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Dioxygenation of Human Serum Albumin Having a Prosthetic Heme Group in a Tailor-Made Heme Pocket

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Human serum albumin (HSA, MW = 66.5 kD) is the most abundant plasma protein in our bloodstream and serves as a transporter for small hydrophobic molecules such as fatty acids, bilirubin, and steroids.[1,2] Hemin dissociated from methemoglobin is also bound within a narrow D-shaped cavity in subdomain IB of HSA with an axial coordination of Tyr-161 and electrostatic interactions between the porphyrin propionates and a triad of basic amino acid residues (Figure 1).[3,4] In terms of the general hydrophobicity of the α-helical pocket, HSA potentially has features similar to the heme-binding site of myoglobin (Mb) or hemoglobin (Hb). However, even if one reduces the ferric HSA−hemin to obtain a ferrous complex, it is immediately oxidized by O_2. This is due to the fact that HSA lacks a proximal histidine, which enables the heme group to bind O_2.[3−5] We have shown that HSA incorporating tetraphenylporphinatoiron derivatives having a covalently linked axial-base can absorb O_2 under physiological conditions with a O_2 binding affinity similar to that of Hb.[6]

In this paper, we report for the first time the introduction of a proximal histidine into the subdomain IB of HSA by site-directed mutagenesis to construct a tailor-made heme pocket, which allows a reversible O_2 binding to the prosthetic heme group. Laser flash photolysis experiments revealed that this artificial hemoprotein appears to have two different geometries of the axial-imidazole coordination and shows rather low O_2 binding affinity.

We designed two recombinant HSA (rHSA) mutants, in which single or double mutations were introduced into subdomain IB: I142H [rHSA(A)] and I142H/Y161L [rHSA(B)] (Figure 1). Replacement of Y161 by histidine was not done because modeling experiments indicated that the distance from N_ϵ(H142) to Fe(heme) would be too great (>4 Å). In our mutants, the N_ϵ(H142)−Fe distance was estimated to be 2.31 Å (compared to 2.18 Å in Mb). The specific mutations were introduced into the HSA coding region in a plasmid vector (pHIL-D2 HSA) using the QuikChange (Stratagene) mutagenesis kit, and the mutants were expressed in the yeast species *Pichia pastoris*.[7] The rHSA(wild-type or mutants)−hemin complexes were prepared essentially according to our previously reported procedures, except that myristate was not added.[4] The resulting hemoproteins exhibited only a single band in SDS-PAGE.

In the absorption spectrum of the rHSA(wt)−hemin solution, the distinct charge-transfer (CT) band of Fe^{3+}−phenolate appeared at 625 nm.[8] A magnetic circular dichroism (MCD) spectrum showed a W-shaped feature in the Soret-band region.[9] These results imply the formation of a high-spin Fe^{3+} complex with the phenolate oxygen ligand of Y161, which is quite consistent with that found in the crystal structure.[3,4]

rHSA(B)−hemin did not exhibit the CT band because of the Y161L mutation and was easily reduced to the corresponding ferrous complex by adding a small molar excess amount of aqueous $Na_2S_2O_4$ under an Ar atmosphere. A single broad absorption band (λ_{max} = 559 nm) in the α,β region was very similar to that of deoxy Mb and indicated the formation of a five-N-coordinate Fe^{2+} complex (Figure 2).[10] The spectral pattern was unaltered in the temperature range of 0−25 °C. The shape of the asymmetric MCD spectrum also resembled that of deoxy Mb.[11] This suggests that the heme is accommodated into the tailor-made heme pocket with an axial coordination involving His-142.

Upon exposure of the rHSA(B)−heme solution to O_2 gas, the UV−vis absorption changed to that of the dioxygenated complex (λ_{max} = 412, 537, 573 nm) at 0−25 °C[10] (lifetime of the O_2−adduct: ca. 10 min). After exposure to flowing CO, the heme produced a typical carbonyl complex (λ_{max} = 419, 538, 565 nm).

On the contrary, rHSA(A)−heme could not bind O_2 even at low temperature (~0 °C). It can be thought that the polar phenolate residue at the top of the porphyrin platform is likely to accelerate the proton-driven oxidation of the Fe^{2+} center. Replacing Y161 by

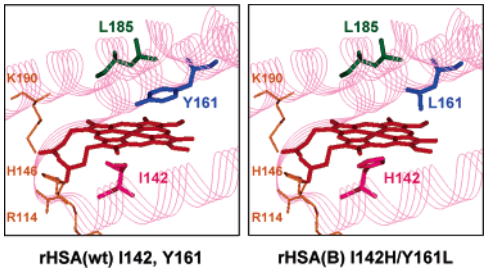

Figure 1. Prosthetic heme group complexed within the heme pocket in subdomain IB of rHSA(wt) and rHSA(B) mutant produced on the basis of the crystal structure coordinate of the rHSA−hemin complex (ref 4).

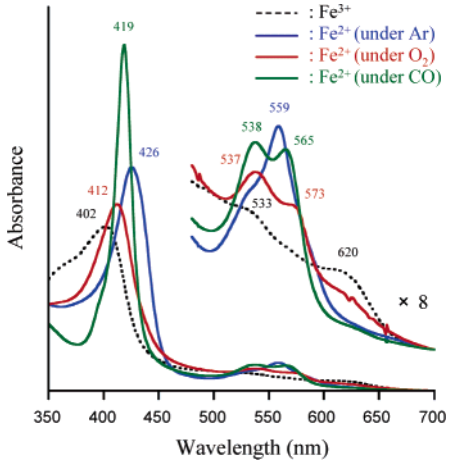

Figure 2. UV−vis absorption spectral changes of rHSA(B)−heme in phosphate buffered solution (pH 7.0, 50 mM) at 8 °C.

