Heme Pocket Architecture in Human Serum Albumin: Regulation of O_2 Binding Affinity of a Prosthetic Heme Group by Site-Directed Mutagenesis

Teruyuki Komatsu,[*1,2] *Akito Nakagawa,*[1] *Eishun Tsuchida*[*1]

Summary: We present the O_2 binding properties of recombinant human serum albumin (rHSA) mutants complexed with an iron(II) protoporphyrin IX as a prosthetic heme group. Iron(III) protoporphyrin IX (hemin) is bound within subdomain IB of HSA with weak axial coordination by Tyr-161. In order to confer O_2 binding capability to this naturally occurring hemoprotein: (i) a proximal histidine was introduced into position Ile-142; and (ii) the coordinated Tyr-161 was replaced with hydrophobic Leu using site-directed mutagenesis. It provided a recombinant HSA double-mutant [rHSA(I142H/Y161L) = rHSA(HL)]. The rHSA(HL)–heme formed a ferrous five-coordinate high-spin complex with axial ligation of His-142 under an Ar atmosphere. This artificial hemoprotein binds O_2 at room temperature. Laser flash photolysis experiments demonstrated that O_2 rebinidng to rHSA(HL)–heme displays monophasic kinetics, whereas the CO recombination process obeyed a double-exponential pattern. This might be attributable to the two different geometries of the axial imidazole coordination arising from the two orientations of the porphyrin plane in the heme pocket. The O_2 binding affinity of rHSA(HL)–heme was considerably lower than those of R-state hemoglobin (Hb) and myoglobin (Mb), principally because of the high O_2 dissociation rate constant. The third mutations have been introduced into the distal side of the heme (at position Leu-185 or Arg-186) to increase the O_2 binidng affinity. The rHSA(HL/L185N)–heme showed high O_2 binding affinity ($P_{1/2}^{O_2}$: 1 Torr), which is 18-fold greater than that of the original double mutant rHSA(HL)–heme and which is rather close to those of Hb (R-state) and Mb. Furthermore, replacement of polar Arg-186 with Leu or Phe adjusted the O_2 binding affinity ($P_{1/2}^{O_2}$) to 10 Torr, which is almost equivalent to value for human red blood cells.

Keywords: biomimetics; heme; human seruma albumin; O_2 binidng; proteins

Introduction

Human serum albumin (HSA), the most abundant plasma protein (4–5 g/dl) in our circulatory system, is characterized by its remarkable ability to bind widely various endogenous and exogenous compounds[1] such as fatty acids, bilirubin, bile acids, thyroxine,[2,3] and a wide range of drugs.[4] Hemin [iron(III) protoporphyrin IX] released from methemoglobin is also captured by HSA with a high binding constant ($K \approx 10^8$ M^{-1}).[5] This strong affinity of HSA for hemin has stimulated efforts to develop albumin as an artificial hemoprotein which can mimic the O_2 binding ability of hemoglobin (Hb) and myoglobin (Mb).[6,7] HSA consists of a helical monomer of 66.5 kDa containing three homologous domains (I–III), each of which comprises of A and B subdomains.[8] Crystallographic

[1] Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan
Fax: (+81) 3-3205-4740;
E-mail: teruyuki@waseda.jp
[2] PRESTO, Japan Science and Technology Agency (JST)

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 1).[9,10] 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, if one reduces the HSA–hemin to obtain the ferrous complex, it is rapidly oxidized by O_2, even at low temperature, because HSA lacks the proximal histidine that enables the prosthetic heme group to bind O_2 and serves to regulate the O_2 binding affinity (Figure 1). In order to confer the O_2 binding capability to this naturally occurring hemoprotein, we have introduced a proximal histidine into the heme binding site of HSA by site-directed mutagenesis; it would provide axial coordination to the central ferrous ion of the heme and thereby promote O_2 binding.[11] Moreover, to modulate its O_2 binding affinity, we have added further modification to the distal side of the heme. The O_2 binding properties of several rHSA(mutant)–heme complexes have been characterized kinetically and compared to those of the natural Hb, Mb, and red blood cells (RBC). We have shown that our mutagenesis approach can create a new class of albumin-based artificial hemoprotein which would serve as an O_2 carrier.

