Review

Structural and Mutagenic Approach to Create Human Serum Albumin-Based Oxygen Carrier and Photosensitizer

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Summary: Human serum albumin (HSA) is a versatile protein found at high concentration in blood plasma and binds a range of insoluble endogenous and exogenous compounds. We have shown that complexation of functional molecules into HSA creates unique proteins never seen in nature. Complexing an iron-protoporphyrin IX into a genetically engineered heme pocket of recombinant HSA (rHSA) generates an artificial hemoprotein, which binds O2 reversibly in much the same way as hemoglobin. A pair of site-specific mutations, (i) introduction of a proximal histidine at the Ile-142 position and (ii) substitution of Tyr-161 with Phe or Leu, allows the heme to bind O2. Additional modification on the distal side of the heme pocket provides rHSA(triple mutant)-heme complexes with a variety of O2 binding affinity. Complexing a carboxy-C60-fullerene (CF) into HSA generates a protein photosensitizer for photodynamic cancer therapy. Energy transfer occurs from a photoexcited triplet-state of HSA-CF (HSA-CF*) to O2, forming singlet oxygen (1O2). This protein does not show dark cytotoxicity, but induceds cell death under visible light irradiation.

Keywords: human serum albumin; oxygen carrier; photosensitizer; heme; site-directed mutagenesis; fullerene; singlet oxygen; photodynamic therapy

Introduction

Human serum albumin (HSA, Mw: 66.5 kDa) is the most prominent plasma protein in our bloodstream and is characterized by remarkable ability to bind a great variety of hydrophobic molecules.1–3 Typical endogenous ligands for HSA are fatty acids, bilirubin, bile acids and thyroxine.4–8 The protein binds a wide range of drugs. Hemin (Fe3+ protoporphyrin IX, Fig. 1) released from methemoglobin (metHb) during the enucleation of red blood cells (RBCs) is also captured by HSA. Free hemin is potentially toxic because it may catalyze hydroxyl radical formation. In 1938, Fairley verified that serum protein observed to bind the hemin was albumin and proposed the name “methemalbumin”.9 Muller-Eberhard and Morgan reported the UV-vis absorption spectrum of the HSA-hemin complex in 1975 and supposed formation of high-spin hemoprotein with an axial coordination of amino acid residue of the protein.10 The binding constant for hemin to HSA was determined to be 1.1 X 108 M−1.11 This strong affinity of HSA for hemin has stimulated efforts to develop HSA as an artificial hemoprotein which mimics the diverse biological reactivities of natural hemoproteins, such as O2 transport of Hb. If the HSA-based O2 carrier is realized, it has the potential of acting not only as an RBC substitute, but also an O2-providing therapeutic reagent. However, it has taken over 60 years to confer the O2 binding capability on the HSA-hemin complex since Fairley’s finding.

HSA is composed of three structurally similar domains (I–III), each containing A and B subdomains.12,13 Crystallographic studies reveal that hemin is bound within a narrow cavity in subdomain IB with an axial coordination of tyrosine to the central Fe3+ ion and electrostatic interactions between the porphyrin propionates and a triad of basic amino acid residues (Fig. 2).14,15 In terms of the general hydrophobicity of this α-helical pocket, the subdomain IB of HSA has similar features to the heme binding site of Hb or myoglobin (Mb), namely “heme pocket”. However, the reduced form of HSA-hemin is rapidly oxidized by O2.16

It is of current interest to prepare albumin-based fake enzymes by exploiting the ligand binding properties of HSA.16–19 Casella et al., demonstrated the HSA-hemin
Fig. 1. Chemical formula of heme and CF

Fig. 2. (A) Crystal structure of HSA-hemin complex (1O9X) from ref. 15. (B) Heme pocket structure in subdomain IB and positions of amino acids where site-specific mutations are introduced. Abbreviations of triple mutants are shown in the table.

Recombinant HSA Mutants Complexed with Heme (rHSA-Heme) as Oxygen Carrier

Naturally occurring HSA-hemin complex: Crystal structure analysis revealed that hemin is bound within a narrow D-shaped hydrophobic cavity in subdomain IB of HSA where the central ferric ion is axially coordinated by Tyr-161 and the two propionate side-chains at the porphyrin periphery form salt-bridges with a triad of basic amino acid residues (Arg-114, His-146, Lys-190) (Fig. 2). The UV-vis absorption spectrum of the HSA-hemin solution showed a Soret band at 405 nm and the pπ-dπ charge-transfer (CT) band at 624 nm (Fig. 3). The dominant features of the spectrum were almost the same as those of previously reported HSA-hemin and H93Y recombinant Mb [rMb(H93Y)], in which the proximal His-93 was changed to tyrosine. Our absorption spectral data imply that the hemin is bound to Tyr-161 of HSA to form a ferric five-coordinate high-spin complex. Nevertheless, CT absorptions of the HSA-hemin appeared at a higher wavelength (λmax: 624 nm) compared to
The magnetic circular dichroism (MCD) spectra also support the formation of a five-coordinate high-spin hemin complex with weak Tyr-161 ligation in HSA-hemin.41)

