Table 6. O_2 Binding Properties of HSA-FePs in Phosphate Buffered Solution (pH 7.3, 25 °C)

FeP	$k_{on}(O_2)$ (μM^{-1} s^{-1})		$k_{off}(O_2)$ (ms^{-1})		$P_{50}(O_2)^a$ (Torr)	refs
	fast	slow	fast	slow		
1	34	9.5	0.75	0.20	13 (33)	139, 149, 150
2	46	7.3	0.98	0.16	13 (35)	150
3	36	6.1	0.059	0.010	1 (3)	150
4	54	8.8	0.089	0.014	1 (3)	150
5	54	6.8	0.02	0.0024	0.2 (1)	151
6	54	8.1	0.62	0.093	7 (22)	151
7	34	4.5	0.045	0.0059	0.8 (2)	152
8	11	1.5	0.50	0.069	28	153
9	11	2.0	0.41	0.076	23	153
10	8.9	2.3	0.34	0.088	23	153
12	29	4.4	1.10	0.16	22 (45)	154
13	–	–	–	–	0.1	155, 156
14	–	–	–	–	0.1	155, 156
15	–	–	–	–	0.4	156
16	–	–	–	–	0.1	155, 156
17	28	–	0.33	–	9 (27)	157

[a] At 37 °C in parentheses.

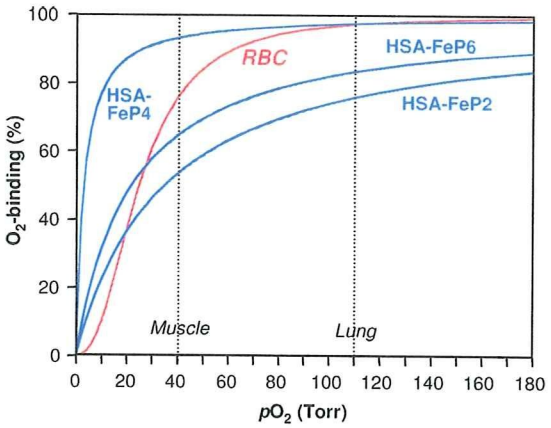


Figure 13. O_2 binding equilibrium curve of HSA-FePs under physiological conditions (pH 7.3, 37 °C).

and COP to those of the nonmodified HSA-FeP2. In contrast, PEG_{M5}(HSA-FeP2) showed a higher viscosity and more pronounced hyperoncotic property relative to those of HSA-FeP2. Nevertheless, PEG_{M5} conjugate may be useful as an efficient plasma expander (*118*, *166*).

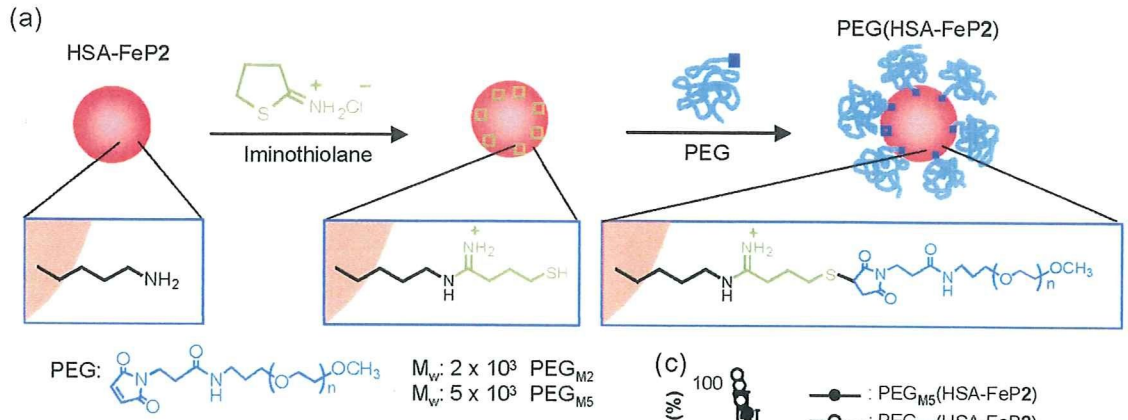
Under physiological conditions, PEG_{Mn}(HSA-FeP2) binds and releases O_2. The $P_{50}(O_2)$ values were almost identical to those of the original HSA-FeP2, indicating that the O_2 binding equilibrium was not influenced by the presence of the PEG chains (Figure 14b). Surface modification by PEG delays proton-driven oxidation of the O_2 adduct complex, giving HSA-FeP2 the $\tau_{1/2}(O_2)$ of 12 h, which is almost equal to that of a natural hemoprotein, Mb [$\tau_{1/2}(O_2)$, 12 h; pH 7, 35 °C] (*167*). The conjugated PEG might change the local proton concentration of the HSA interior compared to the outer aqueous solution.

The circulation persistence of FeP2 in the bloodstream was measured after administration of PEG_{Mn}(HSA-FeP2) to anesthetized rats (*164*). The PEG_{Mn}(HSA-FeP2) solution (20% volume of the circulatory blood) was injected intravenously into rats from the tail vein. The concentration decays of PEG_{Mn}(HSA-FeP2) in the blood showed single exponentials with half-life [$\tau_{1/2}$(FeP2)] of 13−16 h (Figure 14c). These values are considerably longer than those of the corresponding nonmodified HSA-FeP1 (*168*). Surface modification of HSA-FeP2 by PEG prevented the rapid clearance of the incorporated FeP2. On the basis of these findings, we can conclude that surface modification of HSA-FeP2 by PEG comprehensively improved its O_2 transporting ability.

We then proceeded to evaluate physiological responses to an exchange transfusion with PEG_{M2}(HSA-FeP2) in an acute anemia rat model (*169*) (Figure 15). The animals were first placed in a 65 vol % hemodilution with 5 g/dL HSA. They subsequently underwent a 30 vol % blood replacement with the PEG_{M2}(HSA-FeP2) solution. As negative and positive control groups, a 5 g/dL HSA solution (HSA group) and washed RBC suspension (RBC group) were infused, respectively, to similarly operated rats in hemorrhage. The isovolemic 65% hemodilution with HSA reduced the Hb concentration, thereby decreasing the O_2 supply to the tissue. Consequently, the mean arterial pressure (MAP), renal cortical O_2 partial pressure [PtO_2(R)], and O_2 partial pressure of muscle tissue [PtO_2(M)] were decreased. During hemorrhagic shock by 30% bleeding, significant decreases in the MAP, venous O_2 pressure (PvO_2), PtO_2(R), and PtO_2(M) were observed by the loss of the circulation blood volume. The heart rate (HR) and respiration rate were also decreased. In contrast, arterial O_2 pressure (PaO_2) increased to about 160% of the basal value (b.v.). The arterial CO_2 pressure ($PaCO_2$) decreased to about 62% of the b.v.; the pH increased to 7.55.

The injection of the sample solutions increased the blood volume and improved the circulatory flow. Lactate was washed out from the tissues and into the circulatory system, which decreased the pH to the initial level of 7.43 in all groups. The administration of HSA restored no parameters: death occurred within 41 min. In contrast, the infusion of PEG_{M2}(HSA-FeP2) or RBC kept all the rats alive until the end of measurements. After injection of PEG_{M2}(HSA-FeP2), the animals showed marked and rapid recovery in MAP, HR, PaO_2, PvO_2, $PaCO_2$, and pH, resembling that shown in the RBC group. These results demonstrate the O_2 transporting capability of the PEG_{M2}(HSA-FeP2) solution as a resuscitative fluid. We observed that albumin-based oxygen carrier does not induce hypertensive action, because of its low permeability through the vascular endothelium in comparison with that of Hb molecules. The heart rate responses after the injection were also negligibly small. Visualization of the intestinal microcirculatory changes clearly revealed the widths of the venule and arteriole to be fairly constant (*170*).

Reversible oxygenation of PEG_{M2}(HSA-FeP2) was observed even in the solid state (*171*). The aqueous solution of PEG_{M2}(HSA-FeP2(CO)) complex was spread on the glass plate and dried overnight at room temperature, producing a red transparent solid membrane (Figure 16a). In contrast, HSA-FeP2 without PEG decollation yielded a brittle membrane with many cracks. Scanning electron microscopy (SEM) observations of the PEG_{M2}(HSA-FeP2) membrane showed a uniform thickness of 15 μm and a smooth surface (Figure 16b). The $\tau_{1/2}(O_2)$ was 40 h, which is three times longer than the value in water. The



(b)

Solution and O_2 binding properties of PEG_{Mn}(HSA–FeP2)[a]

Samples	M_w of PEG	Viscosity (cP)	COP (Torr)	P_{50} (Torr)
PEG_{M2}(HSA–FeP2)	2 × 10³	1.14	27	32
PEG_{M5}(HSA–FeP2)	5 × 10³	2.34	65	31
HSA–FeP2	-	1.05	21	35

a) In phosphate buffered solution ([HSA]: 5g/dL, PEG_{Mn}/HSA–FeP2= 6, mol/mol) in average, pH 7.3, 37°C.

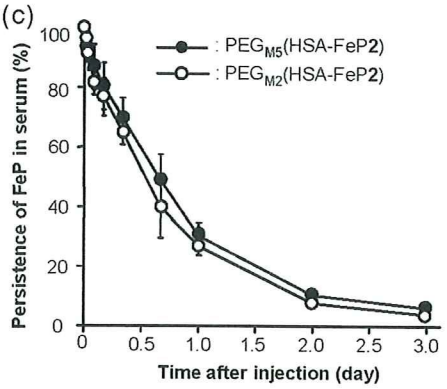


Figure 14. Surface modification of HSA-FeP with poly(ethylene glycol). (a) Synthetic scheme of PEG_{Mn}(HSA-FeP2). (b) Solution and O_2 binding properties. (c) Persistence of FeP2 in serum after administration of PEG_{Mn}(HSA-FeP2) into Wistar rats. Each value represents the mean ± SD of four rats.

