JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Optical Spectroscopic Exploration of Binding of Cochineal Red A with Two Homologous Serum Albumins

Priyanka Bolel, Niharendu Mahapatra, and Mintu Halder*

Department of Chemistry, Indian Institute of Technology Kharagpur, Kharagpur-721302, India

Supporting Information

ABSTRACT: Cochineal Red A is a negatively charged synthetic azo food colorant and a potential carcinogen. We present here the study of binding of Cochineal Red A with two homologous serum albumins, human (HSA) and bovine (BSA), in aqueous pH 7.4 buffer by optical spectroscopic techniques. Protein intrinsic fluorescence quenching by Cochineal Red A occurs through ground-state static interaction and its binding with BSA is stronger than with HSA. The magnitudes of thermodynamic parameters suggest that dye binding occurs principally via electrostatic complexation. Site-marker competitive binding shows that Cochineal Red A binds primarily to site I of serum albumins. Circular dichroic spectra indicate that dye binding results in some conformational modification of serum albumins. Increased ionic strength of the medium results in lowering of binding. This study provides an important insight into possible means of removal of dye toxicity.

KEYWORDS: *fluorescence quenching, serum protein, dye binding, site marker*

INTRODUCTION

Proteins play fundamental roles in sustaining life and are an integral part of origin, evolution, and metabolism. Serum albumin, as the most abundant protein constituent of blood plasma, facilitates the disposition and transportation of various endogenous and exogenous molecules (ligands) including fatty acids, steroids, and metal ions.[1] It has a key role in maintaining blood pH,[2] and it contributes to colloid osmotic blood pressure. Serum albumin often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cell in vivo and in vitro. The absorption, distribution, metabolism, and excretion properties, as well as the stability and toxicity, of dyes and drugs can be significantly affected as a result of their binding to serum albumins. Thus the investigation of these molecules with respect to binding with albumins becomes an important research field in chemistry, life sciences, and clinical medicine.[3,4] Bovine serum albumin (BSA) and human serum albumin (HSA; PDB ID 1AO6) are the major components in plasma protein for cows and humans, respectively. BSA is homologous to HSA, having about 88% sequence homology, and consists of three linearly arranged domains (I–III) that are composed of two subdomains (A and B).[5] There are two tryptophan residues (Trp134 and Trp213), of which Trp134 is located on the surface of the molecule and Trp213 resides in the hydrophobic pocket/fold. Similarly, the only tryptophan residue (Trp214) of HSA is also located in the hydrophobic pocket.[6] The environments of two tryptophan residues in BSA are different from each other, and thus the study of their interactions with small molecules can provide useful insights to understanding the environment-dependent molecular interactions. Moreover, there is evidence of conformational changes of serum albumin induced by its interaction with low molecular weight dyes and drugs, which appear to affect secondary and tertiary structures of proteins.[7]

Nowadays some azo, thiazine, triphenylmethane, and acridine dyes have been used extensively for medicinal purposes[8] and protein purification in protein–dye chromatography.[9] Synthetic azo dyes, with sulfonate groups in a benzene or naphthalene ring, are very often used in industries for coloring foods, cosmetics, and medicines. They are soluble in water and stable over a wide range of pH and temperature. Negative sulfonate groups of azo dyes are known to interact with basic side chain of amino acid residues of proteins through electrostatic force.[10,11] We are curious to know what happens with an azo dye having 2–3 sulfonate groups; we anticipate pronounced electrostatic binding. So we have chosen Cochineal Red A, a well-known synthetic food coloring agent with three negatively charged sulfonates, and two serum albumins. This dye can have a high tendency to be absorbed by the body via food while binding with plasma proteins. Cochineal Red A is also considered carcinogenic and is known to have the ability to enhance asthma. Naturally, the exploration of binding of Cochineal Red A with serum proteins will be important and it would also be interesting to know any possible mode to modulate the strength of interaction, which will have some therapeutic value. The molecular structure of Cochineal Red A is shown in Figure 1.

Fluorescence spectroscopy is a powerful technique to study the reactivity of chemical and biological systems since it allows nonintrusive measurements of substances in low concentration under physiological conditions. Because of its high sensitivity, rapidity, and simplicity, fluorescence technique has been widely used to study dye–protein interactions.[10,12] Here we have investigated the interaction of food dye Cochineal Red A with HSA and BSA at physiological pH 7.4 by means of optical spectroscopic techniques like UV–vis, fluorescence, and circular dichroism (CD) spectroscopy.