Reduction of the ferric HSA-hemin by the addition of aqueous sodium dithionite under an Ar atmosphere gave a ferrous heme complex with a Soret band at 419 nm and two definite Q bands at 538 and 570 nm (Fig. 3). Based on careful inspection of UV-vis and MCD spectra, we concluded that the ferrous HSA-heme is an unusual mixture of a five-coordinate high-spin complex with Tyr-161 and a four-coordinate intermediate-spin state under an Ar atmosphere. Smulevich et al. recently measured resonance Raman spectroscopy of the HSA-hemin complex and strongly supported our interpretation.46)

Upon the addition of O2 gas through this solution, the central ferrous ion was rapidly oxidized even at 5°C. This is due to the fact that HSA lacks the proximal histidine which enables the prosthetic heme group to bind O2 in Hb and Mb.

rHSA(double mutant)-heme complexes: On the basis of the crystal structure of the HSA-hemin complex, we used site-directed mutagenesis to introduce a histidine into the heme binding site of HSA. This should provide axial coordination to the central Fe2+ atom of the heme and thereby promote O2 binding. Tyr-161 was the first candidate for site-directed mutagenesis (Fig. 2), but our simulation results showed that the distance from Nε(Y161H) to Fe(heme) would be too far (≈ 4.0 Å). As an alternative, favorable positions for axial imidazole insertion would be Ile-142. The Nε(I142H)-Fe distance was estimated to be 2.31 Å. We therefore designed and prepared rHSA mutants, rHSA(I142H/Y161F) and rHSA(I142H/Y161L) (Fig. 2) and evaluated the O2 binding properties of the heme complexes.

The rHSA(double mutant)-hemin was easily reduced to the ferrous complex under an Ar atmosphere. A single broad Q band (λmax: 559 nm) in the visible absorption of rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme was similar to that of deoxy Mb47) or the synthetic chelated-heme in N,N-dimethylformamide (Fig. 4),48) indicating the formation of a five-N-coordinate high-spin complex. The heme was incorporated into the artificial heme pocket with axial His-142 coordination. Upon exposure of the rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme solutions to O2, the UV-vis absorptions changed to that of the O2 adduct complex at 22°C (Fig. 4).47,48) After flowing CO gas, these hemoproteins produced very stable carbonyl complexes. The single mutant rHSA(I142H)-heme which retains Y161, could not bind O2. The polar phenolate residue at the top of the porphyrin plane probably accelerates the proton-driven oxidation of the Fe2+ center.

To evaluate the kinetics of O2 and CO binding to rHSA(double mutant)-heme, laser flash photolysis experiments were carried out.41,42,49,50) It is noteworthy that the absorbance decay accompanying CO recombination to rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme was composed of double-exponential profiles normally not seen in Mb. The rebinding of O2 to the hemoproteins followed simple monophasic decay. Based on numbers from investigation on synthetic Fe2+ porphyrin models, it has been shown that a bending strain in the proximal histidine coordination to the central Fe2+ atom, “proximal-side steric effect”, increases the dissociation rate and decreases the association rate for CO, whereas it increases the O2 dissociation rate without changing the kinetics of O2 association.49,50) Our interpretation was that there may be two different geometries for axial
His-142 coordination to the central Fe$^{2+}$ of the heme (species I and II), each one accounting for a component of biphasic kinetics of CO rebinding. The heme molecule appears to bind to subdomain IB in two orientation (180° rotational isomers), giving two different geometries for axial His-142 coordination.

The association and dissociation rate constants for O$_2$ or CO ($k_{on}$, $k_{off}$, $k_{on}^{CO}$, $k_{off}^{CO}$) and binding affinities for O$_2$ or CO ($P_{1/2}^{O_2}$, $P_{1/2}^{CO}$) for the rHSA(mutant)-heme complexes (eq. 1) are summarized in Table 1 and 2.

$$rHSA-heme + \frac{k_{on}}{k_{off}} = rHSA-heme-L \quad (L = O_2 \text{ or CO})$$

$P_{1/2}^{O_2}$ of rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme were determined to be 3–18 and 31–134 Torr for species I (the first phase) and species II (the second phase), respectively. Even the O$_2$ binding affinity of species I was 6–75-fold lower than that of native Hbα (R-state) and Mb.51–53 This low affinity for O$_2$ was kinetically due to an 8–18-fold increase in $k_{off}^{O_2}$.