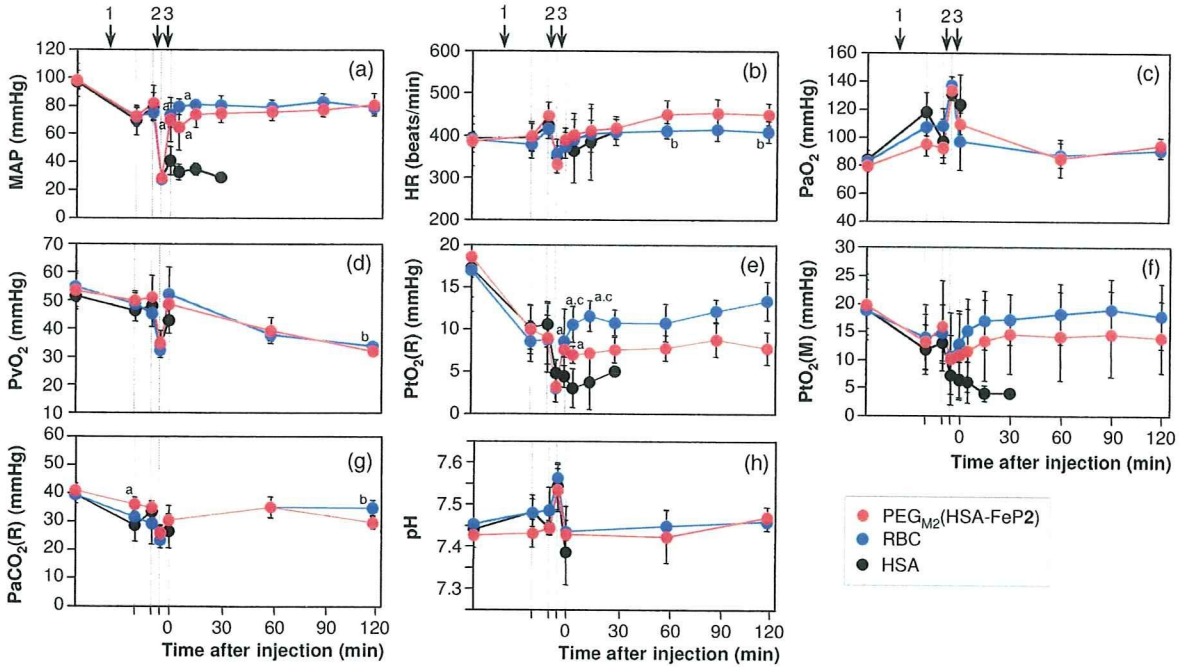


Figure 15. Effect of PEG_{M2}(HSA-FeP2) solutions on (a) MAP, (b) HR, (c) PaO_2, (d) PvO_2 (e) PtO_2(R), (f) PtO_2(M), (g) $PaCO_2$, and (h) pH in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SD of five rats [red, PEG_{M2}(HSA-FeP2) group; blue, washed RBC group; and black, HSA group]. Arrows (1), (2), and (3), respectively, indicate the periods of 65% hemodilution, 30% bleeding, and sample infusion. [a] $p < 0.05$ versus HSA group (Tukey-Kramer test), [b] $p < 0.05$ versus PEG_{M2}(HSA-Fe2) group (unpaired t-test), and [c] $p < 0.05$ versus PEG_{M2}(HSA-FeP2) group (Tukey-Kramer test).

O_2 binding affinity was about a half that of the monomeric PEG_{M2}(HSA-FeP2).

We subsequently added hyaluronic acid (HA) as a supporting polymer to the protein solution and prepared the solid membrane on a poly(styrene) dish. Actually, HA is known as a glycosaminoglycan component of connective tissues, hyaline bodies, and extracellular matrix (*172*). Water evaporation of the PEG_{M2}(HSA-FeP2)/HA mixture ([HSA]: 2.5 wt % and [HA]: 0.2 wt %) produced a uniform red solid membrane that was easily peeled from the dish, yielding a free-standing homogeneous thin film of the PEG(HSA-FeP2)/HA hybrid (Figure 16c,d).

The PEG_{M2}(HSA-FeP2) solution is useful as a valuable O_2-carrying plasma. Membranes of PEG_{M2}(HSA-FeP2) with micrometer thickness can serve as a RBC substitute that can be preserved anywhere and reproduced as a saline solution at any time.

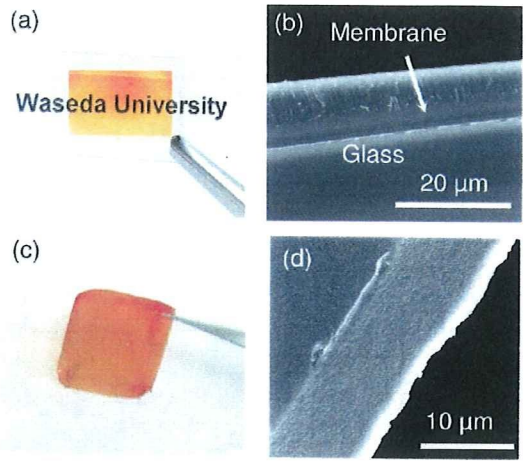


Figure 16. The solid membrane of PEG_{M2}(HSA-FeP). (a) Photograph of the membrane on the glass, (b) SEM of the membrane section, (c) photograph of the flexible film peeled from the poly(styrene) dish, and (d) SEM of the isolated film.

3.3. Recombinant HSA-Heme (rHSA-Heme) Prepared Using Site-Directed Mutagenesis. Hemin [Fe^{3+}protoporphyrin IX] released from metHb during enucleation of RBC or through hemolysis is captured by HSA with a high binding constant ($K \approx 10^8\ M^{-1}$) (*173*). Crystallographic studies have revealed that hemin is bound within a narrow D-shaped hydrophobic cavity in subdomain IB with axial coordination of Tyr-161 to the central ferric ion and electrostatic interactions between the porphyrin propionates and a triad of basic amino acid residues (Arg-114, His-146, and Lys-190) (Figure 17) (*174*, *175*). In terms of the general hydrophobicity of this α-helical heme pocket, the subdomain IB of HSA potentially has similar features to the heme binding site of Hb or Mb. However, when one reduces HSA-hemin to obtain the ferrous complex, it is autoxidized rapidly by O_2, even at low temperature (~0 °C), because HSA lacks the proximal His, which, in Hb and Mb, enables the prosthetic heme group to bind O_2. Knowledge of the detailed architecture of the heme binding site in HSA enables us to design mutagenesis experiments to construct a tailor-made heme pocket for stable O_2 binding. Therefore, we used site-directed mutagenesis to introduce an His into the heme binding site that was expected to provide axial coordination to the central Fe^{2+} atom of the heme and thereby promote O_2 binding.

Results of our modeling experiments suggested that a favorable position for the axial imidazole insertion would be Ile-142 (Figure 17). The N_ϵ(His)-Fe distances were estimated as 2.31 Å for H142 (compared to 2.18 Å for Mb). We therefore designed and produced two single mutants I142H and a double mutant I142H/Y161L (HL) (*176*).

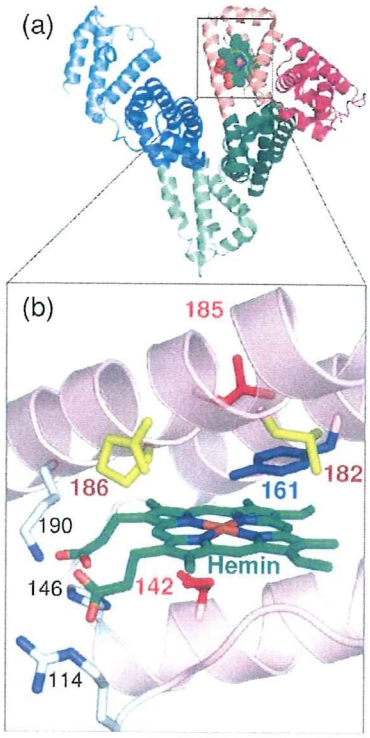
In the UV−vis absorption spectrum of rHSA(HL)-hemin, the ligand-to-metal charge transfer band at 625 nm was weakened because of the Y161L mutation. The MCD spectrum of rHSA(HL)-hemin showed a similar S-shaped pattern in the Soret band region resembling that of ferric Mb (*177*, *178*). These results suggest that rHSA(HL)-hemin is in a predominantly ferric high-spin complex having a water molecule as the sixth ligand. The rHSA-hemin was easily reduced to the ferrous complex by adding a small molar excess of aqueous sodium dithionite under an Ar atmosphere (Figure 18). A single broad absorption band (λ_{max}: 559 nm) in the visible absorption spectrum and the MCD spectrum of rHSA(HL)-heme indicated the formation of a five-N-coordinate high-spin complex (*176*, *177*, *179*). The heme therefore appears to be accommodated in the mutated heme pocket with an axial coordination involving His-142. Upon exposure of rHSA(HL)-heme solution to O_2, the UV−vis absorption changed immediately to that of the O_2 adduct complex (Figure 18). It formed a carbonyl complex under a CO atmosphere. The single mutant rHSA(I142H)-heme, which retains Y161, was unable to bind O_2. The polar phenolate residue at the top of the porphyrin plane is likely to accelerate the proton-driven oxidation of the Fe^{2+} center. The replacement of Tyr-161 in rHSA(I142H)-heme by Leu enhanced stabilization of the O_2 adduct complex.