Received: December 19, 2011
Revised: March 5, 2012
Accepted: March 7, 2012
Published: March 7, 2012

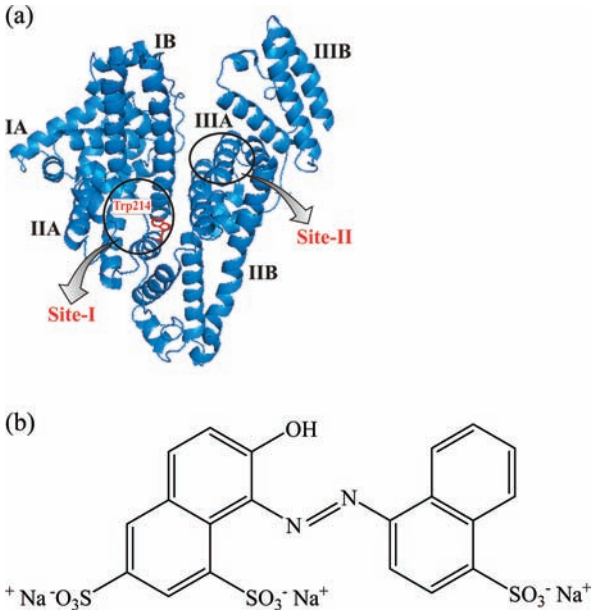

Figure 1. (a) Crystal structure of HSA (PDB ID 1AO6) and (b) molecular structure of Cochineal Red A.

MATERIALS AND METHODS

Materials. HSA (~99%, essentially fatty acid free) was purchased from Fluka. BSA (≥98%), Cochineal Red A (mol wt 604.46), and ibuprofen (≥98%) were obtained from Sigma–Aldrich. Warfarin and hemin were purchased from TCI-Japan and SRL, respectively. All experiments were carried out in 5 mM phosphate buffer, pH 7.4. NaCl (analytical grade, Merck) solution was used to prepare various ionic strengths of the medium, and all solutions were prepared in ultrapure water. The pH of solutions was measured with a Eutech pH 510 ion pH meter.

UV–Visible Absorbance Spectra. UV–vis absorbance spectra are recorded on a Shimadzu UV-1601 absorption spectrophotometer against solvent blank reference in the wavelength range of 400–650 nm. Experiments are performed by keeping the Cochineal Red A concentration constant at 30 μM, and protein is varied from 0 to 45 μM.

Fluorescence Measurements. All steady-state corrected fluorescence spectra were recorded on a Jobin Yvon-Spex Fluorolog-3 spectrofluorometer equipped with a thermostatic cell holder and a 1-cm path length quartz cuvette. Quenching experiments were performed by keeping the concentrations of HSA and BSA fixed at 2 μM and 1.5 μM, respectively, and quencher (Cochineal Red A) concentration was varied from 0 to 6.2 μM. The steady-state fluorescence experiments have been carried out at 293, 303, and 313 K. An excitation wavelength of 295 nm was used in all cases for selective excitation of the tryptophan (Trp) residues of HSA and BSA, and emission spectra are recorded from 310 to 470 nm.

The time-resolved fluorescence decays were measured on a time-correlated single-photon-counting (TCSPC) spectrometer (Horiba Jobin Yvon IBH). Diode laser (λ_{exc} = 295 nm) was used as the excitation source, and the emission was monitored at 349 nm (emission max) selected by an emission monochromator. For both proteins, concentration was kept fixed at 2 μM and the dye concentration was varied from 0 to 2 μM for HSA and from 0 to 3 μM for BSA. The qualities of the fits were assessed by the χ^2 values.

Circular Dichroic Spectra. Circular dichroic measurements were made on a Jasco-810 automatic recording spectropolarimeter with a 0.1-cm path length cell. The spectra were recorded in the range of 190–260 nm with a scan rate of 50 nm/min and a response time of 4 s. Three scans were accumulated for each spectrum. Appropriate baseline corrections in the CD spectra were made. Spectra were recorded as ellipticity (θ) in millidegrees. A 9 μM concentration of protein, at pH 7.4, was maintained in the experiments. Two sets of solutions were prepared with protein:Cochineal Red A molar ratios of 1:0 and 1:2.

Data Analysis. All data are represented as mean ± SD. Experiments were performed in triplicate. All graphical representations and statistical analyses were done with Origin.