In Hb and Mb, the distal His-64 stabilizes bound O$_2$ due to the hydrogen bonding. Rohlf et al. showed that replacement of His-64 in rMb with nonpolar amino acid residues (Leu or Phe) results in loss of hydrogen bonding and increases $k_{off}^{O_2}$ (342–833-fold higher than Mb).53 In rHSA(double mutant)-heme, dioxygenated heme is buried in the core of the hydrophobic cavity without any counterpart for the hydrogen bond; thus the even small $k_{off}^{O_2}$ for species I are greater than those of Hbα and Mb.

In species II, the proximal-side steric effect further enhanced the dissociation rates and caused large decline in O$_2$ binding affinity.

We compared O$_2$ and CO binding properties of the rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme and found an interesting distal-side steric effect on ligand binding.53 The rHSA(I142H/Y161F)-heme complex binds O$_2$ and CO about 4–6 times more strongly than rHSA(I142H/Y161L)-heme, because of high association rate constants. This effect appears due to the concerted steric effects of the residues at positions 161 and 185. In the rHSA(I142H/Y161F)-heme complex, the bulky benzyl side-chain of Phe-161 (137° A$^3$) may prevent rotation of neighboring Leu–185, thereby providing easy access of O$_2$ to the heme (Fig. 5A). In contrast, substitution of Phe-161 by the smaller Leu-161 (102° A$^3$) may allow free rotation of the isopropyl group of Leu-185, which reduces the volume of the distal side (Fig. 5B) and hinders association of O$_2$ and CO with heme.

### Table 1. O$_2$ binding parameters of rHSA(mutant)-heme complexes in 50 mM potassium phosphate buffered solution (pH 7.0) at 22°C

<table>
<thead>
<tr>
<th>Hemoproteins</th>
<th>$k_{on}^{O_2}$ ($\mu M^{-1}$ s$^{-1}$)</th>
<th>$k_{off}^{O_2}$ (ms$^{-1}$)</th>
<th>$P_{1/2}^{O_2}$ (Torr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHSA(I142H/Y161F)-Heme</td>
<td>20</td>
<td>0.10</td>
<td>9</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L)-Heme</td>
<td>7.5</td>
<td>0.22</td>
<td>18</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L/F185N)-Heme</td>
<td>26</td>
<td>0.10</td>
<td>24</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L/L185N)-Heme</td>
<td>14</td>
<td>0.02</td>
<td>14</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L/R186L)-Heme</td>
<td>25</td>
<td>0.49</td>
<td>199</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L/R186F)-Heme</td>
<td>21</td>
<td>0.29</td>
<td>203</td>
</tr>
<tr>
<td>Hbα (R-state)$^6$</td>
<td>33°</td>
<td>0.013$^3$</td>
<td>0.24</td>
</tr>
<tr>
<td>Mb$^6$</td>
<td>14</td>
<td>0.012$^3$</td>
<td>0.51</td>
</tr>
<tr>
<td>RBC$^7$</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$Number I or II indicates species I or II.54,55 $^1$ Human Hb α-subunit. $^1$ In 0.1 M phosphate buffer (pH 7.0, 21.5°C)$^{56}$ 5$^6$ In 10 mM phosphate buffer (pH 7.0, 20°C)$^{56}$ $^6$ In 10 mM phosphate buffer (pH 7.0, 20°C)$^{56}$ $^6$ Human red cell suspension. In isotonic buffer (pH 7.4, 20°C)$^{66}$

### Table 2. CO binding parameters of rHSA(mutant)-heme complexes in 50 mM potassium phosphate buffered solution (pH 7.0) at 22°C