To evaluate the kinetics of O_2 and CO bindings to rHSA-hemes, laser flash photolysis experiments were carried out (Tables 7 and 8). It is noteworthy that the absorbance decay accompanying the CO recombination to rHSA(HL)-heme was composed of double-exponential profiles, which is normally not observed in Mb (the faster phase is defined as species I; the slower phase is defined as species II). The ratio of the amplitude of the species I and the species II was approximately 3:2. On the other hand, the rebinding of O_2 to rHSA(HL)-heme followed a simple monophasic decay. Numerous investigations of synthetic model hemes have helped to reveal the relation between the structure around the hemes and their O_2 and CO binding abilities (*4*, *147*, *148*). A bending strain in the proximal base coordination to the central Fe^{2+} atom, the "proximal-side steric (proximal pull) effect", is known to be capable of both increasing the dissociation rate for CO and decreasing the association rate. Simultaneously, it increases the O_2 dissociation rate without greatly altering the O_2 association kinetics. Consequently, one possible explanation for the existence of the two phases is that two different geometries of the axial His (His-142) coordination to the central ferrous ion of the heme might exist, each one accounting for a component of the biphasic kinetics of CO rebinding.

3.4. Modulation of O_2 Binding Property of rHSA-(mutant)−Heme. To control the O_2 binding affinity of rHSA-heme, we designed and produced diverse rHSA(mutant)−hemes in which bulky hydrophobic or hydrophilic amino acids were introduced around the O_2 binding site (Tyr-161, Leu-182, Leu-185, and Arg-186) (Figure 17). More recently, the beneficial effect of low-dose CO on the microcirculation by a hemoglobin-based artificial oxygen carrier has been discussed (*132*, *182*). Control of the CO binding affinity of rHSA−heme is also tempting.

A. Substitution of Tyr-161 with Leu or Phe. The first, Tyr-161, was substituted to noncoordinating and hydrophobic amino acids (Leu or Phe). The O_2 and CO binding properties of rHSA(HL)−heme and rHSA(I142H/Y161F)−heme [rHSA(HF)−heme] showed that the presence of a Phe rather than a Leu at position 161 results in 6-fold and 4-fold increases in the O_2 binding affinity for species I and II, respectively (Table 7). This enhancement is mainly attributable to an increase in the O_2 association rate constant. The same trend was observed for CO binding [3-fold increase in k_{on}(CO)] (Table 8). The substitution of Leu-161 (102 $Å^3$) by Phe-161 (137 $Å^3$) (*183*) replaces an isopropyl group with a rigid benzyl group within the heme pocket. In rHSA(HL), the small side chain of Leu-161 might enable free rotation of the side chain of neighboring Leu-185, thereby reducing the volume on the distal side of the porphyrin plane (Figure 19a,b). On the other hand, the bulkier aromatic side chain of Phe-161 might prevent rotation of the isopropyl group of Leu-185 and thereby provide greater room of the distal pocket; this effect might provide easier access to the heme Fe^{2+} atom and account for the increased association rates for O_2 and CO.

B. Substitution of Leu-185 with Polar Amino Acid. Leu-185 was substituted with a more hydrophilic amino acid (Asn, Gln, or His), which was expected to interact with the coordinated O_2 by hydrogen bond and to stabilize the O_2 adduct complex similarly to Hb and Mb. In rHSA(mutant)−hemes in which Gln or His was introduced into Leu-185, they formed ferrous six-coordinated low-spin complexes under an Ar atmosphere. That



rHSA–heme	Position				
	142	161	182	185	186
Wild type	Ile	Tyr	Leu	Leu	Arg
I142H	***His***	Tyr	Leu	Leu	Arg
I142H/Y161L (HL)	***His***	***Leu***	Leu	Leu	Arg
I142H/Y161F (HF)	***His***	***Phe***	Leu	Leu	Arg
HL/L185N	***His***	***Leu***	Leu	***Asn***	Arg
HF/L185N	***His***	***Phe***	Leu	***Asn***	Arg
HL/L185Q	***His***	***Leu***	Leu	***Gln***	Arg
HF/L185Q	***His***	***Phe***	Leu	***Gln***	Arg
HF/L185H	***His***	***Phe***	Leu	***His***	Arg
HL/R186L	***His***	***Leu***	Leu	Leu	***Leu***
HL/R186F	***His***	***Leu***	Leu	Leu	***Phe***

Figure 17. (a) Crystal structure of HSA-hemin complex (1O9X) from ref 174. Hemin is shown in a space-filling representation. (b) Heme pocket structure in subdomain IB and positions of amino acids where site-specific mutations were introduced. Abbreviations of rHSA(mutant)s are shown in the table.

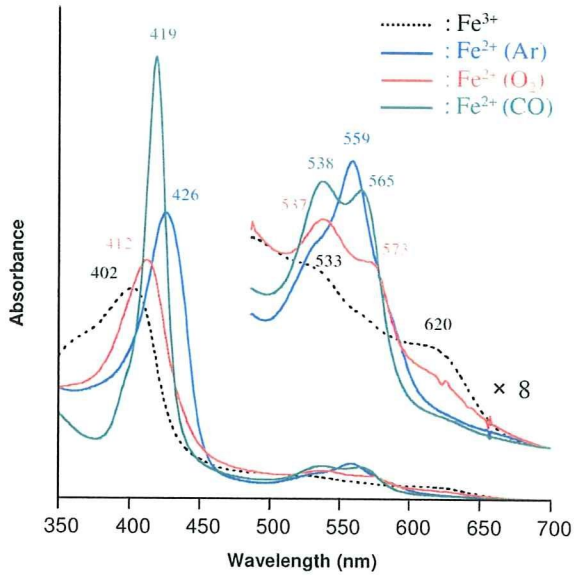


Figure 18. UV−vis absorption spectral changes of rHSA(HL)-heme in potassium phosphate buffered solution (pH 7.0).

result suggests that the introduced amino acid coordinates to the heme iron as a sixth ligand under an Ar atmosphere. Upon exposure of the solutions to O_2, they were oxidized. Bis-histidyl hemochromes are known to be oxidized by O_2 rapidly via an outer sphere mechanism (*184*−*186*). On the other hand, rHSA(HL/L185N)−heme and rHSA(HF/L185N)−heme in which Asn was introduced at Leu-185 formed ferrous five-coordinated high-spin complexes under an Ar atmosphere. They formed O_2 adduct complexes under O_2 atmosphere. The introduced Asn is estimated to be too far to coordinate to the heme.

Marked differences were apparent in a comparison of the O_2 and CO binding parameters for rHSA(HL)−heme and rHSA(HL/L185N)−heme. First, the presence of Asn rather than Leu at

Table 7. O_2 Binding Parameters of rHSA(Mutant)-Heme Complexes in Phosphate Buffered Solution (pH 7.0) at 22 °C

hemoproteins	$k_{on}(O_2)$ ($\mu M^{-1} s^{-1}$)	$k_{off}(O_2)$ ($m\ s^{-1}$)		$P_{50}(O_2)$ (Torr)	
		I	II	I	II
rHSA(HL)-heme	7.5	0.22	1.70	18	134
rHSA(HF)-heme	20	0.10	0.99	3	31
rHSA(HL/L185N)-heme	14	0.02	0.29	1	14
rHSA(HF/L185N)-heme	26	0.10	1.03	2	24
rHSA(HL/R186L)-heme	25	0.41	8.59	10	209
rHSA(HL/R186F)-heme	21	0.29	7.01	9	203
Mb[a]	14	0.012		0.51	
RBC[b]				8	

[a] Sperm whale myoglobin in 0.1 M potassium phosphate buffer (pH 7.0, 20 °C); ref 180. [b] Human red cell suspension in isotonic buffer (pH 7.4, 20 °C); ref 181.

Table 8. CO Binding Parameters of rHSA(Mutant)-Heme Complexes in Phosphate Buffered Solution (pH 7.0) at 22 °C

hemoproteins	$k_{on}(CO)$ ($\mu M^{-1}\ s^{-1}$)		$k_{off}(CO)$ (s^{-1})		$P_{50}(CO)$ (Torr)	
	I	II	I	II	I	II
rHSA(HL)-heme	2.0	0.27	0.013	0.079	0.0053	0.240
rHSA(HF)-heme	6.8	0.72	0.009	0.061	0.0011	0.068
rHSA(HL/L185N)-heme	6.8	1.60	0.008	0.039	0.0010	0.020
rHSA(HF/L185N)-heme	7.7	1.09	0.008	0.043	0.0008	0.032
rHSA(HL/R186L)-heme	5.0	0.57	0.011	0.165	0.0018	0.234
rHSA(HL/R186F)-heme	7.9	1.12	0.010	0.148	0.0010	0.107
Mb[a]	0.51		0.019		0.03	

[a] Sperm whale myoglobin in 0.1 M potassium phosphate buffer (pH 7.0, 20 °C); ref 180.

position 185 caused 2-fold and 3−6-fold increases, respectively, in the $k_{on}(O_2)$ and $k_{on}(CO)$ values. The Asn might partly rotate upward, which provides somewhat greater space of the distal pocket. Second, Asn-185 induced 18-fold and 10-fold increases in the O_2 binding affinity for species I and II, because the $k_{off}(O_2)$

