Binding of heme to human serum albumin: Steady-state fluorescence, circular dichroism and optical difference spectroscopic studies

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The binding of monomeric heme to human serum albumin (HSA) was investigated using steady-state fluorescence, circular dichroism (CD) and optical difference spectroscopic (ODS) techniques. The existence of one strong binding site for heme on HSA was confirmed by titrating heme with HSA and following the quenching of tryptophan (Trp214) fluorescence emission intensity that occurred due to energy transfer. Up to around 1:1 stoichiometric ratio of HSA/heme, the quenching was observed to be very strong, however at higher ratios the quenching progressed very weakly. Similarly, the negative CD band centered at ~397 nm, which appeared on adding heme to HSA, increased in intensity on sequential addition of heme up to [heme]/[HSA]=1. Titration of HSA with heme was followed by ODS and the dissociation constant $K_D = (4.0 \pm 1.0) \times 10^{-5}$ M was deduced. Results have been explained on the basis of Michaelis-Menton type of mechanism for the heme binding, in which heme first binds reversibly to His146 at the surface of the protein to form an intermediate complex, followed by irreversible binding to Tyr161 in the interior of the protein.

**Keywords**: human serum albumin (HSA), HSA-heme, methemalbumin (MHA), steady-state fluorescence, circular dichroism, optical difference spectroscopy, two stage binding.

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Serum albumin, the most abundant protein present in the blood at around 40 mg/ml (~0.6 mM), is the primary transporter of non-esterified fatty acids. In addition, it binds and transports bilirubin, steroid hormones, bile salts, tryptophan, thyroxin, several vitamins, hematin, metal ions and a large number of pharmaceutical agents. Under normal physiological condition, heme [Fe(III) protoporphyrin IX] binds to a specific heme binding protein, hemopexin present in the blood plasma. Under certain conditions such as intra-vascular or massive extra-vascular hemolysis, heme (separated from the hemoglobin) binds HSA after hemopexin gets saturated by heme. It is then transported to the liver for further metabolism and excretion. Binding of heme to HSA to form methemalbumin, therefore, is important for its protein-ligand interaction aspects, as well as from the pathophysiological point of view. In addition, heme-albumin infusions are found to be very safe and effective in repleting deficient heme pools and hemoproteins in patients, suffering from acute porphyria. The peroxidative and catalytic activities of heme are inhibited by the binding of HSA and BSA. HSA-heme complex has been used as an artificial hemoprotein in modelling cytochrome-P 450 catalyzed reactions. Synthetic heme, tetraphenylporphinato iron(II) is found to bind HSA to form a complex that reversibly binds and releases dioxygen under physiological conditions.

In aqueous solution, difficulties have been encountered in studying the HSA-heme interaction, mainly due to the fact that heme exists as aggregates, thus any interpretation of binding data will be complicated by simultaneous de-aggregation of heme oligomers. To prevent such complexities, dimethylsulfoxide/water (3:5 v/v) mixture was used as a solvent, as heme remains monomeric and HSA is undenatured in this composition. Equilibrium study on methemalbumin formation in aqueous solution revealed the existence of one strong binding site for heme ($K_D = 2 \times 10^{-8}$ M$^{-1}$), along with large number of

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**Abbreviations**: HSA, human serum albumin; Heme, Fe(III) protoporphyrin IX; CD, circular dichroism; MHA, methemalbumin; ODS, optical difference spectroscopy; $K_D$, dissociation constant
weaker sites where fatty acids bind. Experimental evidence suggests that the strong heme-binding site is located on domain-I of HSA. We showed recently that the strong heme binding site is a non-histidine amino acid residue, contrary to what was suggested earlier that heme binds to histidine and to water. Crystal structure of methemalbumin (1:1 complex) at 1.9 Å resolution put an end to these controversies and showed that the iron is penta coordinated and the fifth ligand is the hydroxyl oxygen of Tyr161 with phenolic oxygen to heme plane distance of 2.73Å located in a hydrophobic pocket (Fig. 1).

However, the nature of HSA-heme interaction is not clearly understood. From the kinetics of heme binding to HSA, Adams and Berman inferred that the formation of methemalbumin is a two stage, single intermediate process. The first stage of reaction involves ligation of heme to an amino acid residue on the protein surface, which, from pH studies ($pK_a \approx 5.9$) was attributed to histidiyl imidazole. This process with $K_D = (6.0 \pm 3.0) \times 10^{-5} \text{ M}^{-1}$ is followed by a slow internalization of the heme through a conformational folding to a thermodynamically stable complex. They reported an overall $K_D$ value of $9.0 \times 10^{-9} \text{ M}^{-1}$ calculated from the microscopic rate constants for the individual stages, which broadly agrees with the value reported by Beaven et al. However, since histidine is not the binding site of heme as revealed by the recent X-ray crystal structure of methemalbumin, the results of Adams and Berman remain obscure.