Results and Discussion

Double-Mutations to Confer the O_2 Binding Capability

The detailed structure of the heme binding site in HSA revealed by crystallographic studies allows the design of mutagenesis experiments to construct a tailor-made heme pocket for O_2 binding.[9,10] In fact, Tyr-161 was the first candidate to introduce a proximal histidine (Figure 1). However, the Y161H mutation was not done because our simulation indicated that the distance from His-161 to the central Fe would be too great (4.0 Å). Instead, modeling experiments suggested that the favorable positions for the axial imidazole insertion would be Ile-142 (Figure 2a). The N(histidine)–Fe distance was estimated as 2.31 Å for H142 (compared to 2.18 Å for Mb). We therefore designed a recombinant HSA (rHSA) double-mutant I142H/Y161L [=rHSA(HL)].

The specific mutations were introduced into the HSA coding region in the plasmid vector (pHIL-D2 HSA) using the QuikChange mutagenesis kit (Stratagene), and clones were expressed in the yeast *Pichia pastoris* (Invitrogen Corp.). The rHSA–hemin complexes were prepared funda-

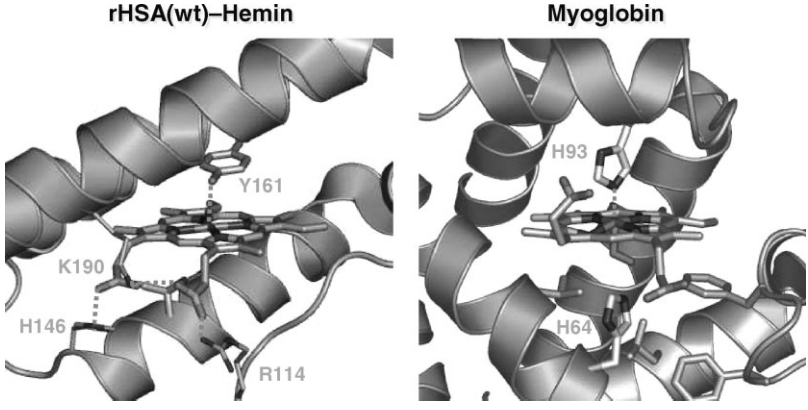

Figure 1.
Heme pocket structure in subdomain IB of HSA (left; 1O9X from ref. [9]) and heme pocket structure of Mb (1MBO).

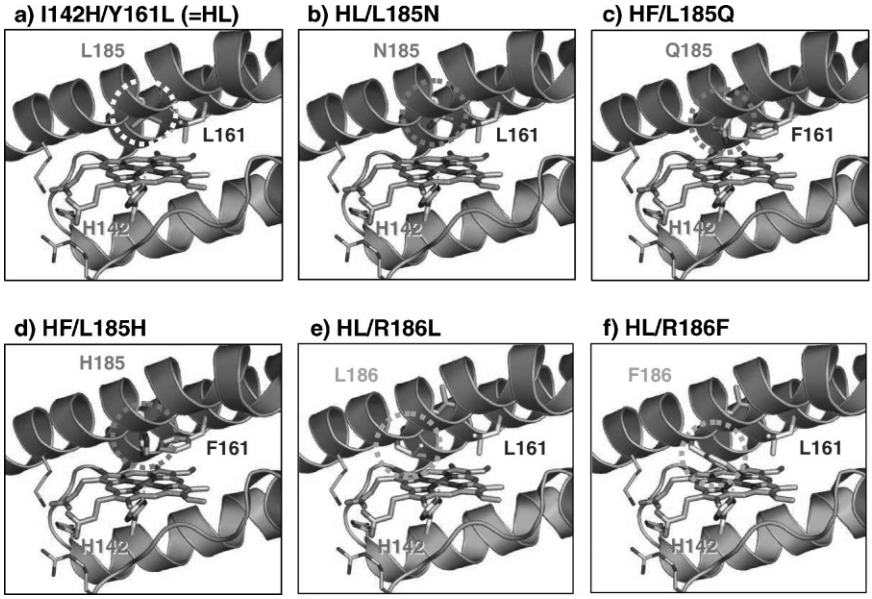

Figure 2.
Structural models of the heme pocket in rHSA(mutant)–heme complexes.