RESULTS AND DISCUSSION

Absorption Spectroscopy. UV–vis absorption measurement is a simple but effective method of detecting complex formation. In the case of static quenching, a dark complex is formed between ground state of fluorescent substance and the quencher, and thereby the fluorescence quantum yield is reduced. Therefore, the absorption spectra of fluorophore would be affected[13] as a result of complexation. The λ_{max} of Cochineal Red A is 507 nm, and that has been red-shifted to 512 nm upon addition of protein. Also, an isosbestic point at 541 nm (Figure 2) is indicative of a ground-state

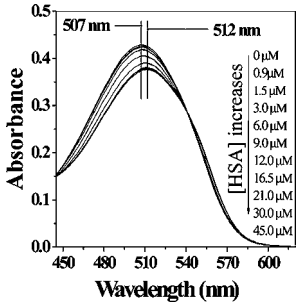

Figure 2. Absorption spectra of Cochineal Red A at pH 7.4 with increasing HSA concentration up to 45 μM at 298 K. The concentration of Cochineal Red A is 30 μM.

equilibrium system. Similar ground-state protein–dye interaction has also been observed with Cochineal Red A–BSA (data is not shown).

Fluorescence Studies. *Steady-State Fluorescence Quenching.* Fluorescence quenching refers to a process that decreases the fluorescence intensity of any fluorescent sample and it occurs due to a variety of molecular interactions such as excited-state reactions, energy transfer, ground-state complex formation, and collisional quenching.[14] The intrinsic fluorescence intensities of tryptophan residues of HSA and BSA are found to decrease with increasing Cochineal Red A concentration (see Figure S1 in Supporting Information).

Fluorescence quenching data are then analyzed by use of the Stern–Volmer equation:

$$\frac{F^0}{F} = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (1)$$

where F^0 and F are the fluorescence intensities of the fluorophore in the absence and in the presence of quencher, respectively, and [Q] is quencher concentration. k_q is the bimolecular quenching constant; τ_0 is the lifetime of the fluorophore in the absence of quencher. The Stern–Volmer (S–V) quenching constant K_{SV} is equal to $k_q\tau_0$. All fluorescence spectra are corrected for the absorption of quencher at the excitation and emission wavelengths of the fluorophore. Optical absorption of the quencher in the excitation region decreases the effective intensity of the exciting light beam, and its absorption in the emission region decreases the measured fluorescence intensity (the inner filter effect). Since the absorption increases with increasing quencher concentration, this induces an apparent quenching and increases the real magnitude of the

dx.doi.org/10.1021/jf205219w | J. Agric. Food Chem. 2012, 60, 3727–3734

quenching constants obtained from steady-state experiments. The correction factor η is given by[15]

$$\eta = \frac{A_{x_0}A_{y_0}(1 - 10^{-A_{x_i}})(1 - 10^{-A_{y_i}})}{A_{x_i}A_{y_i}(1 - 10^{-A_{x_0}})(1 - 10^{-A_{y_0}})} \quad (2)$$

where A_{x_0} and A_{y_0} are the fluorophore absorbances and $A_{x_i} = A_{x_0} + \Delta A_{x_i}$ and $A_{y_i} = A_{y_0} + \Delta A_{y_i}$ are total absorbances of fluorophore and quencher (ΔA_{x_i} and ΔA_{y_i}) at the excitation and emission wavelengths, respectively. The corrected Stern–Volmer (S–V) equation should be

$$\frac{F^0}{F} = \left(\frac{F^0}{F}\right)_{obs} \eta = 1 + K_{SV}[Q] \quad (3)$$

where $(F^0/F)_{obs}$ is the experimentally observed data, which when multiplied by η gives the corrected fluorescence ratio F^0/F. All the corrected S–V plots are connected by smooth lines or curves.

From Figure 3 it is to note that two homologous serum albumins, HSA and BSA, exhibit different quenching behavior.

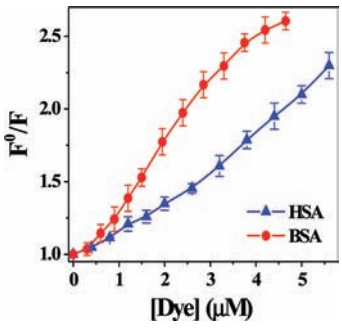

Figure 3. Stern–Volmer plot for Cochineal Red A–HSA and Cochineal Red A–BSA systems in 5 mM phosphate buffer of pH 7.4 at 298 K. Symbols with cap include error bar.

In the case of HSA upward curvature is observed, whereas for BSA the curve starts with an initial upward curvature followed by bending downward at higher quencher concentrations.