HSA contains three domains designated as I-III, each with sub domains A and B and has a single tryptophan (Trp214) located on domain-IIA. The objective of this study was to monitor the binding of monomeric heme to HSA by steady-state tryptophan fluorescence, circular dichroism (CD) spectroscopy and determine the binding constant for the HSA-heme complex (1:1) by optical difference spectroscopy (ODS). The results have been discussed on the basis of Michaelis-Menten type mechanism. The knowledge of changes in spectral properties of HSA on heme binding may be useful to set-up guide lines for drug dosage to patients suffering from serious pathological conditions, such as acute porphyria who are administered heme intravenously (that forms complex with HSA) and may help to reduce incidences of serious side effects, if any.

**Experimental**

**Materials**

Freshly drawn human blood was treated to extract and purify HSA as described. Hemin chloride was purchased from Sigma Chem. Co, USA. Dimethyl sulfoxide was obtained from E Merck. The buffer used was 25 mM sodium phosphate containing 60 mM NaCl, pH 6.8. All solutions were prepared in dimethyl-sulfoxide/buffer (3:5, v/v) mixture. Deionized water (PURITE RO 50) was used in the preparation of the buffer. All experiments were performed at room temperature (25°C).

**Characterization of heme binding to HSA**

Formation of methemalbumin was monitored by fluorescence as well as circular dichroism (CD) measurements. Fluorimetric titration of albumin (10 μm) was carried out at varying heme concentrations (0-85 μm) using a stock heme concentration of 1.7 mm in SPEX Fluorolog-1681T (dM 3000 F) spectrofluorimeter with cuvette length of 10 mm. The excitation wavelength was chosen to be 295 nm to minimize contributions from tyrosine and phenylalanine. CD titrations were carried out in the wavelength region 250-700 nm for HSA (10 μm) with hemc (0-32 μm) using the same stock, in Jasco J-600 spectropolarimeter with a cuvette length of 10 mm.

**Optical difference spectroscopy (ODS)**

For determining the binding constant ($1/K_D$) of HSA and heme, the difference absorption spectrum of

![Fig. 1—Schematic sketch of the heme binding location in human methemalbumin as illustrated in the crystal structure (PDB code: 1n5u) [Domain-1B helical motif (h8-11), which encloses heme is coloured dark grey and the nearest helices (h7 of domain-IA & h12 of domain-IIA) are coloured light grey. The amino acid residues tyrosine 161 (Tyr161) histidine 146 (His146), lysine 190 (Lys190), and tryptphan 214 (Trp214) are shown as ‘ball and stick’ models]
heme-HSA complex versus heme concentration was recorded on Shimadzu UV-2100 spectrophotometer. Initially, both the sample and reference cuvettes were filled with 1.0 ml of heme solution (2.7 µm), followed by baseline tracing. Thereafter, a small volume (15-50 µl) of the HSA stock (86 µm) was successively added to the sample cuvette, with concomitant addition of the solvent, dimethyl-sulphoxide/buffer (3:5, v/v), into the reference cuvette. After each addition, the contents of both cuvettes were stirred well. The apparent dissociation constant $K_D$ is obtained from the resulting difference spectral curves and using the following equation for the formation of 1:1 complex

$$\frac{1}{\Delta A} = \left(\frac{K_D}{\Delta A_{\infty}}\right) \frac{1}{S_0} + \frac{1}{\Delta A_{\infty}} \quad \ldots \quad (1)$$

where $\Delta A$ and $\Delta A_{\infty}$ are the changes in absorption at the observation wavelength at given and saturating substrate concentrations, respectively. $S_0$ is the concentration of the protein added and $K_D$ is the dissociation constant of heme-HSA complex formed. The experiment was repeated with excess HSA in the range of 18-83 µm with the heme concentration of 2.7 µm.

**Results and Discussion**

We have followed the binding of heme to albumin by fluorimetry and CD. The heme group is a fluorescence quencher via energy transfer, due to the extensive overlap of its absorption band with the fluorescence spectra of the aromatic amino acids of the protein. As the [heme]/[protein] ratio increases, tryptophan fluorescence of HSA is quenched, indicating energy transfer to heme (Fig. 2A). The quenching as a function of the mole ratio of heme added, at a series of protein concentrations is shown in Fig. 2B. The quenching of the tryptophan fluorescence is very strong up to around 1:1 stoichiometric ratio of heme/HSA, but at higher ratios, it progresses weakly, indicating that there is a single strong binding site for heme, located not more than about 25 Å away from Trp214. This is in conjunction with the crystal structure of methemalbumin (prepared by 1:1 mixing of HSA and heme). The data further suggest that there are number of far-removed weak binding sites for heme. These observations agree with the inferences of Beaven et al. The progressive change in the CD spectra of HSA, when titrated with heme in the 250-450 nm range is shown in Fig. 3A. HSA does not have a chromophore absorbing at ~ 400 nm, accordingly, no CD band is observed around this wavelength. Further, free heme is CD inactive, due to its symmetric center; however, on binding to the protein, the induced CD arises due to the interaction of heme chromophore with the asymmetric protein environment around heme.