mentally according to our previously reported procedures.[9,11]

The MCD spectra of the rHSA(HL)–hemin showed S-shaped patterns in the Soret band region, which resembled that of ferric Mb.[12] One water molecule is known to coordinate axially to the sixth position of the central ferric ion of the hemin in metMb. Our MCD result suggests that the rHSA(HL)–hemin is also in a ferric high-spin complex, having a water molecule as the sixth ligand.

O_2 Binding Affinity of rHSA(HL)–Heme

The rHSA(HL)–hemin was reduced to a ferrous complex by adding a small molar excess of aqueous sodium dithionate under an Ar atmosphere. A single broad absorption band (λ_{max}: 559 nm) in the visible region of rHSA(HL)–heme resembled that observed for deoxy Mb[13] or the chelated protoheme in DMF,[14] indicating the formation of a five-coordinate high-spin complex. The heme therefore appears to be accommodated in the mutated heme pocket with an axial coordination involving His-142. Upon exposure of the rHSA(HL)–heme solution to O_2, the UV-vis absorption immediately changed to that of the O_2 adduct complex at 0–25 °C.[13,14] After introduction of CO gas, the hemoprotein produced a stable carbonyl complex.

Laser flash photolysis experiments were carried out to evaluate the kinetics of the O_2 binding to the rHSA(HL)–heme.[11,15,16] The absorbance decay accompanying the O_2 recombination to rHSA(HL)–heme was composed of single-exponential. On the other hand, the rebinding of CO followed biphasic decay, which is normally not observed in Mb. Results of numerous investigations of synthetic model hemes have shown that a bending strain in the proximal base coordination to the central ferrous ion, the ''proximal-side steric effect'', can decrease the association rate for CO without greatly altering the kinetics of O_2 association.[15,16] Therefore, a possible explanation is that there are two different geometries of the axial histidine (His-142) coordination to the central ferrous ion of the heme in rHSA(HL), each one accounting for the biphasic kinetics of CO rebinding.

Table 1.
O_2 binding parameters of rHSA(mutant)–heme complexes.[a]

Hemoproteins	$10^{-6}\ k_{on}^{O_2}$ ($M^{-1}s^{-1}$)	$10^{-3}\ k_{off}^{O_2}$ (s^{-1})		$P_{1/2}^{O_2}$ (Torr)	
		I	II	I	II
rHSA(HL)–Heme	7.5	0.22	1.70	18	134
rHSA(HL/L185N)–Heme	14	0.02	0.29	1	14
rHSA(HL/R186L)–Heme	25	0.41	8.59	10	209
rHSA(HL/R186F)–Heme	21	0.29	7.01	9	203
Hb(α) (R-state)[b]	33[c]	0.013[d]		0.24	
Mb[e]	14	0.012		0.51	
RBC[f]				8	

[a] In 50 mM potassium phosphate buffered Solution (pH 7.0) at 22 °C. I or II indicates species I or II.
[b] Human Hb α-subunit.
[c] In 0.1 M phosphate buffer (pH 7.0, 21.5 °C); ref. [17].
[d] In 10 mM phosphate buffer (pH 7.0, 20 °C); ref. [18].
[e] Sperm whale Mb. In 0.1 M potassium phosphate buffer (pH 7.0, 20 °C); ref. [19].
[f] Human red cell suspension. In isotonic buffer (pH 7.4, 20 °C); ref. [23].