In general, the observed positive deviation from linearity of the S–V plot indicates the possibility of a combined quenching (static and dynamic) process, the existence of more than one binding site, or the large extent of quenching.[16] The possibility of a combined quenching process is analyzed by use of eqs 4 and 5:

$$\frac{F^0}{F} = (1 + K_D[Q])(1 + K_S[Q]) \quad (4)$$

Multiplication of the terms in parentheses gives $F^0/F = 1 + K_{app}[Q]$, where

$$K_{app} = \left[\frac{F^0}{F} - 1\right]\frac{1}{[Q]} = (K_D + K_S) + K_DK_S[Q] \quad (5)$$

where K_{app}, K_D, K_S, and $[Q]$ are the apparent, dynamic, and static quenching constants and the quencher concentration, respectively. With knowledge of the static quenching constant from the Stern–Volmer plot, the value of the dynamic quenching constant can be obtained. A graph plotted as K_{app} versus $[Q]$ to find the static and dynamic quenching constants results in imaginary numbers. This indicates the absence of a combined static/dynamic quenching mechanism. Thus, the upward curvature for HSA is most likely due to large extent of quenching and/or involvement of more than one binding site. Estimation of linear K_{SV} values is made by considering only the initial linear portion (initial 3–4 data points) of the S–V plot. The K_{SV} value is $(1.52 \pm 0.03) \times 10^5$ L·mol^{-1} for HSA at 298 K. But for BSA, two different slopes (two K_{SV} values) are found, corresponding to lower and higher quencher concentration range, respectively (Figure 3). The K_{SV} calculated for the first phase [$K_{SV1} = (3.56 \pm 0.24) \times 10^5$ L·mol^{-1}] is 5-fold higher than that for the second phase [$K_{SV2} = (7.22 \pm 0.09) \times 10^4$ L·mol^{-1}]. Similar biphasic dependence has been reported in the literature, where the intrinsic tryptophan fluorescence of BSA is quenched by iodide,[17] and there the difference in accessibility of the two tryptophan residues toward quenching is ascribed to be the reason. Similarly, in our case it may be argued that the two tryptophan residues are not equally accessible to Cochineal Red A. One of them is less available for quenching than the other, which results in the biphasic nature with almost a near-saturation trend in the latter part of the Stern–Volmer plot.

The bimolecular quenching constants (K_q) obtained from the equation $K_{SV} = K_q\tau_0$, where τ_0 is the lifetime of the fluorophore (protein) in the absence of quencher, are on the order of 10^{13} for both the Cochineal Red A–protein systems [$(5.81 \pm 0.39) \times 10^{13}$ and $(2.38 \pm 0.04) \times 10^{13}$ L·mol^{-1}·s^{-1} for BSA and HSA, respectively], which is well above the diffusion limit (10^{10} L·mol^{-1}·s^{-1}) at the experimental conditions,[16] indicating that the binding is primarily static in nature.

For dye–protein interaction, the binding constant (K_b) is calculated by use of eq 6,[18] where n is the number of binding sites:

$$\log\left(\frac{F^0 - F}{F}\right) = \log K_b + n \log [Q] \quad (6)$$

Competitive Binding Studies. The site marker competitive binding experiments are carried out with a drug that specifically binds to a known site or region of serum albumins to locate the precise binding site of Cochineal Red A. HSA and BSA are composed of three homologous domains named I, II, and III, and each domain includes two subdomains called A and B as mentioned earlier.[5] As reported in the literature, warfarin has been demonstrated to bind to subdomain IIA (site I), while ibuprofen and hemin are considered as subdomain IIIA (site II) and IB binders, respectively.[19,20] By monitoring the changes in the binding constant of Cochineal Red A to HSA and BSA brought about in the presence of site markers, information about the specific binding site in the proteins can be obtained. In these experiments, dye concentration is increased in the solution containing a 1:1 complex of protein and site markers. With the addition of warfarin into protein solution, the maximum emission wavelength of proteins has a red shift (data not shown), and the fluorescence intensity has been significantly reduced. Now with the addition of dye, the fluorescence intensity of the protein solution containing warfarin (1:1 with serum albumin) decreases gradually but the extent of quenching is much lower than that without warfarin (see Figure S2 in Supporting Information), indicating that the binding of Cochineal Red A to protein is affected in presence of warfarin. But when ibuprofen is present, quenching of the protein–ibuprofen 1:1 complex by Cochineal Red A is not affected significantly. The binding constants (K_b) at pH 7.4 are $(8.4 \pm 0.40) \times 10^5$ and $(4.6 \pm 0.09) \times 10^6$ for Cochineal Red A–HSA and –BSA, respectively, at 298 K. The binding constant of Cochineal Red A in the presence of warfarin and ibuprofen are found to be $(5.42 \pm 0.10) \times 10^5$ and $(8.5 \pm 0.16) \times 10^5$, respectively, for HSA