On addition of heme, negative CD band, centered at ~397 nm appears. The negative intensity of the band increased on sequential addition of heme up to [heme]/[HSA] = 1 (Fig. 3A & B). On further addition ([heme]/[HSA] >1), however no further increase in the intensity is observed (Fig. 3B). These observations suggest that the strong binding site is located in an asymmetric center of the protein and weak binding sites are not located in asymmetric protein environment.

![Fig. 2](image-url)
Dissociation constant ($K_D$) of HSA and heme for the complexation in the strong binding site is determined by carefully measuring optical difference (OD) spectra. Fig. 4A shows the OD spectra of the complex formation. The double reciprocal plot of $1/\Delta A$ versus $1/[S]_0$ is a straight line (Fig. 4B) and thus follows Eq. 1. Dissociation constant $K_D = (4.0 \pm 1.0) \times 10^{-5}$ M evaluated by the least square’s fit of data to Eq. 1, compares well with $K_D = 1.3 \times 10^{-5}$ M determined by isothermal titration calorimetry (Dhingra K & Behere D V, unpublished data) and that reported by Adams and Berman\textsuperscript{4} ($K_D = 6.0 \times 10^{-5}$ M) for the initial fast step of the reaction for heme binding to the strong binding site\textsuperscript{4}. However, the present value does not compare either with the value reported by Beaven \textit{et al}\textsuperscript{11} ($K_D = 2.0 \times 10^{-8}$ M) or with the overall $K_D$ value ($9.0 \times 10^{-9}$ M) calculated for the entire two-step reaction by Adams and Berman\textsuperscript{4}.

Adams and Berman\textsuperscript{4} calculated the individual rate constants of two reversible steps as $k_1 = 1.7 \pm 0.3 \times 10^5$ M$^{-1}$ s$^{-1}$ and $k_{-1} = 10.1 \pm 0.3$ s$^{-1}$ for the first step and $k_2 = 6.3 \pm 0.3$ s$^{-1}$ and $k_{-2} = 0.00091 \pm 0.00011$ s$^{-1}$ for the second step. The first step is a fast true equilibrium step ($K_D = k_{-1}/k_1$) to form the intermediate complex ({HSA-heme}), followed by almost an irreversible ($k_{-2}/k_2 \approx 0$) slow step to the stable complex (MHA). Such a mechanism is similar to the Michaelis-Menten mechanism\textsuperscript{19} derived for enzyme activity and is given as:

$$E + S \quad \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \quad ES \quad \underset{k_{-2}}{\overset{k_2}{\rightarrow}} \quad E + P \quad \ldots \quad (2)$$

where E is the enzyme, S is the ligand, ES is the enzyme-substrate complex (Michaelis complex) and P is the product. The Eq. 1 is similar to that of double
reciprocal Michaelis-Menten equation\textsuperscript{19}, in which $K_M$ [Michaelis constant, $(k_1 + k_2)/k_1$] replaces $K_D$, and $K_M = K_D$ when $k_2 << k_1$.

We recorded the OD spectra immediately after adding the titrant, and thus the elapsed time before the recording of absorbance at 416 nm is the same in all the titrations. Therefore, the $K_D$ obtained in our case is similar to the $K_M$ of Michaelis-Menten equation, and therefore refers to the first equilibrium step. Accordingly, our $K_D$ value agrees with the reported value\textsuperscript{4} for the first step. However, the $K_D$ value is very high as compared to that reported for heme binding to apo-myoglobin to form myoglobin (10\textsuperscript{-12} - 10\textsuperscript{-15} M)\textsuperscript{20}, indicating low affinity of heme towards albumin, which is consistent with the nature of HSA as a heme transporting protein, rather than being a naturally-occurring heme protein. Thus, a similar mechanism as that of enzyme kinetics can be proposed for the heme binding to HSA as

\[
\text{HSA} + \text{heme} \xrightarrow{k_1} \text{HSA-heme} \xrightarrow{k_2} \text{MHA}
\]

However, the two mechanisms, the Michaelis-Menten mechanism\textsuperscript{19} (Eq. 2) and the mechanism involving the formation of \{HSA-heme\} intermediate followed by the formation of MHA (Eq. 3) slightly differ with each other. For the enzyme kinetics, $k_1$ value is of the order of 10\textsuperscript{8} M\textsuperscript{-1} s\textsuperscript{-1}, which is typical of diffusion-controlled rate and hence the first stage of the enzyme kinetics is diffusion controlled, entropic-driven, enzyme-ligand complexation with van der Waal’s interactions holding the enzyme and ligand\textsuperscript{19}. But for methemalbumin formation, $k_1$ value\textsuperscript{4} is in the order of 10\textsuperscript{5} M\textsuperscript{-1} s\textsuperscript{-1}. Since the $K_D$ value we obtained is independent of HSA concentration (comparable or in excess to heme concentration), the restrictions in the diffusion rate is ruled out and the relatively lower $k_1$ value (~10\textsuperscript{5} M\textsuperscript{-1} s\textsuperscript{-1}) is not arising due to accessibility limitations of an otherwise, diffusion-controlled rate.