By analyzing the CO/O_2 competitive binding following laser flash photolysis,[15,16] we obtained the association rate constants for O_2 ($k_{on}^{O_2}$) and the O_2 binding affinities [$P_{1/2}^{O_2} = (K^{O_2})^{-1}$] for rHSA(HL)–heme (Table 1).[11] The faster phase, defined as species I, and the slower phase, defined as species II, yielded two different O_2 binding affinities. In species I, the proximal His might coordinate to the central ferrous ion without strain, whereas in species II, the ligation might involve some distortion, resulting in weaker O_2 binding (Figure 3).

The $P_{1/2}^{O_2}$ value of rHSA(HL)–heme was determined to be 18 Torr (species I), which was 35–75-fold higher (O_2 binding affinity is lower) than those of Hbα (R-state) and Mb.[17–19] This low affinity for O_2 was kinetically attributable to a 17–18-fold increases in the O_2 dissociation rate constants. The O_2 binding affinity should be adjusted to similar values for Hb and human RBC to develop this artificial hemoprotein as a blood substitute.

Introduction of Distal Base into the 185 Position

The His-64 in Hb and Mb on the distal side of the heme plays an important role for tuning their ligand affinities. A neutron diffraction study of MbO_2 showed that the N–H bond of the distal His-64 is restrained from optimal alignment for strong H–bonding with the coordinated O_2.[20] Olson and co-workers reported that the substitution of Gly for His-64 in Mb and Hb(α) caused a marked decrease in the O_2 binding affinity.[21] In view of these investigations, we reasoned that systematic variation of the

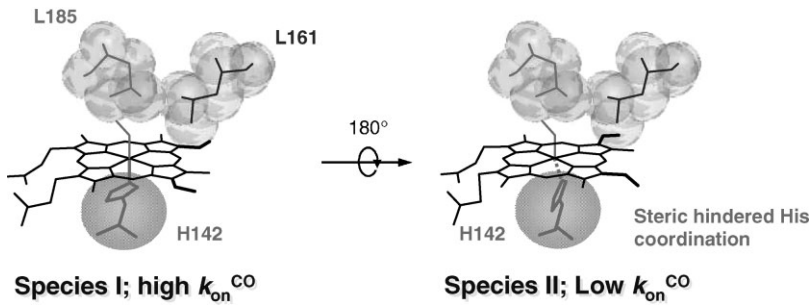

Figure 3.
Structural models of heme-O_2 site of rHSA(HL)–heme complex.

steric hindrance and local polarity of the heme pocket in subdomain IB of HSA would allow modulation of the O_2 binding affinity. One approach to enhancing the O_2 binding affinity of rHSA–heme would be to introduce a basic amino acid into an appropriate position on the distal side of the heme. Our modeling results showed that the favorable position for the distal base insertion was Leu-185. Consequently, we replaced Leu-185 in rHSA(HL) [or rHSA(I142H/Y161F) [=rHSA(HF)]] with Asn, Gln, and His using site-directed mutagenesis (Figure 2b–2d).[22]

The rHSA(HL/L185N)–heme under Ar atmosphere showed a visible absorption band at 559 nm with a small shoulder at 530 nm, which was similar to the spectrum observed for rHSA(HL)–heme,[11] deoxy Mb,[13] and chelated protoheme.[14] The spectral pattern clearly indicated the formation of a five-coordinate high-spin complex. On the contrary, in the spectra of rHSA(HF/L185Q)–heme and rHSA(HF/L185H)–heme, the β band at 528 nm appeared relatively sharp, suggesting partial formation of a six-coordinate heme complex. Upon exposure of the rHSA(HL/L185N)–heme solution to O_2, the UV-vis absorption changed immediately to that of the O_2 adduct complex at 22 °C. In contrast, rHSA(HF/L185Q)–heme and rHSA(HF/L185H)–heme were oxidized by O_2, even at low temperature (5 °C). After introducing CO gas, all the hemoproteins produced stable carbonyl complexes with identical absorption spectral patterns.