<table>
<thead>
<tr>
<th>Hemoproteins</th>
<th>$k_{on}^{CO}$ ($\mu M^{-1}$ s$^{-1}$)</th>
<th>$k_{off}^{CO}$ (s$^{-1}$)</th>
<th>$P_{1/2}^{CO}$ (Torr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHSA(I142H/Y161F)-Heme</td>
<td>6.8</td>
<td>0.09</td>
<td>0.0011</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L)-Heme</td>
<td>2.0</td>
<td>0.013</td>
<td>0.0053</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L/F185N)-Heme</td>
<td>7.7</td>
<td>0.008</td>
<td>0.0008</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L/L185N)-Heme</td>
<td>6.8</td>
<td>0.008</td>
<td>0.0010</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L/R186L)-Heme</td>
<td>5.0</td>
<td>0.011</td>
<td>0.0018</td>
</tr>
<tr>
<td>rHSA(I142H/Y161L/R186F)-Heme</td>
<td>7.9</td>
<td>0.010</td>
<td>0.0010</td>
</tr>
<tr>
<td>Hbα (R-state)$^6$</td>
<td>4.6°</td>
<td>0.009$^3$</td>
<td>0.0016$^3$</td>
</tr>
<tr>
<td>Mb$^6$</td>
<td>0.51</td>
<td>0.019</td>
<td>0.030</td>
</tr>
</tbody>
</table>

$^6$Number I or II indicates species I or II.54,55 $^6$ Human Hb α-subunit. $^6$ In 50 mM potassium phosphate buffer (pH 7.0, 20°C)$^{66}$ 5$^6$ In 0.1 M phosphate buffer (pH 7.0, 20°C)$^{66}$ 5$^6$ Calculated from ($k_{on}^{CO}/k_{off}^{CO}$)$^{-1}$. $^6$ Sperm whale Mb. $^6$ In 0.1 M potassium phosphate buffer (pH 7.0, 20°C)$^{66}$

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Fig. 5. Structural models of the heme pocket in dioxygenated rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme. Distal-side steric effect of Leu-185 on O₂ and CO association.

(R-state). To develop this O₂ carrying albumin as a blood substitute, it is required to regulate O₂ binding affinity suitable for Hb, Mb, and human RBC. One approach to increasing O₂ binding affinity of rHSA(double mutant)-heme would be to introduce another histidine into an appropriate position on the distal side of the heme pocket. The Ne atom of histidine may act as a proton donor to form an hydrogen bond with the coordinated O₂.

In Hb and Mb, His-64 on the distal side of the heme plays a crucial role for tuning their ligand affinity, as shown by a neutron diffraction study and high-resolution X-ray structural analysis. A number of systematic investigations on site-directed mutants of Hb and Mb show that the overall polarity and packing of the distal residues are key factors in regulating the equilibrium constants for ligand binding. Then, we generated new rHSA(triple mutant)-heme complexes, in which the specific third mutation was introduced near the O₂ binding site.

Another important point in this design is to prevent the formation of a six-coordinate low-spin ferrous complex. Bis-histidyl hemochromes are generally autoxidized by O₂. Therefore, the distal amino acid must be located relatively far (> 4 Å) from the central iron. Our simulation indicated that the favorable position for the distal His insertion was Leu-185. The polarity of the distal side of the heme in rHSA(I142H/Y161F) and rHSA(I142H/Y161L) was increased by replacing Leu-185 with asparagines, glutamine, and histidine using site-directed mutagenesis. Five triple mutants [rHSA(I142H/Y161F/L185N), rHSA(I142H/Y161F/L185Q), rHSA(I142H/Y161L/L185N), rHSA(I142H/Y161L/L185Q), and rHSA(I142H/Y161F/L185H)] were thus cloned and their heme complexes were prepared.

The rHSA(I142H/Y161F/L185N)-heme, rHSA(I142H/Y161F/L185Q)-heme, and rHSA(I142H/Y161L/L185Q)-heme showed a visible absorption band at 558–559 nm with a small shoulder at 530 nm, that was similar to the spectrum observed for the rHSA(I142H/Y161F)-heme, rHSA(I142H/Y161L)-heme, deoxy Mb and synthetic chelated-heme. The spectral patterns indicated the formation of a five-N-coordinate high-spin complex. In the spectra of the rHSA(I142H/Y161F/L185Q)-heme and rHSA(I142H/Y161F/L185H)-heme, the β band at 528 nm appeared slightly sharp, suggesting partial formation of a six-N-coordinate heme complex. On the basis of all the UV-vis absorption and MCD spectral results, we concluded that the heme is axially coordinated by His-142 in rHSA(triple mutant) and forms a five-N-coordinate high-spin ferrous complex under an Ar atmosphere in the case of I142H/Y161F/L185N, I142H/Y161F/L185Q, and I142H/Y161L/L185Q mutants (Fig. 6 A, B, D). In addition to the His-142 ligation, Gln-185 and His-185 partially interact with the sixth coordinate position of the central Fe²⁺ ion of the heme in I142H/Y161F/L185Q and I142H/Y161F/L185H mutants in spite of the bulky Phe-161 (Fig. 6 C, E). We suppose that the rHSA(I142H/Y161F/L185Q)-heme may also produce a six-coordinate low-spin complex, because the small Leu-161 allows free rotation of Gln-185. However, it gave a five-coordinate high-spin ferrous complex. This suggests that the long Gln-185 may interact with neighboring amino acids (Fig. 6 D).