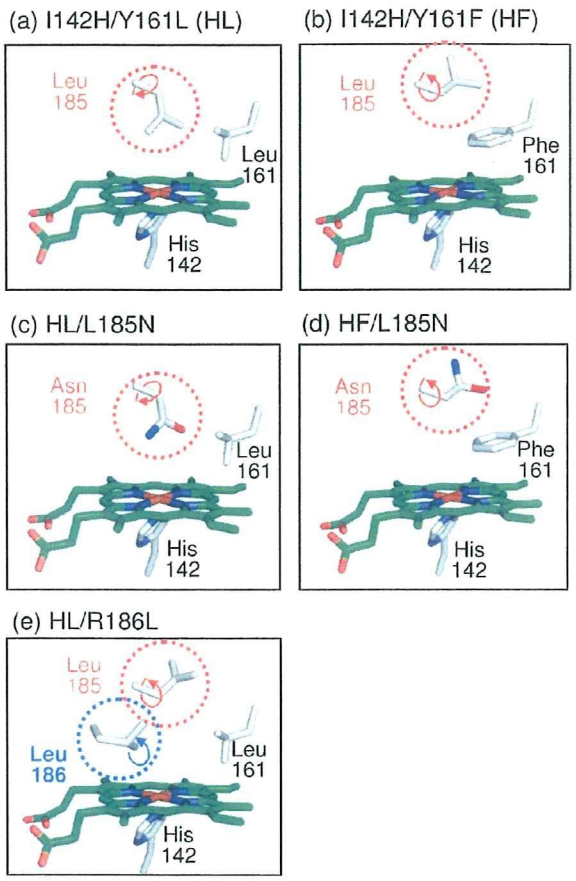


Figure 19. The proposed configuration of Leu-185 in (a) rHSA(HL)−heme and (b) rHSA(HF)−heme, Asn-185 in (c) rHSA(HL/L185N)−heme and (d) rHSA(HF/L185N)−heme, and Leu-186 in (e) rHSA(HL/R186L)–heme.

values were 1/6−1/11 of their former values. This corresponds to a free energy difference of −1.8 kcal mol^{-1} at 22 °C. The magnitude of the effect seems to be reasonable considering that, in HbO_2 and MbO_2, the distal His-64 stabilizes the coordinated O_2 by −0.6 to −1.4 kcal mol^{-1} because of the hydrogen bond (*187*). In contrast, The O_2 and CO binding parameters for rHSA(HF)−heme and rHSA(HF/L185N)−heme showed no significant differences. The bulky benzyl side chain of Phe-161 can prevent rotation of the polar amide group of Asn-185 and thereby decrease the effect of polarity and size on O_2 and CO binding parameters (Figure 19c,d) (*188*).

C. Substitution of Arg-186 with Leu or Phe. For administration into the human circulatory system, it would be better if the affinity were similar to the human RBC [$P_{50}(O_2)$: 8 Torr, 25 °C]. It is expected that providing a certain degree of hydrophobicity into the distal side of the heme by insertion of a nonpolar residue would reduce the O_2 binding affinity of the rHSA−heme complex. The most suitable position for that introduction might be at Arg-186, which is the entrance of the heme pocket and which is rather close to the central Fe(II) ion. Therefore, rHSA(HL/R186L)−hemin and rHSA(HL/R186F)−hemin were prepared. The O_2 dissociation rate constants of rHSA(HL/R186L)−heme and rHSA(HL/R186F)−heme were 3−4-fold higher than that of rHSA(HF)−heme, which reduced the O_2 binding affinities [larger $P_{50}(O_2)$]. This reduction might be attributable to the increased hydrophobicity in the distal pocket. The O_2 binding affinities of rHSA(HL/R186L)−heme [$P_{50}(O_2)$: 10 Torr] and rHSA(HL/R186F)−heme [$P_{50}(O_2)$: 9 Torr] have become equivalent to those of human RBC. The important structural factor in these mutants is Y161L, which enables the rotation of the isopropyl group of Leu-185 above the O_2 coordination site. Unexpectedly, the $k_{on}(O_2)$ and $k_{on}(CO)$ values of rHSA(HL/R186L)−heme and rHSA(HL/R186F)−heme were 3-fold and 3−4-fold higher than those of rHSA(HL)−heme and in the same range as that of rHSA(HF)−heme. In fact, Leu-161 is small, but the hydrophobic Leu-186 or Phe-186 might be integrated into the heme pocket from the entrance and might push up the neighboring Leu-185 residue (Figure 19e) (*188*).

We have engineered mutant rHSA−heme complexes that can bind O_2. Principal modifications to the heme pocket that are necessary to confer reversible O_2 binding are (i) replacement of Tyr-161 by hydrophobic amino acid (Leu or Phe), and (ii) introduction of His as a proximal base at position Ile-142. Furthermore, (iii) modification of the distal amino acid has a considerable effect on the modulation of O_2 and CO binding affinities.

4. CONCLUSIONS

The structures of our artificial O_2 carriers differ greatly from those of sophisticated RBCs. However, clear advantages of simplified artificial O_2 carriers are readily apparent: the absence of blood-type antigens and infectious viruses, stability for long-term storage at room temperature for any emergency, all of which overwhelm the functionality of RBCs. The shorter half-life of artificial O_2 carriers in the bloodstream (ca. 3 days) limits their use, but they are applicable as a transfusion alternative for shorter periods of use. Easy manipulation of physicochemical properties such as $P_{50}(O_2)$ and viscosity supports their possible development of tailor-made O_2 carriers to suit various clinical indications. The achievements of ongoing research described above give us confidence in advancing the further development with the expectation of its eventual realization.

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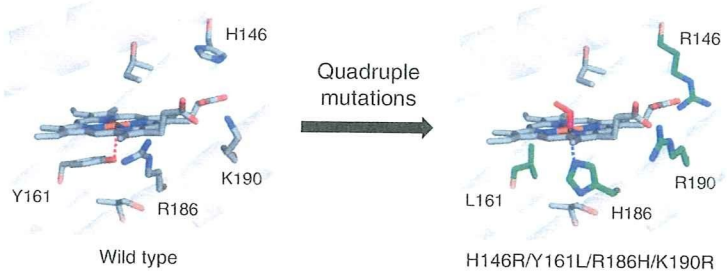
O_2 Binding Properties of Human Serum Albumin Quadruple Mutant Complexed Iron Protoporphyrin IX with Axial His-186 Coordination

Akito Nakagawa,[1] Teruyuki Komatsu,*[1,2] Stephen Curry,[3] and Eishun Tsuchida[1]
[1]*Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555*
[2]*PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi 332-0012*
[3]*Biophysics Section, Blackett Laboratory, Imperial College London, Exhibition Road, London SW7 2AZ, U.K.*

(Received May 18, 2009; CL-090494; E-mail: teruyuki@waseda.jp)



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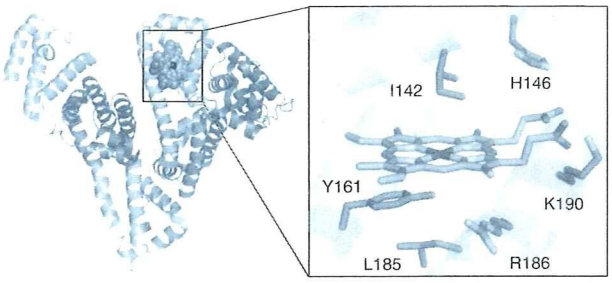
Akito Nakagawa,[1] Teruyuki Komatsu,*[1,2] Stephen Curry,[3] and Eishun Tsuchida[1]
[1]*Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555*
[2]*PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi 332-0012*
[3]*Biophysics Section, Blackett Laboratory, Imperial College London, Exhibition Road, London SW7 2AZ, U.K.*

(Received May 18, 2009; CL-090494; E-mail: teruyuki@waseda.jp)

The O_2 binding properties of complexes of iron(II) protoporphyrin IX with quadruple mutants of recombinant human serum albumin (rHSA) that provide axial His-186 coordination have been characterized; their O_2 binding parameters were similar to those of analogues having proximal His-185 and of human red blood cells.

In our bloodstream, iron(III) protoporphyrin IX (hemin) dissociated from methemoglobin (metHb) is captured by human serum albumin (HSA) and transported to liver cells for catabolism. Crystal structure analysis of this naturally occurring hemoprotein revealed that hemin is bound within a narrow D-shaped cavity in subdomain IB of HSA with a weak axial coordination by Tyr-161 and electrostatic interactions between the porphyrin propionate side-chains and three basic amino acid residues (Arg-114, His-146, and Lys-190) (Figure 1).[1,2] The axial phenolate ligation by Tyr-161 of HSA keeps the hemin group physiologically silent. In fact, the reduced ferrous HSA–heme is immediately oxidized by O_2.[3] We previously demonstrated that a pair of site-specific mutations in subdomain IB of HSA conferred O_2 binding capability on the heme: (i) introduction of a proximal His at Leu-185 position and (ii) substitution of Tyr-161 with noncoordinating Leu.[4a,4b] The resulting artificial hemoprotein can reversibly bind O_2 in much the same way as Hb and myoglobin (Mb). The albumin-based O_2 carrier has attracted medical interest because of its potential acting as a red blood cell (RBC) substitute. Interestingly, the proximal His introduced into the opposite side of the porphyrin plane (Ile-142 position) also allows O_2 binding to the heme.[4] These results suggest that there may be other sites where the proximal His can be inserted in the coordination sphere of the central iron. Our modeling and experimental results showed that Arg-186 is the third candidate because rHSA(I142H/Y161L/R186H)–heme formed a bishistidyl low-spin hemochrome.[4c] Furthermore, we have recently found that replacing His-146 and Lys-190 at the entrance of the heme pocket with Arg (H146R, K190R) resolved the structural heterogeneity of the two orientations of the porphyrin plane and afforded a single O_2 binding affinity.[4d]



rHSA	Position				
	146	161	185	186	190
Wild type (wt)	His	Tyr	Leu	Arg	Lys
H146R/Y161G/R186H/K190R (1G)	**Arg**	**Gly**	Leu	**His**	**Arg**
H146R/Y161L/R186H/K190R (1L)	**Arg**	**Leu**	Leu	**His**	**Arg**
H146R/Y161G/L185H/K190R (2G)	**Arg**	**Gly**	**His**	Arg	**Arg**
H146R/Y161L/L185H/K190R (2L)	**Arg**	**Leu**	**His**	Arg	**Arg**

Figure 1. Structure of the heme pocket in the rHSA(wt)–hemin complex (PDB ID: 1O9X from ref 2).[5] Positions of the amino acids where site-specific mutations were introduced and abbreviations of the mutants are shown in the table. Structural models of the four rHSA(quadruple mutant)–heme complexes are demonstrated in Figure S1.[5,6]

In this study, we generated new rHSA(quadruple mutant)–heme complexes involving axial His-186 coordination and kinetically characterized their O_2 binding properties. The steric effect of the neighboring amino acid at the 161 position to the O_2 binding parameters is also investigated.

