=Electronic supplementary information=

Genetically and chemically tuned haemoglobin-albumin trimers with superior O_2 transport efficiency

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Materials and apparatus

1,6-Bis(maleimido)hexane (BMH) was purchased from Tokyo Chemical Industry Co., Ltd. Bis(3,5-dibromosalicyl) fumarate was purchased from Abcam PLC. Human serum albumin (HSA, Albumin 25%, Benesis) was purchased from the Japan Blood Products Organization. The other special-grade chemicals were used without additional purification unless otherwise noted. Water was deionized (18.2 MΩ cm) using two water purification systems (Elix UV and Milli Q Reference; Millipore Corp.). SDS-PAGE was performed using a 15% poly(acrylamide) precast gel (SuperSep Ace 15%, Fujifilm Wako Pure Chemical Corp.). Isoelectric focusing (IEF) was performed using a pH 3–10 IEF protein gel (Novex; Thermo Fischer Scientific Inc.). The UV-vis absorption spectra were obtained using a UV-vis spectrophotometer (V-650; Jasco Corp.). CD spectra were recorded using a CD spectrometer (J-820; Jasco Corp.). The Size exclusion chromatography (SEC) profiles were obtained using a high-performance liquid chromatography (HPLC) system (Prominence LC-20AD/CTO-20A/SPD-20A; Shimadzu Corp.) that was equipped with an SEC column (Superdex 200 Increase, 5/150 GL; Cytiva); a PBS solution (pH 7.4) was used as the mobile phase. The ^{1}H NMR spectrum was obtained with a Varian Mercury 400 NMR spectrometer at 400 MHz.

Expression and preparation of rHb(Cβ120/X) variants

The expression plasmids for the rHb(Cβ120/X) variants [pHIL-D2-rHb(Cβ120/X), X = Tβ66, Kβ108, Fβ28, XLα99, Acα99] were constructed according to the standard protocol of the QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies Inc.) using a pHIL-D2-rHb(Cβ120) vector[3e] and following oligonucleotide primer sets.

Tβ66 forward:	5′-GTCAAAGCACATGGGAAGACTGTCCTGGGCGCGTTTTCC-3′
Tβ66 reverse:	5′-GGAAAACGCGCCCAGGACAGTCTTCCCATGTGCTTTGAC-3′
Kβ108 forward:	5′-CGCTTGCTGGGCAAGGTGCTGGTCTGTGTTCTC-3′
Kβ108 reverse:	5′-GAGAACACAGACCAGCACCTTGCCCAGCAAGCG-3′
Fβ28 forward:	5′-GATGAAGTGGGTGGTGAAGCATTTGGTCGTCTGCTGGTAGTG-3′
Fβ28 reverse:	5′-CACTACCAGCAGACGACCAAATGCTTCACCACCCACTTCATC-3′

DNA sequencing confirmed that the desired mutations were definitely introduced in the plasmid vectors. The obtained pHIL-D2-rHb(Cβ120/X) vectors were linearized with *Sal*I and used to transform the GS115-strain *Pichia pastoris* (Thermo Fisher Scientific K.K.) via electroporation.

Each transformed clone cell was grown in a buffered mineral glycerol complex (BMGY) medium (4 L total) in a shaking incubator (Bio-Shaker G·BR-200; Taitec Corp; 200 rpm, 30 °C) and subsequently in a buffered mineral methanol complex (BMMY) medium (1.6 L total) containing haemin (0.3 mM) for 5 days. During cultivation, 100% methanol at 1.5% of the medium volume was

