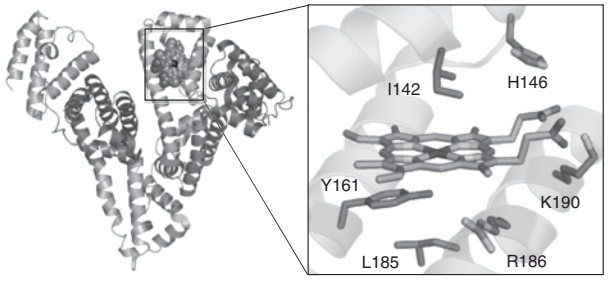
O_2 Binding Properties of Human Serum Albumin Quadruple Mutant Complexed Iron Protoporphyrin IX with Axial His-186 Coordination

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)

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 (**1**G)	**Arg**	**Gly**	Leu	**His**	**Arg**
H146R/Y161L/R186H/K190R (**1**L)	**Arg**	**Leu**	Leu	**His**	**Arg**
H146R/Y161G/L185H/K190R (**2**G)	**Arg**	**Gly**	**His**	Arg	**Arg**
H146R/Y161L/L185H/K190R (**2**L)	**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.

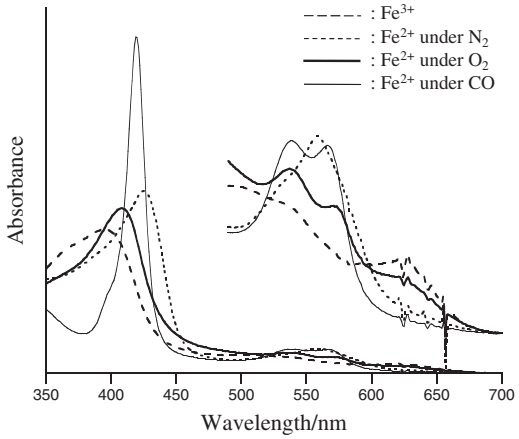


Figure 2. UV–vis. absorption spectral changes of rHSA**1**L–heme in 50 mM potassium phosphate buffered solution (pH 7.0) at 22 °C.

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.

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

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

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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