HSA(wt)$_3$ cluster and simple mixture of Hb/HSA(Y161H)–MnPP (1/3, molar ratio).

Results

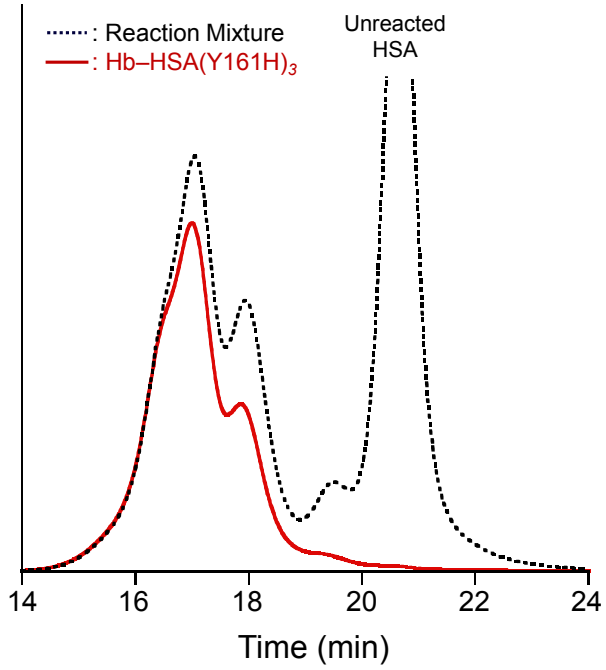

Fig. S1. SEC curve of Hb–HSA(Y161H)$_3$ cluster. Mobile phase buffer: PB solution (pH 7.4).

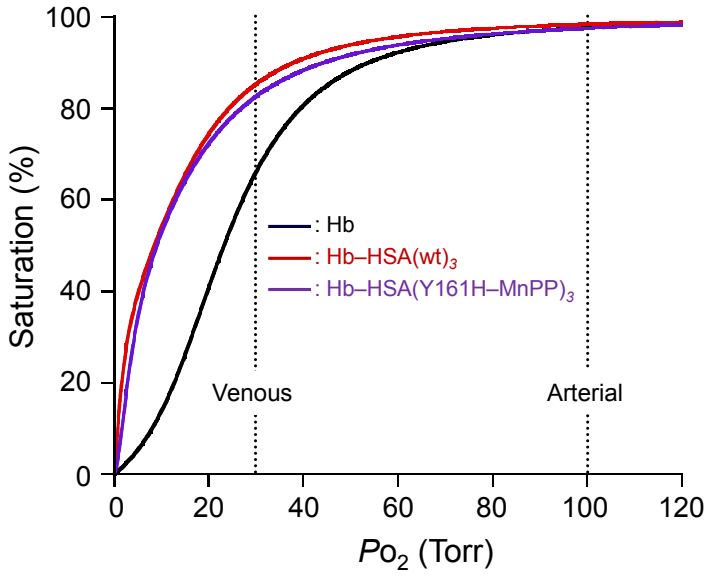

Fig. S2. O_2 equilibrium curve of Hb–[HSA(Y161H)–MnPP]$_3$ cluster. In PBS solution (pH 7.4) at 37 °C.

Table S1. UV-Vis absorption spectral data of Hb–[HSA(Y161H)–MnPP]$_3$ cluster in PBS solution (pH 7.4) at 25 °C

	λ_{max} (nm)		
Hemoproteins	deoxy	oxy	carbonyl
Hb–HSA(wt)$_3$ [a]	430, 556	413, 541, 577	420, 538, 569
Hb–HSA(Y161H)$_3$	430, 556	413, 541, 577	420, 538, 569
Hb–[HSA(Y161H)–MnPP]$_3$	430, 470, 557	413, 470, 543, 576	419, 470, 537, 568
Hb [a]	430, 555	414, 541, 577	420, 538, 569
Hb [b]	430, 555	415, 541, 577	419, 540, 569

[a] From ref. 14. [b] Hb A, from ref. 24.

Table S2. O_2-Binding parameters of Hb–[HSA(Y161H)–MnPP]$_3$ clusters in PBS solution (pH 7.4) at 37 °C

Hemoproteins	P_{50} (Torr)	n
Hb–HSA(wt)$_3$ [a]	9	1.5
Hb–HSA(Y161H)$_3$	9	1.5
Hb–[HSA(Y161H)–MnPP]$_3$	9	1.5
Hb [a]	23	2.6
RBC	28	2.8

[a] From ref. 14.