added every 24 h. The cells were harvested by centrifugation at 3,000 *g* for 10 min. The obtained cells were then washed with water (300 mL × 2) and resuspended in 100 mL of sodium PB solution (10 mM, pH 6.0) containing 1 mM phenylmethanesulfonyl fluoride. After the addition of glass beads (150 mL, $d = 0.5$ mm), the cells were lysed using a BeadBeater (Biospec Products, Inc.) with six cycles of disruption (2 min) and incubated while cooling on ice (2 min). After centrifugation at 12,000 *g* for 1.5 h, the supernatant was equilibrated with CO. The solution was loaded onto a cation exchange chromatography (CEC) column (SP Sepharose Big Beads; Cytiva), which was equilibrated with 10 mM PB (pH 6.0). After washing with the same buffer solution, the target protein was eluted using 20 mM Tris-HCl buffer solution (pH 8.0). Next, the resulting protein solution was subjected to an anion exchange chromatography (AEC) column (Q Sepharose Big Beads column; Cytiva) with 20 mM Tris-HCl (pH 8.0) as the running buffer. After washing with 20 mM Tris-HCl (pH 8.0), the rHb(Cβ120/X) was eluted using PBS solution (pH 7.4). The purity was checked by SDS-PAGE analysis and SEC on the HPLC system. The concentration of the rHb(Cβ120/X) was measured using a protein assay kit (Pierce 660 nm; Thermo Fisher Scientific K.K.). The sulphhydryl group assay of the rHb(Cβ120/X) was conducted by reaction with 4,4'-dithiodipyridine (4,4'-DTP). The rHb(Cβ120/X) variant solutions were stored at −80 °C.

Preparation of MA-HSA

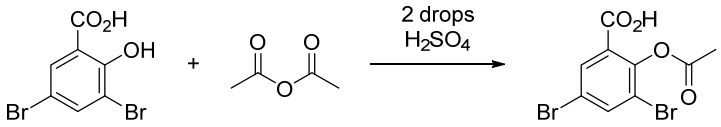
MA-HSA was prepared using our previously reported procedure.[3e] The 25% HSA solution (3.8 mM, 4 mL) was diluted with PBS solution (14 mL, pH 7.4). A DMSO solution of BMH (7.5 mM, 2 mL) was added dropwise to the HSA solution (0.84 mM, 18 mL). After stirring for 3 h at 25 °C, the reactant was subjected to SEC column (Sephadex G25 superfine; Cytiva) to remove the unreacted cross-linker. The protein concentration was measured using a protein assay kit (Pierce 660 nm), whereas the sulphhydryl group assay of MA-HSA was conducted by reaction with 4,4'-DTP. The MA-HSA solution was stored at −80 °C.

Preparation of rHb(Cβ120/XLα99)

To the rHb(Cβ120) solution (0.1 mM, 1 mL, 0.1 M Bis-Tris, pH 7.2), 4,4'-DTP solution (10 mM, 0.1 mL, PBS) was added. After stirring for 2 h at 25 °C, unreacted 4,4'-DTP was removed using SEC (PD-10; Cytiva) equilibrated with 0.1 M Bis-Tris buffer solution (pH 7.2), yielding the protected rHb(Cβ120) [rHb(Cβ120/4-DTP)]. The oxygenation was performed using our previously reported technique.[3d] To the oxy rHb(Cβ120/4-DTP) solution (0.1 mM, 1 mL), the phytic acid (IHP) solution (40 mM, 25 μL) was added, and then N_2 gas was blown into the solution to yield deoxy rHb(Cβ120/4-DTP). The solution was transferred to 5 mL flask containing bis(3,5-dibromosalicyl) fumarate (DBBF, 0.2 mg) under N_2 atmosphere. After stirring for 2 h at 37 °C, glycine solution (1 M, 10 μL) was added to the mixture solution for quenching the unreacted crosslinker. The solution was equilibrated with CO and heated for 30 min at 75 °C to remove the unreacted rHb(Cβ120/4-DTP). The precipitates were

filtrated by a 0.22 μm filter. The crosslinker and glycine were removed using SEC (PD-10; Cytiva) equilibrated with 0.1 M Bis-Tris buffer solution (pH 7.2). To the rHb(Cβ120/4-DTP) solution, the tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) solution (0.1 M, 50 μL) was added, and the mixture was stirred for 20 h at 25 °C. After buffer exchanging to glycine buffer (0.2 mM, pH 8.0), the solution was loaded into an AEC column (DEAE Sepharose Fast Flow; Cytiva) and rHb(Cβ120/XLα99) was eluted using the PBS solution (pH 7.4). The purity was checked by SDS-PAGE analysis. The sulphhydryl group assay was conducted by reaction with 4,4'-DTP.