dx.doi.org/10.1021/jf205219w | J. Agric. Food Chem. 2012, 60, 3727–3734

and $(2.92 \pm 0.12) \times 10^6$ and $(4.09 \pm 0.14) \times 10^6$, respectively, for BSA. The decrease in binding constant in the presence of warfarin implies that the dye competes with warfarin while binding to the same site of protein. But the magnitude of the binding constants for dye−protein complex are almost the same in the presence and absence of ibuprofen, which indicates that site II marker ibuprofen does not compete with Cochineal Red A at its binding site. These results suggest that Cochineal Red A binds preferentially to site I (subdomain IIA) for both serum albumins, where warfarin binds. Control experiments have also been performed for both warfarin and ibuprofen in the absence of proteins, and it is found that Cochineal Red A does not quench site marker fluorescence. We have also studied the effect of subdomain IB binder hemin for both proteins at pH 7.4. The partially solvent-exposed Trp134 is located in the subdomain IB of BSA. For BSA, the binding constant (K_b) is decreased from $(4.6 \pm 0.09) \times 10^6$ to $(3.5 \pm 0.12) \times 10^6$ in the presence of hemin. This observation suggests that Cochineal Red A also binds in subdomain IB. So the two environmentally dissimilar tryptophans[6] in BSA both undergo interaction with Cochineal Red A, but their accessibility for quenching is different. This is also evident from the binding constants obtained for the first (K_{b1}) and second (K_{b2}) phases of S−V plot of Cochineal Red A−BSA system employing eq 6. The calculated binding constants are $K_{b1} = (4.6 \pm 0.09) \times 10^6$, from initial portion, and $K_{b2} = (3.03 \pm 0.06) \times 10^4$, from the later portion at higher dye concentrations, of the plot. On the other hand, in the case of hemin−HSA (1:1) complex, the binding constant for Cochineal Red A does not change significantly; that is, Cochineal Red A does not seem to bind to subdomain IB. Thus it is clear that Cochineal Red A binds only to subdomain IIA of HSA.

Time-Resolved Measurements. Lifetime measurements have also been carried out to get more insight into the quenching of dye−protein systems. Time-resolved data reveal that for both BSA and HSA the fluorescence decay profiles (Figure 4) are biexponential. For BSA the longer lifetime component is assigned to water-exposed Trp134 and the other component is due to buried Trp213.[21] Although HSA has only one tryptophan residue, it shows biexponential decay. The heterogeneity in the fluorescence lifetime of tryptophan residue has also been ascribed to the position of various other amino acid residues relative to tryptophan, that is, the local environment of tryptophan residue in the protein matrix.[22]

Both the decay constants are found to decrease with Cochineal Red A concentration for BSA and HSA (see Table 1).

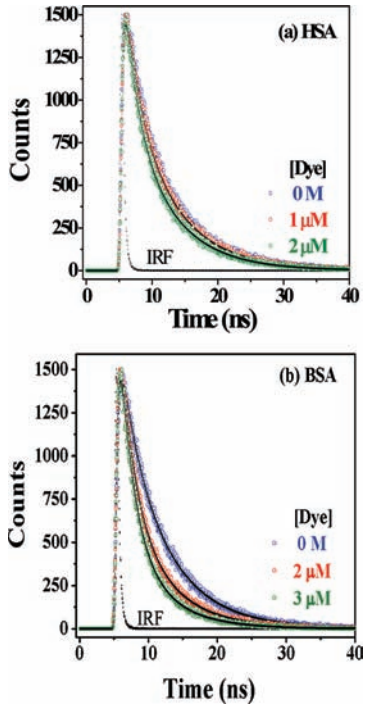

Figure 4. Fluorescence decay profiles of HSA and BSA in the absence and presence of Cochineal Red A in 5 mM phosphate buffer of pH 7.4 at 298 K. λ_{ex} = 295 nm and λ_{em} = 349 nm; [HSA] = [BSA] = 2 μM.