Marked differences are apparent in the comparison of the O_2 binding parameters for rHSA(HL)–heme and rHSA(HL/L185N)–heme. The presence of Asn rather than Leu at position 185 resulted in 18-fold and 10-fold increases in the O_2 binding affinity, respectively, for species I and II (Table 1). These increases were predominantly attributable to the 6–11-fold diminution of the $k_{off}^{O_2}$ values. The high O_2 binding affinity ($P_{1/2}^{O_2}$: 1 Torr) for rHSA(HL/L185N)–heme is now close to that of Hb (R-state) (0.24 Torr) and Mb (0.5 Torr) (Table 1).

Introduction of Leu or Phe into the 186 Position

For rHSA–heme to provide effective O_2 transport *in vivo*, the affinity should be more similar to that of human RBC ($P_{1/2}^{O_2}$: 8 Torr).[23] This requires an O_2 binding affinity that is intermediate between the values of rHSA(HL)–heme and rHSA(HL/L185N)–heme. An effective means to control the O_2 binding affinity of the heme is introduction of a different polar amino acid around the O_2 binding site. A polar Arg-186 exists at the entrance of the heme pocket; we expected that insertion of a nonpolar residue at this position would change the O_2 binding affinity of rHSA–heme. Consequently, we designed new triple mutants, rHSA(HL/R186L) and rHSA(HL/R186F) (Figure 2e, 2f).

The MCD in the Soret band region of the ferric rHSA(HL/R186L)–hemin and rHSA(HL/R186F)–hemin both showed low intensity, which is fundamentally equivalent to that observed for rHSA(HL)–hemin. The reduced ferrous form demonstrated the characteristic UV-vis absorption and MCD spectra of the five-coordinate high-spin complex under an Ar atmosphere. Upon bubbling O_2 gas through the solutions, the spectral patterns were shifted to that of the O_2 adduct complex. The distinct features of all the spectra were quite similar to those of the rHSA(HL)–heme. Fortunately, the O_2 binding affinities of rHSA(HL/R186L)–heme and rHSA(HL/R186F)–heme were more similar to that of human RBC ($P_{1/2}^{O_2}$: 8 Torr) (Table 1). We can conclude that the Arg-186 is an important key amino acid to control the O_2 binding property of the heme and the obtained triple mutants could become RBC substitutes.

Conclusion

We have shown clearly that rHSA–heme can be engineered to bind O_2 reversibly. However, the complex did not display optimal O_2 binding affinity. By emphasizing modification on the distal side of the heme pocket in rHSA, we have prepared distinct

rHSA(triple mutant)–heme complexes with a broad range of O_2 binding affinities. The highest affinity mutant rHSA(HL/L185N) contains Asn-185, which has a short amide side-chain that enhances the O_2 binding affinity. On the other hand, introduction of the larger Gln and His side-chains at position 185 partly provided a six-coordinate heme character, and did not stabilize O_2 binding. In a different approach, substitution of Arg-186 at the entrance of the heme pocket with Leu or Phe provided a useful reduction in the O_2 binding affinity, yielding $P_{1/2}^{O_2}$ values that are closely resemble that of the human RBC.

The transport of O_2 by rHSA–heme could be of great clinical importance, not only as a blood substitute, but also as an O_2-providing therapeutic reagent. If the HSA-based O_2 carrier is realized, it has the potential of acting not only as a RBC substitute, but also as an O_2 providing therapeutic reagent.

This work was supported by PRESTO "Control of Structure and Functions", JST, and Health Science Research Grants (Regulatory Science) from MHLW Japan. The work at Imperial College London was partially carried out through the Japan-UK Research Cooperative Program (Joint Project) of JSPS. The authors acknowledge to Dr. Stephen Curry and Dr. Patricia A. Zunszain for their valuable suggestions related to molecular designs of the rHSA mutants and site-directed mutagenesis.