Preparation of 3,5-dibromoaspirin (DBA)



To a mixture of 3,5-dibromosalicylic acid (5 g, 17 mmol) and acetic anhydride (10 mL, 106 mmol), 2 drops of concentrated sulphuric acid was added. After stirring for 15 min at 55 °C, the mixture was cooled to 25 °C. Then the solution was added to the 100 mL of crashed ice, and stirred at 25 °C for 30 min. The precipitate was collected by filtration and recrystallized from toluene to give DBA (2.0 g, 36%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.18 (d, 2.4 Hz, 1H), 8.01 (d, 2.4 Hz, 1H), 2.40 (s, 3H).

Preparation of rHb(Cβ120/Acα99)

The IHP solution (40 mM, 25 μL) was added to the oxy rHb(Cβ120/4-DTP) solution (0.1 mM, 1 mL) and N_2 gas was blown into the solution to yield deoxy rHb(Cβ120). The solution was transferred to 5 mL flask containing the DBA (0.2 mg, 0.6 μmol) under N_2 atmosphere. After stirring for 4 h at 37 °C, the unreacted DBA was removed using a SEC column (PD-10; Cytiva) equilibrated with 0.1 M Bis-Tris buffer solution (pH 7.2). To the rHb(Cβ120/Acα99) solution, TCEP-HCl solution (0.1 M, 10 μL) was added. The mixture was stirred for 3 h at 25 °C to reduce the disulphide bonds of Cys-β120 residues. After buffer exchanging to glycine buffer (0.2 mM, pH 8.0), the solution was loaded into an AEC column (DEAE Sepharose Fast Flow; Cytiva) and rHb(Cβ120/Acα99) was eluted using the PBS solution (pH 7.4). The sulphhydryl group assay was conducted by reaction with 4,4'-DTP.

Preparation of rHb(Cβ120/X)–HSA$_2$ trimers

The MA-HSA and rHb(Cβ120/X) solutions were mixed and concentrated to 6 mL ([MA-HSA] = 0.4 mM, [rHb(Cβ120/X)] = 0.1 mM). The obtained mixture was stirred under dark conditions for 24 h at 4 °C. The resultant product was purified by AEC column (Q Sepharose Fast Flow; Cytiva). The mixture solution was diluted by the same volume of water and loaded onto the column equilibrated

with PBS solution. The column was then washed using 10 mM PB (pH 7.4) solution. The rHb(Cβ120)–HSA_2 trimer was eluted with a 10 mM PB (pH 6.0) + 120 mM NaCl solution. In this condition, the HSA_2 dimer remained in the column. The collected solution was concentrated and subjected to SEC column (Superdex 200 pg column; Cytiva) to isolate the rHb(Cβ120/X)–HSA_2 trimers (0.29 μmol, 49%). The total protein and Hb concentrations were measured using a protein assay kit (Pierce 660 nm; Thermo Fischer Scientific Inc.) and the extinction coefficient of cyano-metHb ($\varepsilon_{541} = 4.4 \times 10^4\ M^{-1}\ cm^{-1}$). The average [HSA]/[rHb(Cβ120/X) unit] ratio of the product was estimated to be 2.0. The sulphhydryl group assay was conducted by reaction with 4,4'-DTP.

Preparation of oxy and deoxy forms of rHb(Cβ120/X)–HSA_2 trimers

The oxy (O_2 complex) rHb(Cβ120/X)–HSA_2 trimer solutions (3 μM, 3 mL, PBS, pH 7.4) were prepared using our previously reported technique.[3d] The solutions were transferred to an optical quartz cuvette (10 mm path length) with a rubber septum cap. N_2 gas was blown into the oxy-form solution to yield deoxy rHb(Cβ120/X)–HSA_2 trimer. The UV-vis absorption of these species were recorded at 25 °C.

O_2 binding parameters

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 the O_2 equilibrium curve (Hemox Analyzer; TCS Scientific Corp.) at 37 °C. The oxy rHb(Cβ120/X)–HSA_2 trimer solution (PBS, pH 7.4, approximately 5 μM, 4 mL) was used for the measurements. The trimers in PBS solution (pH 7.4) were deoxygenated by flushing with N_2 and oxygenated by increasing the O_2 partial pressure.