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Table 1. O_2 and CO binding Parameters of rHSA(B)−heme in Phosphate Buffered Solution (pH 7.0, 50 mM) at 22 °C

hemoprotein	k_{on}^{CO} (M^{-1} s^{-1})	$k_{on}^{O_2}$ (M^{-1} s^{-1})	$k_{off}^{O_2}$ (s^{-1})	$P_{1/2}^{O_2}$ (Torr)
rHSA(B)−heme[a]	2.0×10^6 (I), 2.7×10^5 (II)	7.5×10^6 (I and II)	221 (I), 1.7×10^3 (II)	18 (I), 134 (II)
Mb[b]	5.1×10^5	1.4×10^7	12	0.51
Hb (*R*-state)[c]	4.6×10^6	3.3×10^7	13	0.24

[a] Number in parentheses (I or II) indicates species I or II. [b] At 20 °C; ref 17. [c] At 20−21.5 °C; ref 18.

hydrophobic leucine greatly enhanced the stabilization of the O_2−adduct complex.

We then employed laser flash photolysis (Nd:YAG SHG; λ = 532 nm; 6 ns pulse width) to evaluate the kinetics of O_2 and CO bindings to rHSA(B)−heme.[12−14] Interestingly, the time dependence of the absorption change accompanying the CO recombination showed double-exponential profiles; the ratio of the amplitude of the fast and slow phases was always 3:2. On the other hand, the rebinding process of O_2 obeyed monophasic decay. On the basis of studies on synthetic model hemes, it has been shown that the proximal-side steric effect is the only primary factor that influences the association rate for CO but not for O_2.[12−14] One possible explanation is that there may be two different geometries of the axial His-142 coordination and that each one shows the individual kinetics of the CO rebinding. Marden and co-workers also found a similar behavior in CO association with HSA−heme and interpreted it as indicating that there are two orientations of the heme plane in the albumin scaffold.[5] In our case, the alternative geometries may arise because the heme molecule binds into the pocket of subdomain IB in two orientations related by a 2-fold rotation about its center (180° rotational isomers). Asymmetric 3,8-divinyl groups at the porphyrin periphery, in particular, would occupy different positions that result in a small divergence of the porphyrin ring and its iron center. The crystal structure analyses could not resolve this ambiguity, because the two configurations exist as a mixture.[3,4]

Our hypothesis is consistent with infrared spectroscopy data. The CO coordinated with rHSA(B)−heme showed a broad ν_{CO} at 1962 cm^{-1} with a shoulder at 1942 cm^{-1}. We therefore suggest that there are two different modes of π-back-donation from the central Fe^{2+} to the bound CO. It is remarkable that the lower stretching frequency is very close to that of Mb (ν_{CO} = 1943 cm^{-1}).[15] Attempts to determine the ratio of the two hemin orientations by ^1H NMR spectroscopy unfortunately failed.[16] The downfield spectra of rHSA(B)−hemin in met and met-azido forms did not show sharp resonances of the four porphyrin CH_3 groups.

The two geometries of the His-142 ligation in rHSA(B)−heme should yield two different O_2 binding affinities. By analyzing CO/O_2 competitive binding following laser flash photolysis, we obtained the association and dissociation rate constants for O_2 ($k_{on}^{O_2}$, $k_{off}^{O_2}$), and the O_2 binding affinities ($P_{1/2}^{O_2}$) for these two species (I and II) (Table 1).[12−14] The $P_{1/2}^{O_2}$ values were determined to be 18 and 134 Torr, respectively; this means that the O_2 binding affinities were 2.8 and 0.4%, respectively, of that observed for Mb.[17,18] Kinetically, for species I with $P_{1/2}^{O_2}$ of 18 Torr, an 18-fold increase in the $k_{off}^{O_2}$ value leads to the low affinity for O_2. Repeated studies with synthetic model hemes and engineered mutants of Mb or Hb have shown that low polarity of the microenvironment around the heme site decreases the O_2-binding affinity by increasing the dissociation rate constant.[12−14,17] In this albumin-based hemoprotein, the porphyrin ring is buried in the core of the pocket entirely made of hydrophobic residues. Therefore, the O_2 binding affinity becomes significantly lower than those of Mb and Hb. In species II with $P_{1/2}^{O_2}$ of 134 Torr, the proximal pull effect could further increase the $k_{off}^{O_2}$ value and may cause a large decline in the O_2 binding affinity.

We have recently found that heme is accommodated into the different architecture of a tailor-made heme pocket in rHSA (Y161L/L185H) [rHSA(C)] and that the rHSA(C)−heme complex also binds O_2 as well. In this case, the proximal histidine coordinates with the heme plane from the roof side, and the O_2 molecule binds from the floor side (Figure 1). Our combined structural and mutagenic approach allows us to significantly enhance the O_2 binding properties of rHSA−heme complexes and thereby mimic the diverse biological reactivities of natural hemoproteins. From the viewpoint of clinical applications, "O_2-carrying plasma albumin" could be of extreme medical importance not only for red blood cell substitutes but also for O_2-therapeutic reagents. The crystal structure analysis of this new class of artificial hemoproteins is now underway.

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Supporting Information Available: Time dependence of the absorption change accompanying the CO or O_2 rebinding to rHSA(B)−heme after the laser flash photolysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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