With exposure of the rHSA(I142H/Y161F/L185N)-heme and rHSA(I142H/Y161L/L185N)-heme solutions to O₂, the UV-vis absorptions changed to that of the O₂ adduct complex at 22°C. However, the rHSA(I142H/Y161L/L185Q)-heme complex bound O₂ only at 5°C and was oxidized at 22°C. This rapid oxidation may suggest that the distal side of the heme has an open structure, which allows easy access of water to the heme. The rHSA(I142H/Y161F/L185Q)-heme and rHSA(I142H/Y161F/L185H)-heme complexes could not bind O₂ even at low temperature. After introducing CO gas, all hemoproteins produced stable carbonyl complexes with identical absorption spectral patterns.

In rHSA(double mutant)-heme complexes, there exists...
two geometries of axial His-142 coordination to the central Fe$^{2+}$ ion of the heme (species I and II). In species I, the proximal His coordinates to the heme without strain, while in species II, the His-Fe ligation involves some distortion, resulting in weaker O$_2$ binding. In rHSA(triple mutant)-hemes, this alternative geometry of the heme plane would also arise in the same manner. As expected, the binding behavior of O$_2$ for rHSA(I142H/Y161F)-heme, rHSA(I142H/Y161L)-heme, rHSA(I142H/Y161F/L185N)-heme, and rHSA(I142H/Y161L/L185N)-heme was almost the same as that of the original double mutants.

The $k_{d_{CO}}$ is normally an indicator of bending strain in the proximal His coordination to the central Fe$^{2+}$ ion. 49,50 The rHSA(I142H/Y161F)-heme, rHSA(I142H/ Y161L)-heme, rHSA(I142H/Y161F/L185N)-heme, and rHSA(I142H/Y161L/L185N)-heme exhibited similar $k_{d_{CO}}$ in species I (0.008–0.013 s$^{-1}$) which were identical to that of Hb$\alpha$ (R-state) (0.009 s$^{-1}$) (Table 2). 61 This indicates that the axial His-142 ligation to the heme in these artificial hemoproteins has the same features as that of Hb.

O$_2$ and CO binding parameters for the rHSA (I142H/Y161F)-heme and rHSA(I142H/Y161F/L185N)-heme complexes did not show any significant differences. The bulky benzyl side-chain of Phe-161 may retard rotation of the polar amide group of Asn-185 (Fig. 6A). In contrast, there are significant differences in the O$_2$ and CO binding parameters for rHSA(I142H/Y161L)-heme and rHSA(I142H/Y161L/L185N)-heme. The Asn-185 induced 18-fold and 10-fold increases in the O$_2$ binding affinity for species I and II. The increase was mainly due to the 6–11-fold reduction of $k_{d_{CO}}$. It is noteworthy that the high O$_2$ binding affinity ($P_{1/2}$: 1 Torr) for rHSA (I142H/Y161L/L185N)-heme is close to that of natural Hb$\alpha$ (0.24 Torr) 51,52 and Mb (0.5 Torr) 53 (Table 2).

Further tuning of O$_2$ binding affinity of rHSA(triple mutant)-heme complexes: The O$_2$ binding equilibrium and kinetics of rHSA-heme complexes are significantly enhanced by site-directed mutagenesis. However, for artificial rHSA-heme solutions to provide effective O$_2$ transport from lungs to tissues in the body, the O$_2$ binding affinity of HSA(I142H/Y161L/L185N)-heme should be reduced to that of human RBC ($P_{1/2}$: 8 Torr). 62 This requires an O$_2$ binding affinity intermediate between the values for rHSA(I142H/Y161L)-heme and rHSA(I142H/Y161L/L185N)-heme. We thus designed new triple mutants, rHSA(I142H/Y161L/R186L) and rHSA(I142H/Y161L/R186F) (Fig. 2). An important structural factor in these mutants is Y161L, which allows rotation of the isopropyl group of Leu-185 above the O$_2$ coordination site. The reduced ferrous forms produced the five-N-coordinate high-spin
complexes under an Ar atmosphere and formed the O₂ adduct complex after bubbling O₂ gas. The distinct features of all the spectra were similar to those of the rHSA(I142H/Y161L)-heme.