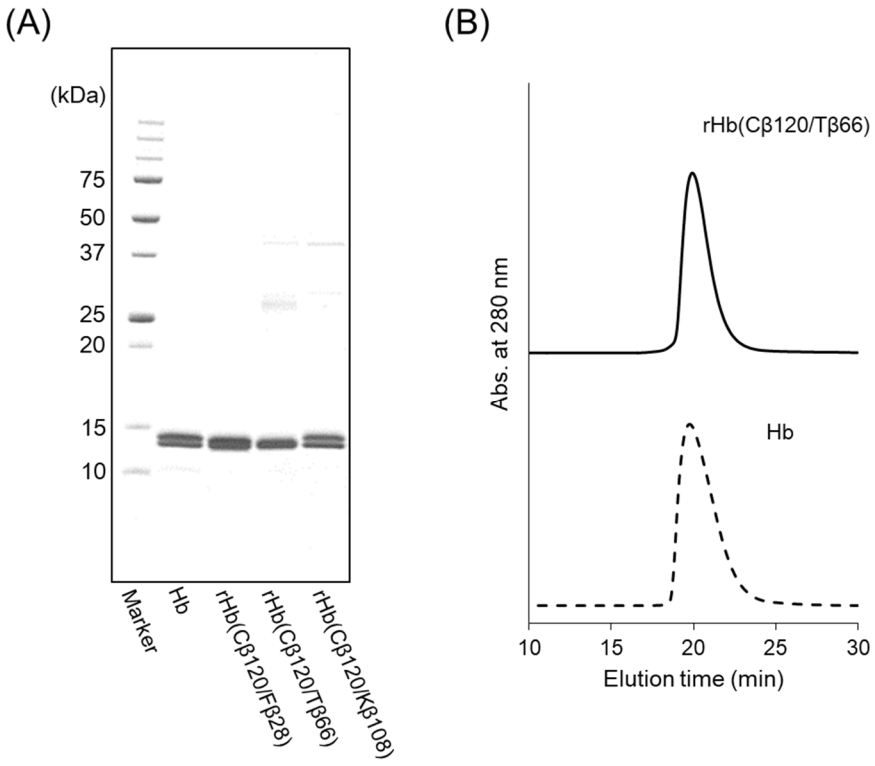


Fig. S1 (A) SDS-PAGE analysis of Hb and rHb(Cβ120/X) variants (X = Fβ28, Tβ66, Kβ108), and (B) SEC profiles of rHb(Cβ120/Tβ66) and Hb.

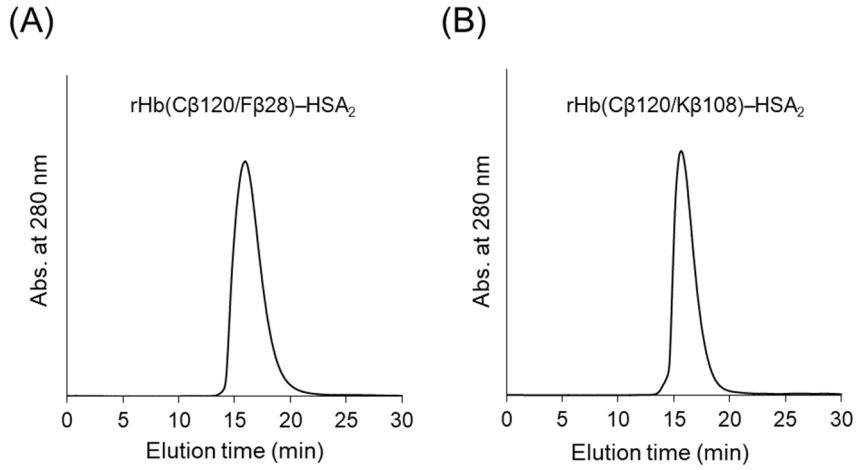


Fig. S2 SEC profiles of rHb(Cβ120/X)–HSA_2 trimers (X = Fβ28, Kβ108).