Table 1. Lifetimes of Fluorescence Decay of HSA and BSA at Different Concentrations of Cochineal Red A[a]

	lifetime (ns)		amplitude		
dye (μM)	τ_1	τ_2	A_1 (%)	A_2 (%)	χ^2
	HSA, 2.0 μM				
0	2.3	7.0	13	87	1.08
1.0	2.2	6.9	14	86	1.09
2.0	1.9	6.7	19	81	1.06
	BSA, 2.0 μM				
0	2.4	6.5	9	91	1.03
2.0	2.1	6.1	30	70	1.05
3.0	1.9	5.6	42	58	1.10

[a]In 5 mM phosphate buffer, pH 7.4, at 298 K.

Competitive ligand binding studies have also shown that Cochineal Red A binds to both subdomains IIA and IB near Trp213 and Trp134, respectively, of BSA, and as a result of this, τ_1 and τ_2 can be affected. The decrease of decay constants of HSA can be ascribed to the interaction of Cochineal Red A near the tryptophan residue and the extent of decrease is less than that of BSA, indicating weaker interaction between HSA and Cochineal Red A compared to Cochineal Red A−BSA system.

Thermodynamic Analysis. The quenching experiments were carried out at three different temperatures (293, 303, and 313 K) for both proteins. Within this temperature range, the conformation of BSA and HSA is known to remain unaltered.[23] Corresponding binding constants (K_b) and number of binding sites (n) were calculated by use of eq 6.[18] It has been found that there is a decrease in the binding constant with rising temperature (Figure 5) which indicates that the Cochineal Red A−protein complex dissociates with temperature. This is again indicative of a static quenching mechanism. The binding constant (K_b) for BSA ($\sim 10^6$) is 1 order of magnitude higher than that for HSA ($\sim 10^5$), indicating the stronger binding of BSA to Cochineal Red A than that of HSA. The values of n are found to be 1.2 and 1.1 at 298 K for BSA and HSA, respectively (see Table 2).

Signs and magnitudes of the thermodynamic parameters indicate the nature of forces actually taking part in the dye−protein interaction. The following two thermodynamic equations are used in this case:

$$\ln K_b = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad (7)$$

and

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (8)$$

Equations 7 and 8 are called the van't Hoff equation and Gibbs−Helmholtz equation, respectively. ΔH^0 and ΔS^0 are obtained from the slope and intercept of the plot of ln K_b versus

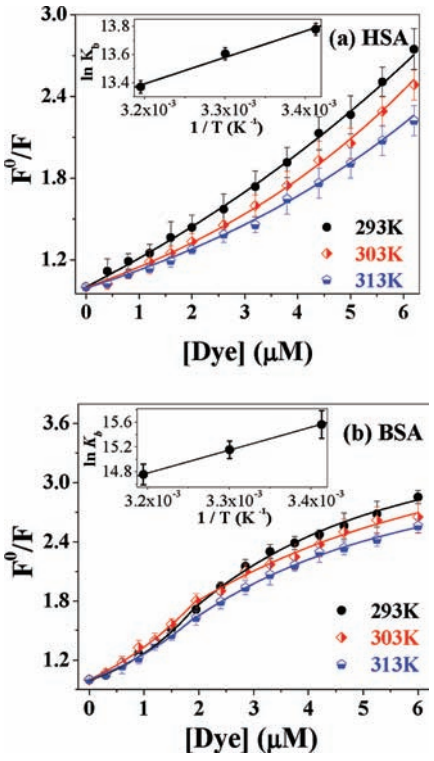

Figure 5. Temperature variation studies for Cochineal Red A–HSA and –BSA systems in 5 mM phosphate buffer of pH 7.4. Insets show the corresponding ln K_b vs $1/T$ plot. Symbols with cap include error bar.

$1/T$, respectively. The magnitude of ΔG^0 at a temperature is obtained from eq 8 after the values of ΔH^0 and ΔS^0 are plugged in.

Ross and Subramanian[24] have summed up the relevant thermodynamic parameters, such as ΔH^0 and ΔS^0, that can be used to determine the types of binding associated with various interactions as follows. If $\Delta H^0 < 0$ and $\Delta S^0 < 0$, van der Waals interactions and hydrogen bonds play major roles in the binding reaction. If $\Delta H^0 > 0$ and $\Delta S^0 > 0$, hydrophobic interactions are dominant. Electrostatic forces are more important when $\Delta H^0 < 0$ and $\Delta S^0 > 0$. Slight negative value of ΔH^0 and positive ΔS^0 for both the Cochineal Red A–HSA and Cochineal Red A–BSA systems (see Table 3) indicate that the interaction is largely driven by electrostatic attractions. Negative value of ΔG^0 indicates the spontaneity of binding interaction. The higher ΔG^0 for Cochineal Red A–BSA indicates stronger binding than for Cochineal Red A–HSA.