We designed rHSA quadruple mutants; rHSA(H146R/Y161G/R186H/K190R) [rHSA**1**G], rHSA(H146R/Y161L/R186H/K190R) [rHSA**1**L], rHSA(H146R/Y161G/L185H/K190R) [rHSA**2**G], and rHSA(H146R/Y161L/L185H/K190R) [rHSA**2**L] (Figure 1). Site-specific mutations were introduced into the HSA coding region in a plasmid vector (pHIL-D2 HSA) using the QuikChange (Stratagene) mutagenesis kit. All mutations were confirmed by DNA sequencing. The proteins were expressed in the yeast species *Pichia pastoris*. The corresponding ferric rHSA–hemin complexes were prepared according to our previously reported procedures.[4]

UV–vis absorption spectra of the four rHSA(quadruple mutant)–hemin complexes were essentially identical regarding their general features (Figure 2, Table S1).[6] They were easily reduced to the ferrous complexes by adding a small molar excess of aqueous $Na_2S_2O_4$ under an N_2 atmosphere (Figure S1).[6] A broad absorption band (λ = 557–559 nm) in the visible region was similar to that observed for deoxy Mb, indicating the formation of a five-N-coordinate high-spin ferrous complex.[7,8] Upon exposure of the rHSA–heme solution to O_2, the UV–vis absorption changed to that of the O_2 adduct complex (Figure 2).[4,7,8] After flowing CO gas, these hemoproteins produced stable carbonyl complexes. It can be concluded that the histidyl group at position 186 acts as a proximal base for dioxygenation of the prosthetic heme group. In contrast, rHSA(single mutant)–heme [rHSA(L185H)–heme and rHSA(R186H)–heme] could not bind O_2. In these complexes, Tyr-161 appears to coordinate to the central ferrous ion of the heme in competition with His-186 or His-185.

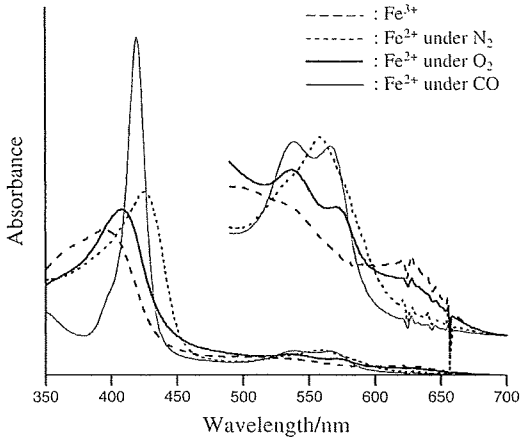


Figure 2. UV–vis. absorption spectral changes of rHSA**1**L–heme in 50 mM potassium phosphate buffered solution (pH 7.0) at 22 °C.

To determine the association and dissociation rate constants (k_{on} and k_{off}) for O_2 binding to rHSA(quadruple mutant)–heme, laser flash photolysis experiments were carried out.[4b] The O_2 recombination to the heme after the laser pulse irradiation occurs according to eq 1.

$$\text{Heme} + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \text{Heme-}O_2 \quad (1)$$

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

The time dependences of the absorbance decays accompanying the O_2 and CO recombinations to rHSA(quadruple mutant)–heme complexes were clearly monophasic (Figure S2).[6] This can be attributed to a uniform heme orientation in the subdomain IB by introduction of Arg into the His-146 and Lys-190 positions.[4] As a result, each hemoprotein showed a single O_2 binding affinity (Table 1). It is noteworthy that all the rHSA(quadruple mutant)–heme complexes exhibited similar O_2 binding parameters independent of the position of the axial base (His-185 or His-186) and the size of the hydrophobic amino acid residue at 161 (Gly or Leu). We had postulated that the small Gly-161 would provide greater room for the proximal His-186 (or His-185), thereby loosening the spatially confined axial ligation. In general, such fluctuation decreases the k_{off} value and enhances the O_2 binding.[4b,4c] However, this was not observed in dioxygenation of rHSA**1**G–heme and rHSA**2**G–heme. The O_2 binding affinities ($P_{1/2}$) of the rHSA(quadruple mutant)–heme complexes (5–8 Torr) are very close to that of the human RBC ($P_{1/2}$ = 8 Torr) and, therefore, well adapted for O_2 transport in the circulatory system.

In conclusion, we have prepared rHSA(quadruple mutant)–heme complexes, in which (i) the proximal His was introduced at position 186 (or 185), (ii) Tyr-161 was substituted with Gly or Leu, and (iii) His-146 and Lys-190 at the heme pocket entrance were replaced with Arg. These artificial hemoproteins formed O_2 adduct complexes with a similar O_2 binding affinity. On the basis of our systematic investigations on rHSA–heme,[4] we conclude that the favorable positions for proximal His insertion are 142, 185, and 186; in all cases Tyr-161 must be replaced with noncoordinating amino acid (e.g., Gly, Leu, Phe, though Ala, Val, or Ile may also be tolerated). This structural flexibility of the heme pocket architecture in HSA has enabled the creation not only of an artificial O_2 carrier using the most abundant plasma protein but may also allow engineering of various hemoprotein enzymes.

Table 1. O_2 binding parameters of rHSA(quadruple mutant)–heme in 50 mM potassium phosphate buffered solution (pH 7.0) at 22 °C

Hemoproteins	k_{on} /μM^{-1} s^{-1}	k_{off} /ms^{-1}	$P_{1/2}$ /Torr
rHSA**1**G–heme	39	0.36	6
rHSA**1**L–heme	67	0.54	5
rHSA**2**G–heme	36	0.46	8
rHSA**2**L–heme[a]	42	0.41	6
Hbα (R-state)	33[b]	0.013[c]	0.24
Mb[d]	14	0.012	0.51
RBC[e]			8

[a]Ref 4d. [b]In 0.1 M phosphate buffer (pH 7.0, 21.5 °C), ref 9. [c]In 10 mM phosphate buffer (pH 7.0, 20 °C), ref 10. [d]In 0.1 M phosphate buffer (pH 7.0, 20 °C), ref 11. [e]Human RBC suspension, in isotonic buffer (pH 7.4, 20 °C), ref 12.

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The role of an amino acid triad at the entrance of the heme pocket in human serum albumin for O_2 and CO binding to iron protoporphyrin IX†

Teruyuki Komatsu,*[a,b] Akito Nakagawa,[a] Stephen Curry,[c] Eishun Tsuchida,[a] Kenichi Murata,[d] Nobuhumi Nakamura[d] and Hiroyuki Ohno[d]

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Complexation of iron(II) protoporphyrin IX (Fe^{2+}PP) into a genetically engineered heme pocket on recombinant human serum albumin (rHSA) creates an artificial hemoprotein which can bind O_2 reversibly at room temperature. Here we highlight a crucial role of a basic amino acid triad the entrance of the heme pocket in rHSA (Arg-114, His-146, Lys-190) for O_2 and CO binding to the prosthetic Fe^{2+}PP group. Replacing His-146 and/or Lys-190 with Arg resolved the structured heterogeneity of the possible two complexing modes of the porphyrin and afforded a single O_2 and CO binding affinity. Resonance Raman spectra show only one geometry of the axial His coordination to the central ferrous ion of the Fe^{2+}PP.

Introduction

Hemin [iron(III) protoporphyrin IX (Fe^{3+}PP), Fig. 1] dissociated from methemoglobin (metHb) is potentially toxic in the human body, because it intercalates in phospholipid membranes and participates in Fenton's reaction to produce hydroxyl radicals.[1] Hemopexin (Hpx, 60,000 Da), a β-glycoprotein in plasma (< 17 μM), captures the Fe^{3+}PP with an extraordinarily high binding affinity ($K > 10^{12}$ M^{-1}) and transports it to the liver for catabolism.[2] When Hpx becomes saturated (*e.g.* as a result of serious hemolytic injuries), the Fe^{3+}PP is first bound by human serum albumin (HSA, 66,500 Da) ($K = 1.1 \times 10^8$ M^{-1}),[3,4] the most abundant plasma protein (*ca.* 650 μM), and then transferred to Hpx. The biological function of the HSA–Fe^{3+}PP complex has attracted considerable interest for many years. However, Casella *et al.* reported little peroxidase or catalase activity,[5] so this naturally occurring hemoprotein may not play any significant role in vivo. If anything, HSA may serve to keep the incorporated hemin group physiologically silent.