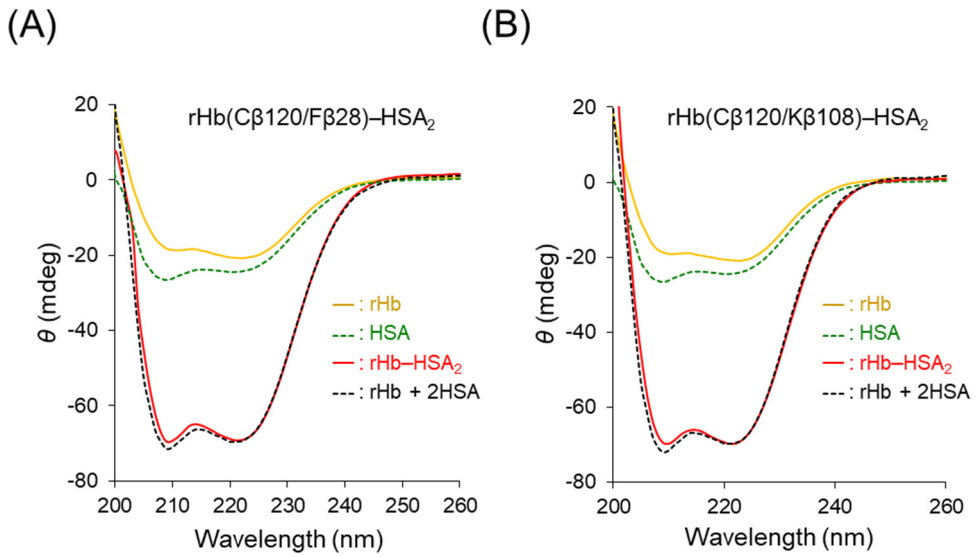


Fig. S3 CD spectra of rHb(Cβ120/X), HSA, and rHb(Cβ120/X)–HSA_2 trimers in PBS at 25 °C ([protein] = 0.2 μM): (A) X = Fβ28 and (B) X = Kβ108.

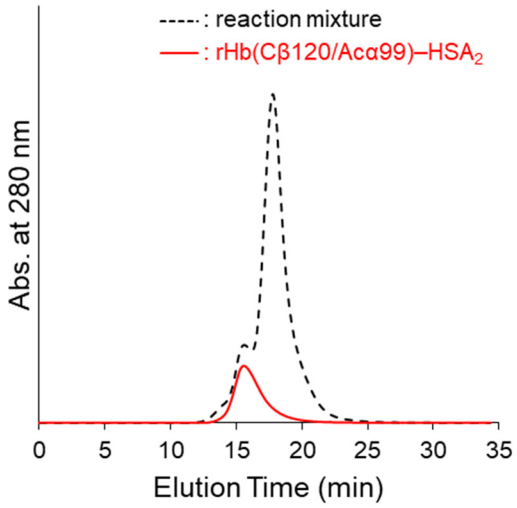


Fig. S4 SEC profiles of reaction mixture and isolated rHb(Cβ120/Acα99)–HSA_2 trimer.

Table S1 UV-vis absorption spectral data of native Hb and rHb(Cβ120/X)–HSA_2 trimers at 25 °C ([protein] = 3 μM, X = Tβ66, Kβ108, Fβ28, XLα99, Acα99).

Haemprotein	λ_{max} (nm)		
	Carbonyl	Oxy	Deoxy
Hb	420, 539, 569	415, 541, 577	430, 555
rHb(Cβ120/Tβ66)–HSA_2	419, 539, 569	414, 541, 577	430, 555
rHb(Cβ120/Kβ108)–HSA_2	419, 538, 569	414, 541, 577	430, 555
rHb(Cβ120/Fβ28)–HSA_2	419, 538, 569	414, 541, 577	430, 555
rHb(Cβ120/XLα99)–HSA_2	419, 538, 568	415, 541, 577	430, 555
rHb(Cβ120/Acα99)–HSA_2	419, 538, 568	415, 541, 576	430, 554

Table S2 Autoxidation rate constants of native Hb and rHb(Cβ120/X)–HSA_2 trimers at 37 °C in PBS solution (pH 7.4).

Haemprotein	k_{ox} (h^{-1})
Hb	0.02
rHb(Cβ120)–HSA_2	0.03
rHb(Cβ120/Tβ66)–HSA_2	0.05
rHb(Cβ120/Kβ108)–HSA_2	0.06
rHb(Cβ120/Fβ28)–HSA_2	0.08
rHb(Cβ120/XLα99)–HSA_2	0.04
rHb(Cβ120/Acα99)–HSA_2	0.05