Effect of Ionic Strength. A closer examination of the results presented in the preceding section indicates that serum albumin fluorescence undergoes static quenching by Cochineal Red A in buffered aqueous solution, and the sign and magnitude of thermodynamic parameters point to electrostatic interactions. To further confirm that the interaction is electrostatic in origin, we have carried out binding experiments in media of varying ionic strengths. The increase in ionic strength should not in principle affect the strength of hydrophobic interaction, but electrostatic attraction should be disfavored.[25] To prepare different ionic strengths, concentration of NaCl has been varied up to 0.2 M in the buffer solutions. Figure 6 indicates that the extent of quenching decreases significantly with increasing ionic strength, and it is also reflected in the decrease of binding constant (Table 2). The characteristic quenching patterns (upward-only for HSA and biphasic for BSA) indeed disappear at highest studied ionic strength (0.2 M) with a little residual quenching. This is a clear indication that electrostatic force is primarily responsible for binding of Cochineal Red A with HSA and BSA. Three sulfonate groups in Cochineal Red A attract the positively charged side chains of lysine (Lys; $pK_a = 10.4$) and arginine (Arg; $pK_a = 12$) residues present in the surrounding of the binding site, as expected, and this interaction becomes weaker with increasing ionic strength of the medium. So the variation of salt concentration (ionic strength) can strongly affect the extent of dye–protein binding. So it

Table 2. Determination of Binding Constants and Number of Binding Sites of Cochineal Red A–HSA and –BSA Systems with Increasing NaCl Concentration[a]

	HSA			BSA		
NaCl (M)	K_b[b]	n	R[c]	K_b[b]	n	R[c]
0	$(8.40 \pm 0.40) \times 10^5$	1.10 ± 0.02	0.9959	$(4.60 \pm 0.09) \times 10^6$	1.20 ± 0.02	0.9824
0.06	$(5.97 \pm 0.32) \times 10^4$	0.98 ± 0.01	0.9911	$(2.40 \pm 0.14) \times 10^5$	0.99 ± 0.05	0.9896
0.2	$(9.02 \pm 0.69) \times 10^3$	0.91 ± 0.01	0.9728	$(3.30 \pm 0.16) \times 10^4$	0.97 ± 0.09	0.6884

[a]Data are represented as mean ± SD of experiments performed in triplicate in 5 mM phosphate buffer, pH 7.4, at 298 K. [b]Concentrations of dye and proteins are expressed as moles per liter. [c]R is the correlation coefficient for K_b values.

Table 3. Binding and Thermodynamic Parameters for the Complexation of Cochineal Red-A with HSA and BSA in 5 mM Phosphate Buffer of pH 7.4 at Different Temperatures[a]

temp (K)	K_b[b]	n	R[c]	ΔG^0 ($kJ \cdot mol^{-1}$)	ΔH^0 ($kJ \cdot mol^{-1}$)	ΔS^0 ($J \cdot mol^{-1} \cdot K^{-1}$)	R[d]
HSA							
293	$(9.65 \pm 0.13) \times 10^5$	1.10 ± 0.03	0.9941	-33.58 ± 0.03			
303	$(8.11 \pm 0.35) \times 10^5$	1.11 ± 0.01	0.9915	-34.20 ± 0.03	-15.54 ± 0.23	61.62 ± 0.91	0.9969
313	$(6.41 \pm 0.09) \times 10^5$	1.10 ± 0.01	0.9899	-34.82 ± 0.04			
BSA							
293	$(5.74 \pm 0.26) \times 10^6$	1.21 ± 0.02	0.9919	-37.91 ± 0.62			
303	$(3.81 \pm 0.24) \times 10^6$	1.18 ± 0.02	0.9816	-38.16 ± 0.12	-30.73 ± 0.52	24.52 ± 1.41	0.9975
313	$(2.56 \pm 0.10) \times 10^6$	1.17 ± 0.02	0.9855	-38.40 ± 0.11			

[a]Data are represented as *mean ± S.D.* of triplicately performed experiments. [b]Concentrations of dye and proteins are expressed as mol lit^{-1}. [c]R is the correlation coefficient for K_b values. [d]R is the correlation coefficient for van't Hoff plots.

dx.doi.org/10.1021/jf205219w | J. Agric. Food Chem. 2012, 60, 3727–3734

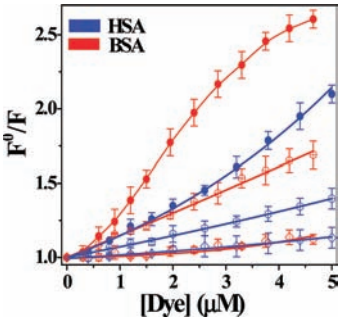

Figure 6. Quenching plot of Cochineal Red A–HSA and –BSA systems in the presence of (solid circles) 0 M, (open circles) 0.06 M, and (open tilted squares) 0.2 M NaCl at pH 7.4 and 298 K. Symbols with cap include error bar.

is important to note that higher salinity should reduce the possibility of absorption of Cochineal Red A by plasma proteins.