Following laser flash photolysis, the absorption decay associated with O₂ recombination to the rHSA(I142H/ Y161L/R186L)-heme and rHSA(I142H/Y161L/R186F)-heme was monophasic. The kinetics for CO rebinding were still composed of two single-exponentials, consistent with the existence of two different geometries of the axial His-142 coordination to the central Fe²⁺ ion of the heme.

The rHSA(I142H/Y161F)-heme binds O₂ with higher affinity than rHSA(I142H/Y161L) because of the presence of Leu at position 161, which allows a downward rotation of the L185 side chain and reduces the affinity. However, insertion of Leu or Phe at position 186 in the presence of Leu-161 yielded $k_{on}^{O_2}$ and $k_{off}^{CO}$ 3-4-fold higher than those of rHSA(I142H/Y161L)-heme. The presence of a hydrophobic residue at position 186 may restrict the downward rotation of the isopropyl group of Leu-185 (Fig. 7). Overall, the O₂ and CO binding parameters of rHSA(I142H/Y161L/R186L)-heme and rHSA(I142H/Y161L/R186F)-heme were more similar to those of rHSA(I142H/Y161F)-heme, but their O₂ dissociation rate constants were 3-4-fold higher than found for rHSA(I142H/Y161F)-heme, which modestly reduced O₂ binding affinity. This may be due to increase in hydrophobicity in the distal pocket. It is noteworthy that O₂ binding affinity of the rHSA(I142H/Y161L/R186L)-heme ($P_{O_2}^{10}$: 10 Torr) and rHSA(I142H/Y161L/R186F)-heme ($P_{O_2}^{10}$: 9 Torr) is essentially the same to that of human RBC ($P_{O_2}^{10}$: 8 Torr).

Circulation life of rHSA complexes: It would be of great importance to study the in vivo circulation behavior of rHSA(mutant)-heme complex for practical medical applications. This investigation is currently undergoing, but we have several results on the HSA-FeP complex. In general, the ligand molecule complexed with HSA gradually dissociates from the protein when infused into the body, since the ligand is noncovalently bound into the hydrophobic cavity of HSA. We found that surface modification of HSA-FeP by poly(ethylene glycol) (PEG) significantly improved the circulation lifetime of FeP in animals and thereby retained its O₂-transporting ability for a long period. Interestingly, the linkage form of the PEG chain dramatically affects the circulation persistence of FeP. Maleimide-PEG conjugates showed 6-8-fold longer lifetime compared to the succinimide-PEG analogue. PEG modified rHSA(mutant)-heme would also remain in the circulatory system with long persistence, which could be a potential advantage for O₂ delivery to the tissues.

HSA Incorporating C₆₀ Fullerene as Photosensitizer for Photodynamic Therapy

Structure and photophysical properties of HSA-fullerene complex: Photodynamic therapy (PDT) is advanced cancer treatment involving a photosensitizer, visible light and tissue. Singlet oxygen ($^{1}O_2$) formed by energy transfer from photoexcited state of sensitizer is highly cytotoxic and has been implicated as an intermediary species leading to cell death in tumors. To accelerate this $^{1}O_2$ formation, various organic dyes, especially porphyrin derivatives, have been designed as photosensitizers. The most widely used reagent in clinical PDT is Photofrin, which is a mixture of water soluble hematoporphyrin oligomers. Several porphyrin or chlorin compounds are also being tested. Another potential regent is 5-aminolevulinic acid (ALA). ALA enters into cancer cells and induces the biosynthesis of protoporphyrin IX (PP). Buckminsterfullerene produces $^{1}O_2$ by energy transfer with extremely high quantum yield ($Φ_{exc} = 532$ nm)$^{72,73}$ and shows strong resistance against laser irradiation. Consequently, various water-soluble fullerenes and fullerene-polymer hybrids have been synthesized as new photosensitizing reagents. However, to evaluate the biological function of fullerene, one must investigate the structure and properties of the HSA-fullerene complex, because exogenous compounds administered into the bloodstream are generally captured by HSA. We prepared HSA complexed with a tris(dicarboxymethylene)[60]fullerene $C_3$-isomer (HSA-CF) and characterized its photoinduced energy transfer to O₂ to produce $^{1}O_2$ and its cytotoxicity to cancer cells under visible light.

The HSA-CF complex was prepared essentially as described previously for HSA-hemin complex. The gel permeation chromatogram of the orange-colored protein exhibited a single elution peak. This indicates that CF efficiently binds to HSA. The UV-vis absorption spectroscopic features of the HSA-CF solution are the sum of those from the individual HSA and CF (Fig. 8), which implies that CF is monomolecularly incorporated into HSA and no specific interaction occurs between the two molecules at the ground state. Gozin et al. reported that CF is incorporated into the subdomain IIA of HSA with a binding constant of $1.2 \times 10^7$ M⁻¹ by fluorescent quenching experiments.