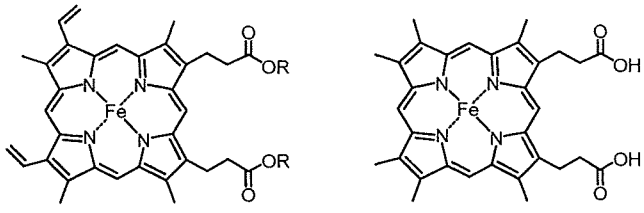


Fig. 1 Chemical formula of Fe porphyrins.

Crystal structure analysis of HSA–Fe^{3+}PP revealed that Fe^{3+}PP is bound within a deep hydrophobic slot in subdomain IB of HSA with axial coordination to the side-chain hydroxyl of Tyr-161 and salt bridges between the porphyrin propionates and a triad of basic amino acid residues at the pocket entrance (Arg-114, His-146, and Lys-190) (Fig. 2).[6,7] While the reduced ferrous HSA–Fe^{2+}PP is immediately autoxidized by O_2,[5] we found that a pair of site-specific mutations into the subdomain IB of HSA allows the Fe^{2+}PP to bind O_2: introduction of a proximal His at the Leu-185 position and substitution of the coordinated Tyr-161 with non-polar Leu (Y161L/L185H [rHSA**1**]) (Fig. 2).[9b,d] Remarkably, introduction of the proximal His at the Ile-142 position (on the opposite side of the porphyrin ring plane) also confers O_2 binding capability to the Fe^{2+}PP (I142H/Y161L [rHSA**2**]).[9a,c,d] These albumin O_2 transporters may serve as an effective red blood cell (RBC) substitute if the O_2 binding affinity is sufficient for clinical use. However, rHSA**1**–Fe^{2+}PP and rHSA**2**–Fe^{2+}PP both show two O_2 binding affinities ($P_{1/2}{}^{O2}$). The major component (species I, 60–75%) exhibits similar $P_{1/2}{}^{O2}$ to that of human RBC ($P_{1/2}{}^{O2} = 8$ Torr), but the minor component (species II, 25–40%) shows only a seventh to a tenth of the affinity (Table 1).[9] Our explanation for this observation is that the porphyrin plane of Fe^{2+}PP binds in the pocket in either of two alternative orientations (180° rotational isomers) that have slightly different geometries of axial His coordination to the central ferrous ion, only one of which confers high affinity O_2 binding.[9] Since less than 20% of species II of rHSA**2**–Fe^{2+}PP ($P_{1/2}{}^{O2} = 134$ Torr) is dioxygenated in the human lung's conditions ($P_{O2} = ca.$ 110 Torr, 37 °C), the low O_2 binding affinity component cannot effectively deliver O_2 to the tissues and should be excluded to develop this promising O_2 carrying plasma protein as an RBC substitute. Interestingly, a similar dependence of O_2 binding affinities on the orientations of the porphyrin ring

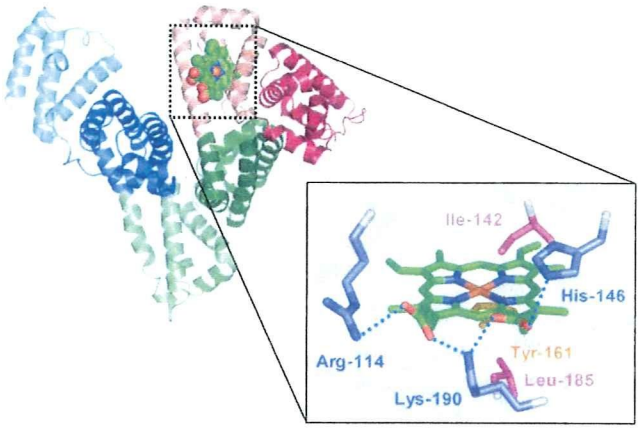
[a]*Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo, 169-8555, Japan. E-mail: teruyuki@waseda.jp; Fax: (+81) 3 5286 3148*
[b]*PRESTO, Japan Science and Technology Agency (JST), Japan. 4-1-8 Honcho, Kawaguchi-shi, Saitama, 332-0012, Japan*
[c]*Biophysics Section, Blackett Laboratory, Imperial College London, Exhibition Road, London, SW7 2AZ, United Kingdom*
[d]*Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei-shi, Tokyo 184-8588, Japan*

† Electronic supplementary information (ESI) available: UV-vis absorption spectral data of rHSA–FePP and rHSA–FeDMDP, and absorption decay of CO rebinding to rHSA**2**–Fe^{2+}DMDP after laser flash photolysis. See DOI: 10.1039/b909794e

Table 1 O_2 and CO binding parameters of rHSA–Fe^{2+}PP in 50 mM potassium phosphate buffered solution (pH 7.0) at 22 °C

	k_{on}^{O2} ($\mu M^{-1}s^{-1}$)	k_{off}^{O2} (ms^{-1})		$P_{1/2}^{O2}$ (Torr)		k_{on}^{CO} ($\mu M^{-1}s^{-1}$)		k_{off}^{CO} (s^{-1})		$P_{1/2}^{CO}$ (Torr)	
		I	II	I	II	I	II	I	II	I	II
rHSA**1**–Fe^{2+}PP[a]	31	0.20	2.1	4	41	3.7	0.35	0.012	0.077	0.0026	0.18
rHSA**2**–Fe^{2+}PP[a]	7.5	0.22	1.7	18	134	2.0	0.27	0.013	0.079	0.0053	0.24
rHSA**1**(H146R)–Fe^{2+}PP	43	0.37	—	6	—	5.1	—	0.013	—	0.0033	—
rHSA**1**(L190R)–Fe^{2+}PP	24	0.35	—	9	—	4.0	—	0.010	—	0.0031	—
rHSA**1**(H146R/K190R)–Fe^{2+}PP	42	0.41	—	6	—	6.1	—	0.011	—	0.0022	—
rHSA**2**(H146R/K190R)–Fe^{2+}PP	11	0.30	—	17	—	1.7	—	0.012	—	0.0058	
Mb[b]	14	0.012		0.51		0.51		0.019		0.030	

[a] Ref. 9b. [b] Sperm whale Mb in 0.1 M potassium phosphate buffer (pH 7.0, 20 °C); ref. 17.



rHSA	Position				
	142	146	161	185	190
Wild type (WT)	Ile	His	Tyr	Leu	Lys
1	Ile	His	Leu	His	Lys
2	His	His	Leu	Leu	Lys
1(H146R)	Ile	Arg	Leu	His	Lys
1(K190R)	Ile	His	Leu	His	Arg
1(H146R/K190R)	Ile	Arg	Leu	His	Arg
2(H146R/K190R)	His	Arg	Leu	Leu	Arg

Fig. 2 Structure of the heme pocket in the rHSA(WT)–hemin complex (PDB ID: 1O9X from ref. 7).[8] Positions of the amino acids where site-specific mutations were introduced and abbreviations of the mutants are shown in the table.

plane is found in insect Hb.[10] If one could prepare a desired heme pocket architecture to distinguish the two possible binding modes of the asymmetric Fe^{2+}PP, it would provide new insights into the modulation of hemoprotein chemistry.

In this paper we report for the first time a role for the basic amino acid triad at the entrance of the heme pocket in rHSA in regulating O_2 and CO binding to the prosthetic Fe^{2+}PP group. Replacing His-146 and/or Lys-190 with Arg in rHSA**1**–Fe^{2+}PP and rHSA**2**–Fe^{2+}PP resolved the structural heterogeneity of the porphyrin plane orientation and afforded a single high-affinity O_2 and CO binding equilibrium. Moreover, the O_2 binding affinities of these hemoproteins are all similar to that of RBC. Resonance Raman (RR) spectra clearly show one geometry of the axial His coordination to Fe^{2+}PP.

Results and discussion

Design of the heme pocket

To bind the hemin molecule tightly, HSA exploits multiple electrostatic interactions between three basic amino acid residues and the hemin propionates at the wide entrance of the heme pocket (Fig. 2). Lys-190 adopts a central position and makes salt bridges to both propionic acid side chains. His-146 and Arg-114 provide a second electrostatic coordination with each carboxylate. Notably, the UV-vis absorption spectrum of HSA complexed with an iron(III) protoporphyrin IX dimethylester (Fe^{3+}PPDM, Fig. 1) showed very broad Soret and Q bands, suggesting that Fe^{3+}PPDM without peripheral carboxylic acids may not be bound stably within subdomain IB. This suggested that modification of this key basic amino acid triad involved in coordinating the hemin propionates could be used to regulate the orientation of the porphyrin ring plane in subdomain IB. We designed four new rHSA mutants based on the existing pair of double mutants that contain the substitutions necessary to confer O_2 binding to the Fe^{2+}PP (Y161L/L185H or I142H/Y161L).[9] His-146 and Lys-190 were replaced by more bulky and basic Arg: H146R, K190R, and H146R/K190R mutations were combined with the O_2 binding mutations (see Fig. 2 for details). We postulated that the introduction of Arg residues would reduce the space available at the entrance to the cavity and might thereby restrict the binding of Fe^{2+}PP to a single conformation.

Site-specific mutations were introduced into the HSA coding region in a plasmid vector (pHIL-D2 HSA). The proteins were expressed in the yeast species *Pichia pastoris*. The rHSA–Fe^{2+}PP complexes were prepared according to our previously reported procedures (see Experimental).