[1] T. Peters, Jr., "*All about Albumin, Biochemistry, Genetics and Medical Applications*", Academic Press, San Diego **1997**.
[2] [2a] U. Kragh-Hansen, *Pharmacol. Rev.* **1981**, *33*, 17; [2b] U. Kragh-Hansen, *Danish Med. Bull.* **1990**, *37*, 57.
[3] [3a] S. Curry, H. Madelkow, P. Brick, N. Franks, *Nat. Struct. Biol.* **1998**, *5*, 827; [3b] A. A. Bhattacharya, T. Grune, S. Curry, *J. Mol. Biol.* **2000**, *303*, 721.
[4] J. Ghuman, P. A. Zunszain, I. Petipas, A. A. Bhattacharya, M. Otagiri, S. Curry, *J. Mol. Biol.* **2005**, *353*, 38.
[5] P. A. Adams, M. C. Berman, *Biochem. J.* **1980**, *191*, 95.
[6] M. C. Marden, E. S. Hazard, L. Leclerc, Q. H. Gibson, *Biochemistry* **1989**, *28*, 4422.
[7] T. Komatsu, Y. Matsukawa, E. Tsuchida, *Bioconjugate Chem.* **2002**, *13*, 397.
[8] X. M. He, D. C. Carter, *Nature* **1992**, *358*, 209–215.
[9] P. A. Zunszain, J. Ghuman, T. Komatsu, E. Tsuchida, S. Curry, *BMC Struct. Biol.* **2003**, *3*, 6.
[10] M. Wardell, Z. Wang, J. X. Ho, J. Robert, F. Ruker, J. Rubel, D. C. Carter, *Biochem. Biophys. Res. Commun.* **2002**, *291*, 813.
[11] [11a] T. Komatsu, N. Ohmichi, P. A. Zunszain, S. Curry, E. Tsuchida, *J. Am. Chem. Soc.* **2004**, *126*, 14304; [11b] T. Komatsu, N. Ohmichi, A. Nakagawa, P. A. Zunszain, S. Curry, E. Tsuchida, *J. Am. Chem. Soc.* **2005**, *127*, 15933.
[12] L. Vickery, T. Nozawa, K. Sauer, *J. Am. Chem. Soc.* **1976**, *98*, 343.
[13] E. Antonini, M. Brunori, "*Hemoglobin and Myoglobin in Their Reactions with Ligands*", North-Holland Pub, Amsterdam **1971**, pp 18.
[14] T. G. Traylor, C. K. Chang, J. Geibel, A. Berzinis, T. Mincey, J. Cannon, *J. Am. Chem. Soc.* **1979**, *101*, 6716.
[15] J. P. Collman, J. I. Brauman, B. L. Iverson, J. L. Sessler, R. M. Moris, Q. H. Gibson, *J. Am. Chem. Soc.* **1983**, *105*, 3052.
[16] T. G. Traylor, S. Tsuchiya, D. Campbell, M. Mitchel, D. Stynes, N. Koga, *J. Am. Chem. Soc.* **1985**, *107*, 604.
[17] Q. H. Gibson, *J. Biol. Chem.* **1970**, *245*, 3285.
[18] J. H. Olson, M. E. Andersen, Q. H. Gibson, *J. Biol. Chem.* **1971**, *246*, 5919.
[19] R. Rohlfs, A. J. Mathews, T. E. Carver, J. S. Olson, B. A. Springer, K. D. Egeberg, S. G. Sliger, *J. Biol. Chem.* **1990**, *265*, 3168.
[20] S. E. V. Phillips, B. P. Schoenborn, *Nature* **1981**, *292*, 81.
[21] J. S. Olson, A. J. Mathews, R. J. Rohlfs, B. A. Springer, K. D. Egeberg, S. G. Sligar, J. Tame, J.-P. Renaud, K. Nagai, *Nature* **1988**, *336*, 365.
[22] T. Komatsu, A. Nakagawa, P. A. Zunszain, S. Curry, E. Tsuchida, *J. Am. Chem. Soc.* **2007**, *129*, in press.
[23] K. Imai, H. Morimoto, M. Kotani, H. Watari, W. Hirata, M. Kuroda, *Biochim. Biphys. Acta.* **1970**, *200*, 189.