The HSA-CF complex was sufficiently stable to apply to HPLC measurement. In the elution profile, only a single peak was observable. The ratio of peak intensity monitored at 280 nm (based on HSA) and at 490 nm (based on CF) ($I_{280}/I_{490} = 30$) was exactly the same as the absorbance ratio at 280 nm and 490 nm in the UV-vis absorption spectrum ($A_{280}/A_{490}$) of HSA-CF, which suggests that all CF molecules are eluted within the HSA fraction. The ESI-TOF mass spectroscopy showed a distinct ion
Fig. 7. Structural models of rHSA(I142H/Y161L)-heme and rHSA(I142H/Y161L/R186L)-heme complexes. Introduction of R186L mutation may induce upward rotation of the L185 residue.

Fig. 8. UV-vis absorption spectrum of HSA-CF, CF, and HSA in 50 mM phosphate buffered solution (pH 7.0). Inset photographs are 5 g dL⁻¹ HSA (left) and HSA-CF (right) solutions.

peak at 67,587 Da, which corresponds to the mass of the equivalent complex of HSA-CF. CD spectral patterns and intensities of HSA-CF were identical to those of HSA. We inferred that fullerene binding did not change the highly ordered structure of the protein. The incorporation of negatively charged CF may influence the surface charge distribution of albumin. However, isoelectric focusing of HSA-CF indicated the same isoelectric point with HSA.

We used small-angle X-ray scattering (SAXS) to evaluate the globular particle structure and protein-protein interactions of HSA-CF. The pair-distance distribution functions [p(r)] of HSA-CF and HSA were almost identical to the curve calculated from crystallographic data of HSA (Fig. 9A). This demonstrates that the maximum diameter (Dmax: ca. 8 nm) and three-dimensional particle shape of HSA are not changed by complexation of the CF molecule. The extrapolated structure factors [S(q→0)] reflect the net repulsive forces between the protein molecules. Plots of S(q→0) for HSA-CF and HSA were lower than that predicted for hardsphere with an identical volume fraction (Fig. 9B), this being due to the strong electrostatic repulsion between the proteins. The perfectly same lines of HSA-CF and HSA suggest that the HSA molecule preserves its surface net charge upon CF binding. We conclude that CF is accommodated into the deep hydrophobic cavity of HSA with internal charge neutralization, and it does not induce marked change in the globular particle size or surface charge distribution of HSA.

Photoexcited triplet state of the HSA-CF complex and O₂ production: Photoexcitation of fullerenes generates the singlet state, which undergoes intersystem crossing to the triplet state in high yield. Laser
Fig. 9. (A) Pair-distance distribution functions \([p(r)]\) obtained from SAXS measurements of HSA-CF and HSA in 150 mM PBS solutions. (B) The extrapolated structure factors \([S(q)\]) of HSA-CF and HSA.

Fig. 10. Living cell numbers of LY80 with HSA-CF and PBS after visible light irradiation (20 mW cm\(^{-2}\), 2 h, 36 ± 1°C). Each value represents the mean ± SD (n = 4).

Flash photolysis of the HSA-CF solution under an N\(_2\) atmosphere gave a triplet-triplet (T-T) absorption spectrum of the \(3\)CF\(^*\) chromophore (\(\lambda_{\text{max}} = 740\) nm).\(^{73,75,78}\) The time course of the absorbance decay was composed of a single exponential kinetics with a lifetime (\(\tau_T\)) of 46 \(\mu\)s. In the presence of O\(_2\), the triplet lifetime of HSA-CF markedly decreased. Energy transfer took place from HSA-3CF\(^*\) to the O\(_2\) molecule to generate active \(1\)O\(_2\). The Stern-Volmer plot depicts a linear correlation for O\(_2\) concentrations (0–1.0 mM), giving the quenching rate constant \([k_q(O_2) = 2.2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}]\). The intensity of the visible band of HSA-CF (\(\lambda_{\text{max}} = 490\) nm) did not change after 10\(^3\)-times laser flash photolysis under air. In contrast, Soret band of HSA-PP was 6% bleached after identical flash photolysis. The light resistance of CF chromosphere is significantly higher than that of PP.