O_2 and CO binding properties of rHSA–Fe^{2+}PP

The UV-vis absorption spectra of all six rHSA(mutant)–Fe^{3+}PP were essentially identical (Fig. 3, Table S1†). They were easily reduced to the corresponding ferrous complexes by adding a small amount of degassed aqueous $Na_2S_2O_4$ under an N_2 atmosphere. A single broad absorption band (λ = 558–559 nm) in the visible region signified the formation of a five-*N*-coordinate high-spin complex similar to deoxy Mb[11] or the synthetic chelated heme in DMF.[12] The spectral features and amplitude did not change in the temperature range of 5–25 °C. These observations show that the guanidinium groups of Arg-146 or Arg-190 do *not* interact with

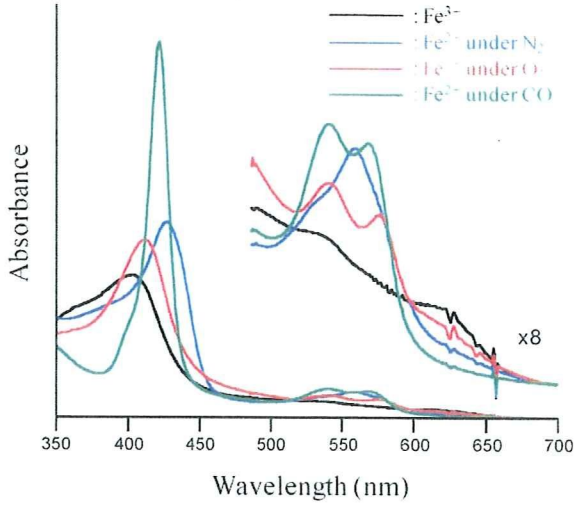


Fig. 3 UV-vis absorption spectral changes of rHSA**1**(H146R/K190R)–Fe^{2+}PP in 50 mM potassium phosphate buffered solution (pH 7.0) at 22 °C.

the ferrous iron of the Fe^{2+}PP, since the resulting formation of a six-*N*-coordinate low-spin complex would have yielded sharp and split α, β bands in the visible region[9c] and been sensitive to rapid oxidation by O_2 *via* an outer sphere mechanism.[13]

Upon exposure of the rHSA–Fe^{2+}PP solution to O_2 gas, the UV-vis absorption changed to that of the dioxygenated complex (Fig. 3).[9,11] After exposure to flowing CO, the Fe^{2+}PP produced a typical carbonyl complex.

We then used laser flash photolysis spectroscopy to determine association and dissociation rate constants (k_{on}, k_{off}) for O_2 and CO binding to rHSA–Fe^{2+}PP.[9] The time dependence of the absorption change accompanying the CO recombination to rHSA**1**–Fe^{2+}PP and rHSA**2**–Fe^{2+}PP obeyed double-exponentials, although the O_2 binding kinetics followed a single-exponential.[9] The slow phase (species II) of the CO rebinding showed 7–11-fold lower k_{on}^{CO} and 6-fold higher k_{off}^{CO} than those of the fast phase (species I) (Table 1). We interpreted this to mean that the low O_2 binding affinity conformers of rHSA**1**–Fe^{2+}PP and rHSA**2**–Fe^{2+}PP have bending strain in the proximal His coordination.[9,14–16] In contrast, the rebinding kinetics of O_2 and CO to rHSA**1**(H146R)–Fe^{2+}PP, rHSA**1**(K190R)–Fe^{2+}PP, rHSA**1**(H146R/K190R)–Fe^{2+}PP and rHSA**2**(H146R/K190R)–Fe^{2+}PP were strictly monophasic (Fig. 4). As a result, these hemoproteins showed single O_2 and CO binding affinity ($P_{1/2}^{O2}$ and $P_{1/2}^{CO}$), which were all similar to the higher affinities (species I) of the original double mutants (Table 1). We can conclude that the introduction of Arg into the entrance of the heme pocket of rHSA**1** and rHSA**2** is effective at excluding the low O_2 binding affinity conformer.

CO binding to rHSA–Fe^{2+}DMDP

To verify our interpretation that the replacement of H146 and/or K190 by Arg resolved the structural heterogeneity of the two complexing modes of the Fe^{2+}PP and gave a single O_2 and CO binding affinity, we examined the incorporation of a symmetrical iron(II) 2,4-dimethyl-deuteroporphyrin (Fe^{2+}DMDP, Fig. 1) as an active site. The UV-vis absorption spectrum of

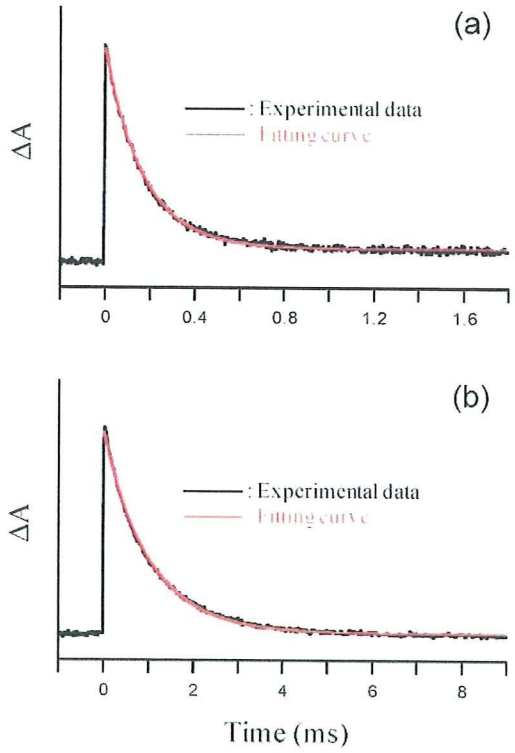


Fig. 4 Absorption decay of O_2 and CO rebinding to rHSA**1**(H146R/K190R)–Fe^{2+}PP after the laser flash photolysis at 22 °C; (a) O_2 and (b) CO. Both kinetics were composed of monophasic phases. A relaxation curve was fitted single exponential (red line).

the ferric rHSA**2**–Fe^{3+}DMDP showed a very similar pattern to that of rHSA**2**–Fe^{3+}PP though each λ_{max} value was hypsochromic (8–11 nm) shifted (Table S1†). The reduced ferrous form of rHSA**2**–Fe^{2+}DMDP under an N_2 atmosphere exhibited a slightly broadened Soret band absorption, but the main species was a five-*N*-coordinate high spin complex involving axial His-142 coordination. Upon introduction of O_2 gas through the solution, rHSA**2**–Fe^{2+}DMDP bound O_2 only at 5 °C and was observed to autoxidize at 22 °C. In general, the stability of the O_2 adduct complex of a heme derivative is sensitive to the electron density at Fe^{2+} and thus to the substituents at the porphyrin periphery.[18,19] Our attempt to determine the O_2 binding parameters of rHSA**2**–Fe^{2+}DMDP unfortunately failed. However, after introduction of CO gas, rHSA**2**–Fe^{2+}DMDP produced a stable carbonyl complex. We again used laser flash photolysis to characterize the CO binding properties of this hemoprotein. As expected, the absorption decay associated with CO recombination with rHSA**2**–Fe^{2+}DMDP was clearly monophasic (Fig. S1†). This result implied that the symmetric Fe^{2+}DMDP molecule is accommodated in subdomain IB of rHSA**2** in a single orientation and there is only one geometry of the axial His-142 coordination to the central ferrous ion of Fe^{2+}DMDP. Interestingly, the CO rebinding to rHSA**2**–Fe^{2+}DMDP (k_{on}^{CO}: 0.42 $\mu M^{-1}s^{-1}$) was relatively slow compared to that of rHSA**2**–Fe^{2+}PP and similar to Mb.[17]

RR and IR spectroscopies

The RR and infrared (IR) spectra of these artificial hemoproteins also supported the results described above. The stretching

frequencies of the carbonyl complex [ν(Fe–CO) and ν(CO)] provide crucial information about the Fe–trans ligand bond.[20,21] The high-frequency region of the RR spectra of rHSA**1**–Fe^{2+}PP(CO) and rHSA**1**(H146R/K190R)–Fe^{2+}PP(CO) both exhibited an intense peak at 1373 cm^{-1} (λ_{ex}: 413.1 nm), which indicates a deformed pyrrole-ring breathing-like mode (ν_4) and corresponds well to the value of the 6-coordinate low-spin carbonyl complex.[21a] However, while the low-frequency RR spectra of rHSA**1**–Fe^{2+}PP(CO) exhibited two ν(Fe–CO) bands at 493 and 525 cm^{-1}, rHSA**1**(H146R/K190R)–Fe^{2+}PP (CO) showed only a single ν(Fe–CO) band at 493 cm^{-1} (Fig. 5).[22] Since it is known that the weaker the Fe-trans ligand coordination, the stronger the Fe–CO bond in the carbonyl complex,[20] we assigned the higher 525 cm^{-1} band of rHSA**1**–Fe^{2+}PP(CO) to the low CO binding affinity conformer. The 493 cm^{-1} band was therefore assigned to the high affinity conformer, in which the proximal His-185 coordinates to the Fe^{2+}PP without unfavourable strain.