Adams and Berman\textsuperscript{4} obtained a $pK_a$ of 5.9 for the pH dependent $k_t$ and attributed heme binding to histidine in the \{HSA-heme\} complex. The crystal structure\textsuperscript{13} indicated tyrosine (Tyr161) being the binding site of heme in MHA. For tyrosine, the $pK_a$ is 10.13\textsuperscript{15}, which does not agree with the $pK_a = 5.9$ suggested by Adams and Berman\textsuperscript{4} and hence Tyr161 cannot be the binding site for the intermediate \{HSA-heme\} complex. This suggests that the nature of HSA-heme complex formation is poorly understood.

Earlier, we reported a detailed characterization of the purified MHA and its carbon monoxide derivative\textsuperscript{16} and found that heme in MHA binds to a non-histidine amino acid and the binding is weaker than a histidine-heme bond, a result in corroboration with the findings of crystal structure\textsuperscript{16}. We also observed that the reduced heme, upon adding CO, in the absence and presence of HSA and in the presence of 2-methyl-imidazole, produced Soret ($\gamma$) bands at the same wavelength (418 nm). The $\alpha$ and $\beta$ bands also resembled each other\textsuperscript{16}. This indicates the heme iron contains histidine as the fifth ligand in the six coordinated CO derivative of MHA. These results coupled with those of Adams and Berman\textsuperscript{4} indicate that there is an alternative potential binding site, histidine, close to Tyr161 that is probably playing an important role in the fast binding step as well as for the formation of CO derivative.

Crystal structure of methemalbumin\textsuperscript{13} shows that His146 is the closest to heme, out of the sixteen histidine moieties, but is located diagonally opposite to Tyr161. This means, a rotation of heme along the vertical plane (plane normal to the heme plane) is necessary prior to switching to His146 (for the formation of CO complex), which may be sterically and energetically unfavourable. The only possibility seems to be a free flipping motion of propionyl and vinyl side chains, simultaneous with the iron binding to Tyr161 or His146, such that the side chains are oriented away from the binding site to meet the steric requirements. Crystal structure\textsuperscript{13} also reveals that the heme propionic acid residues form salt bridges with His146 and Lys190, which together with a series of hydrophobic interactions, enclose and secure the heme within the IB helical motif (helices 8-11, Fig. 1). Thus, the flipping of propionic side chain requires that their salt bridges have to be broken which is an energy consuming process. We assume that the breaking of salt bridges and flipping is driven in order to have large enthalpic and entropic gain when heme binds to Tyr161. This hypothesis has few supporting evidences from the crystal structure\textsuperscript{13}. Heme is located within the helical motif (Fig. 1), one end of which is partially blocked by helix 8, and the other is exposed to water. His146 is located right at the entrance of this hydrophobic region in helix 9. Therefore, the first stage of binding to His146 at the surface is followed by the internalization of heme through a translational motion to bind to Tyr161, where it is further stabilized by salt bridges, hydrogen
bonds and hydrophobic interactions. Binding to Tyr161 is accompanied by the flipping motions of heme side chain groups. We previously observed\textsuperscript{16} that the dithionite reduced form of heme in MHA resembles more to a four coordinated species. Further, heme-Tyr161 bond is weak, compared to heme-His146 bond due to higher electronegativity of oxygen (3.44) than nitrogen (3.04). Therefore, it appears that heme iron is four coordinated at the ferrous state Fe(II) and establishes a stronger fifth ligation to histidine, when CO binds.

Conclusion

Heme binding to HSA to form the methemalbumin (MHA) observed here is consistent with the two-stage process mechanism of Adams and Berman\textsuperscript{4}. A reversible fast binding to a surface residue with an equilibrium dissociation constant ($K_D$) of $(4.0 \pm 1.0) \times 10^{-5}$M precedes the slower irreversible binding to an interior residue, which is Tyr161 from crystal studies\textsuperscript{13}. The surface residue involved in the fast step is suggested to be His146. We also argue that heme switches its fifth ligation site from Tyr161 to His146 for the formation of carbon monoxide complex, possibly due to the requirement of having a stronger coordinate bond.

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