The quantum yield of \(1\)O\(_2\) production (\(\Phi_{1}\)) for HSA-CF was determined from the value of emission intensity of the \(1\)O\(_2\) (\(\lambda_{\text{max}} 1270\) nm).\(^{79}\) \(\Phi_{1}\) of HSA-CF (0.46) was in the same range as that of monomeric CF (0.48), methylene blue (0.52), protoporphyrin IX dimethyl ester in benzene (0.59),\(^{79}\) and substituted fullerenes in organic solvent,\(^{80,81}\) but somewhat lower than for rose bengal (0.75), hematoporphyrin in methanol (0.74),\(^{82}\) and pristine C\(_{60}\) (0.96) in benzene.\(^{72}\) Substitution of the C\(_{60}\) fullerene causes perturbation of the electronic structure of the fullerene core, thereby decreasing the quantum yield for \(1\)O\(_2\) formation.\(^{80,81}\)

Cytotoxicity of HSA-CF complex: The cytotoxicity and photodynamic activity of the HSA-CF complex to LY80 tumor cells was evaluated. The cell cultures were first incubated for 24 h in the dark with HSA-CF (20 \(\mu\)M) at 37°C under 5% CO\(_2\). Cell numbers after incubation were identical to those of the control group with the phosphate buffered saline (PBS, pH 7.4) solution. The HSA-CF complex showed no dark cytotoxicity. The cell culture plate was then exposed to visible light of 350–600 nm (20 mW cm\(^{-2}\)) for 2 h at 36 ± 1°C. Some cells mixed with HSA-CF showed morphological change after light irradiation, whereas the PBS group did not show any morphological change of the cells. The living cell numbers of the PBS groups with and without light were almost the same (Fig. 10), which means that light exposure did not affect the LY80 tumor cells in this experimental condition. The living cell numbers of the HSA-CF group were lower than that of the PBS group; 57% cell death occurred by visible light irradiation. This clearly implies that the HSA-CF complex acts as a photosensitizer for PDT.

Conclusions

Transport of O\(_2\) by the rHSA(mutant)-heme complex may be of great medical importance not only as a blood alternative, but also as an O\(_2\)-therapeutic fluid. The first generation of rHSA(double mutant)-heme complexes can
be successfully engineered to bind to O₂. However, these complexes did not show optimal O₂ binding affinity. We attempted to modify the heme pocket architecture to refine O₂ binding properties. By focusing on modification on the distal side of the heme pocket, we prepared rHSA(triple mutant)-heme complexes with a broad range of O₂ binding affinity. The highest affinity mutant rHSA(I142H/Y161L/L185N) contains Asn-185, which has a short amide side-chain that enhances O₂ binding affinity. In a different approach, substitution of the polar Arg-186 with Leu or Phe caused useful reduction in O₂ binding affinity, yielding $P_{1/2}O₂$ almost identical to that of human RBC.

Amphiphilic C₆₀ fullerene also be adopted as functional ligand for HSA. The HSA-CF complex is easily excited by visible light and shows a high charge transfer rate constant for O₂. The efficiency of $^1O₂$ production of this artificial protein is in the same range as common dyes. The HSA-CF complex does not have dark cytotoxicity, but engenders cell death under visible light irradiation.

In both the HSA-based O₂ carrier and photosensitizer, the protein plays a crucial role for solubilizing the heme and CF in aqueous medium up to ca. 3 mM. Even at high concentration, shielding of the chromophore by negatively charged HSA inhibits oxidation of the dioxygenated heme via $μ$-oxo dimer formation and a bimolecular Triplet-Triplet annihilation of excited $^3CF^*$. Furthermore, in the case of the rHSA-heme complex, a pair of site-specific mutations is essential to confer O₂ binding capability on the heme. In contrast, no mutation is required for photosensitizing HSA-CF; replacement of some amino acid around the CF binding site may enhance the photophysical properties of the CF molecule. rHSA is now manufactured on an industrial scale using yeast species *Pichia pastoris*, which allows these functional proteins for use in practical applications.

Acknowledgments: This work was supported by PRESTO “Control of Structure and Functions”, JST, Grant-in-Aid for Scientific Research (No. 20350058) from JSPS, and Health Science Research Grants (Regulatory Science) from MHLW Japan. The authors thank to Prof. Dr. Eishun Tsuchida for his precious suggestions and Prof. Dr. Stephen Curry (Imperial College London) for his valuable comments and cooperation on protein structure and site-directed mutagenesis. Prof. Koichi Kobayashi and Prof. Hirohsa Horinouchi (Keio University) are also gratefully acknowledged for their supports on animal experiments. We are grateful to Dr. Takaaki Sato for his skillful experiments and evaluation on SAXS measurements. X.Q. thanks a JSPS Postdoctoral Fellowship for Foreign Researchers.

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