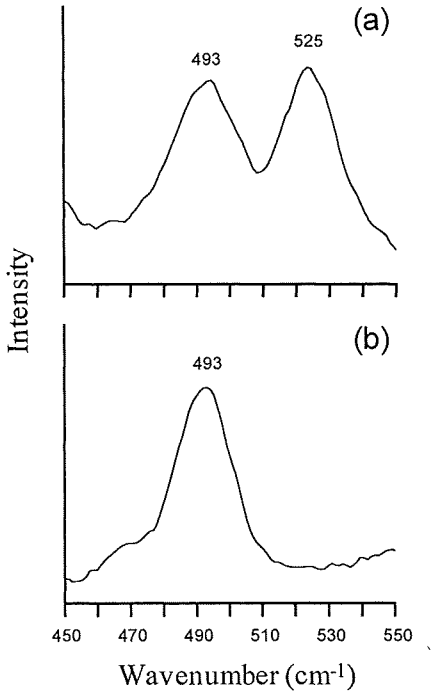


Fig. 5 Resonance Raman spectra of (a) rHSA**1**–Fe^{2+}PP(CO) and (b) rHSA**1**(H146R/K190R)–Fe^{2+}PP(CO) in 50 mM potassium phosphate buffered solution (pH 7.0) at 22 °C.

Regarding IR spectra, the ν(CO) vibration appeared at 1963 cm^{-1} for rHSA**1**–Fe^{2+}PP(CO) and at 1967 cm^{-1} for rHSA**1**(H146R/K190R)–Fe^{2+}PP(CO). Spiro *et al.* prepared a systematic plot of ν(Fe–CO) *versus* ν(CO) for a large number of carbonyl heme complexes and found a single inverse correlation when imidazole is the axial ligand.[21b,c] This is attributed to back donation of Fe^{2+} dπ electrons to the CO π* orbital. The relationship between ν(Fe–CO) and ν(CO) for rHSA**1**(H146R/K190R)–Fe^{2+}PP(CO) fits on the line for the imidazole complexes.[21b,c] On the other hand, the low O_2 binding component of rHSA**1**–Fe^{2+}PP(CO) showed a positive deviation from the line. This result again indicates a very weak electron donation from the proximal His-185 in the low O_2 binding conformer.

Conclusions

We prepared rHSA–Fe^{2+}PP complexes having a single O_2 and CO binding affinity by introducing Arg into the His-146 and/or Lys-190 positions. These artificial hemoproteins have a uniform Fe^{2+}PP orientation and His ligation (His-185 or His-142) geometry to the central ferrous ion without inclination. The key triad of the basic amino acid residues (Arg-114, His-146 and Lys-190) at the entrance of the heme pocket of HSA plays an important role in stabilizing the porphyrin molecule *via* salt-bridge formation and might also discriminate the two sides of the porphyrin ring. In mammals, His-146 is universally conserved, but Lys-190 is present only in primate albumin. The wild-type HSA statistically accommodates the hemin in alternative orientations; the discrimination of the porphyrin plane by serum albumin might be unnecessary for the evolution process. But the engineering of an rHSA–Fe^{2+}PP complex with a single O_2 binding affinity is potentially of tremendous clinical importance for blood substitutes and O_2-transporting therapeutic reagents.

Experimental

Materials and apparatus

All materials were used as purchased without further purification. Iron(III) protoporphyrin IX chloride (Fe^{3+}PP) was purchased from Fluka. Iron(III) protoporphyrin IX dimethyl ester chloride (Fe^{3+}PPDM) was synthesized from protoporphyrin IX dimethyl ester (Sigma). Iron(III) 2,4-dimethyl-deuteroporphyrin chloride (Fe^{3+}DMDP) was synthesized from 2,4-dimethyl-deuteroporphyrin dimethyl ester (Frontier Scientific).[23] UV-vis absorption spectra were obtained on an Agilent 8453 UV-visible spectrophotometer equipped with an Agilent 89090A temperature control unit. Kinetic measurements for the O_2 and CO bindings were carried out on a Unisoku TSP-1000WK time-resolved spectrophotometer with a Spectron Laser Systems SL803G-10 Q-switched Nd:YAG laser, which generated a second-harmonic (532 nm) pulse of 6-ns duration (10 Hz). A 150 W xenon arc lamp was used as the probe light source. The gas mixture with the desired partial pressure of O_2/CO/N_2 was prepared by a Kofloc Gasblender GB-3C. Resonance Raman spectra of the carbonyl rHSA–Fe^{2+}PP complexes were obtained on a JASCO NRS-1000 spectrophotometer using a Kaiser Optical Holographic Notch-Plus filter and a liquid N_2-cooled CCD detector. The excitation source was a Coherent Innova 90C Kr^+ laser. Infrared spectra of the carbonyl rHSA–Fe^{2+}PP complex were obtained on a JASCO FT/IR-4200 spectrophotometer.

Preparation of rHSA

The designed rHSAs were prepared according to our previously reported techniques.[9b] The mutations (H146R and/or K190R) were introduced into the rHSA coding region in a plasmid vector encoding the double mutant [rHSA**1** or rHSA**2**] by use of the Stratagene QuikChange mutagenesis kit. All mutations were confirmed by DNA sequencing. The plasmid was then digested by NotI and introduced into yeast (*Pichia pastoris* GS115) by electroporation. The expression protocols and media formulations were as previously described.[9b] The expressed proteins were harvested from the growth medium by precipitation with ammonium sulfate

and purified by a Cibacron Blue column of Blue Sepharose 6 Fast Flow (Amersham Pharmacia Biotech). After concentration using a Vivaspin 20 ultrafilter (10 kDa M_w cutoff), the samples were applied to a Superdex 75 column (Amersham Pharmacia Biotech) using 50 mM potassium phosphate as the running buffer. The purification steps were followed by SDS-PAGE analysis. The purified rHSA was lyophilized and stored in the freezer at −20 °C.

Preparation of rHSA–Fe^{2+}porphyrin

Typically 5 mL of 0.1 mM rHSA in 50 mM potassium phosphate (pH 7.0) was mixed with 0.8 mL of 0.688 mM Fe^{3+}PP in DMSO (Fe^{3+}PP : rHSA was molar ratio of 1:1) and incubated overnight with rotation in the dark at room temperature. The complex was then diluted with 50 mM potassium phosphate (*ca.* 15 mL) and concentrated to the initial volume (5.8 mL) using a Vivaspin 20 ultrafilter (10 kDa M_w cutoff). These dilution and concentration cycles were repeated to reduce the final concentration of DMSO to *ca.* <0.001 vol%. The rHSA–Fe^{2+}PPDM and rHSA–Fe^{2+}DMDP were also prepared in the same manner. The 50 mM phosphate buffered solution (pH 7.0) of rHSA–Fe^{3+}PP ([Fe^{3+}PP]: *ca.* 10 μM) in a 10 mm path length optical quartz cuvette sealed with a rubber septum was purged with N_2 for 30 min. A small excess amount of degassed aqueous sodium dithionite was added by microsyringe to the sample under an N_2 atmosphere to reduce the central ferric ion of the Fe^{3+}PP, generating the deoxy ferrous rHSA–Fe^{2+}PP.

Determination of O_2 and CO binding parameters

The O_2 and CO recombination with rHSA–Fe^{2+}PP after nanosecond laser flash photolysis of the dioxygenated or carbonyl complex occurs according to eqn (1) with the association rate constant ($k_{on}{}^{L}$) and dissociation rate constant ($k_{off}{}^{L}$) (where L: O_2 or CO).

$$Fe^{2+}PP\text{-}L \xrightarrow{h\nu} Fe^{2+}PP + L \underset{k_{off}{}^{L}}{\overset{k_{on}{}^{L}}{\rightleftharpoons}} Fe^{2+}PP\text{-}L \qquad (1)$$

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

The $k_{on}{}^{CO}$ was measured by following the absorption at 436 nm for Fe^{2+}PP(CO) or 411 nm for Fe^{2+}DMDP(CO) after laser pulse irradiation to the carbonyl complex at 22 °C. The $k_{on}{}^{O2}$ and O_2 binding equilibrium constant [$K^{O2} = (P_{1/2}{}^{O2})^{-1}$)] can be determined by a competitive rebinding technique by use of gas mixtures with different partial pressures of O_2/CO/N_2 at 22 °C. The relaxation curves that accompanied the O_2 or CO recombination were analyzed by single or double exponential profiles with Unisoku Spectroscopy & Kinetics software. The $k_{off}{}^{O2}$ was calculated from $k_{on}{}^{O2}/K^{O2}$. The $k_{off}{}^{CO}$ was measured by displacement with NO for the carbonyl complex at 22 °C. The time course of the UV-vis absorption change that accompanied the CO-dissociation was fitted to single or double exponential. The CO binding constants [$K^{CO} = (P_{1/2}{}^{CO})^{-1}$] were calculated from $k_{off}{}^{CO}/k_{on}{}^{CO}$.

Raman spectroscopy

Spectra of the carbonyl complexes of rHSA–Fe^{2+}PP ([Fe^{2+}PP]: 2–4 mM in 50 mM phosphate buffered solution (pH 7.0)) were collected using back-scattering geometry at an excitation wavelength of λ_{ex}: 413.1 nm. The laser power for the samples was 1.8 mW. Each spectrum was recorded with 20 s accumulation time at 22 °C, and ten repetitively measured spectra were averaged to improve the signal to noise ratio. Peak frequencies were calibrated relative to indene and CCl_4 as a standard and were accurate to 1 cm^{-1}.

IR spectroscopy

The IR spectra of the carbonyl complexes of rHSA–Fe^{2+}PP ([Fe^{2+}PP]: 2–4 mM in 50 mM phosphate buffered solution (pH 7.0)) were obtained in CaF_2 cells (JASCO, path length: 0.025 mm). The cell containing water was used for the reference. The spectrum was accumulated 64 times to improve its signal-to-noise ratio.

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