We have also carried out ionic strength variation in the quenching of BSA fluorescence by iodide—a system where quenching in known to occur via dynamic mechanism.[26] It is found that there is hardly any effect of ionic strength on the extent of quenching (data not shown). So like hydrophobic interaction, a dynamic mode of quenching is insensitive to ionic strength. Thus the possibility of a dynamic mode of quenching in Cochineal Red A–serum protein system can be ruled out.

Urea Denaturation Study. Urea acts by increasing the aqueous solubility around the hydrophobic domains of protein and/or by weakening the hydrogen bonds in the 3D protein structure leading to unfolding. In the presence of urea (4 M) it is found that quenching is greatly reduced (see Figure S3 in Supporting Information) due to a consequent unfolding of the protein and resulting water exposure of Trp residues. Since water-exposed tryptophan residues have lower F^0, this can lead to reduction of F^0/F. Also for water-exposed amino acid residues in the denatured protein, the dye seems to have considerably reduced interaction. Hence the extent of quenching and binding decreases for both proteins in the presence of 4 M urea.

Effect of Ethanol Addition. Ethanol affects binding of exogenous molecules in subdomain IIA[27] that is the warfarin binding site. With increasing ethanol content (up to 10% v/v), fluorescence quenching is drastically reduced (see Figure S4 in Supporting Information) and corresponding binding constants (K_b) are also reduced significantly, from $(8.40 \pm 0.40) \times 10^5$ to $(2.56 \pm 0.12) \times 10^5$ for HSA and from $(4.60 \pm 0.09) \times 10^6$ to $(3.4 \pm 0.16) \times 10^5$ for BSA. This further confirms that the dye must have bound near Trp214 and Trp213 of HSA and BSA, respectively; that is, site I.

Energy Transfer between Cochineal Red A and Serum Albumin Proteins. The nonradiation energy transfer theory has been introduced to study the distance between amino acid residues in BSA and quencher. According to Förster nonradiation energy transfer theory,[16] the energy transfer efficiency E is related not only to the distance (r) between donor and acceptor but also to the critical energy transfer distance (R_0):

$$E = 1 - \frac{F}{F^0} = \frac{{R_0}^6}{{R_0}^6 + r^6} \tag{9}$$

R_0 is defined as the critical distance at 50% energy transfer efficiency and is given by

$${R_0}^6 = (8.8 \times 10^{-25})\kappa^2 N^{-4} \phi J \tag{10}$$

where κ^2 is the spatial orientation factor. N is the refractive index of medium and φ is the fluorescence quantum yield of the donor in the absence of acceptor. J is the spectrum overlap integral of the donor fluorescence emission spectrum and the acceptor absorption spectrum and is given by

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{11}$$

where $F(\lambda)$ is the fluorescence intensity of donor at wavelength λ. $\varepsilon(\lambda)$ is the molar absorption coefficient of acceptor at the same wavelength λ. It is reported elsewhere that generally for proteins $N = 1.4$[28] and $\kappa^2 = {}^2/_3$ and $\varphi = 0.15$.[18] Now by plugging in these numbers in eqs 9, 10, and 11, J, E, R_0 and r for both the Cochineal Red A–protein systems are calculated to be 6.41×10^{-15} cm^3·M^{-1}, 0.65 nm, 2.29 nm, and 2.07 nm, respectively, for HSA and, 7.83×10^{-15} cm^3·M^{-1}, 0.65 nm, 2.38 nm, and 2.14 nm, respectively, for BSA. The donor-to-acceptor distance lies within the range of $(0.5–2)R_0$,[16] indicative of an efficient energy transfer from serum albumins to Cochineal Red A.

Conformational Investigation: Circular Dichroism Spectroscopy. Circular dichroism (CD) spectroscopy is a sensitive technique to monitor the secondary structural change of protein upon interaction with ligands. The CD spectra of BSA and HSA in the absence and presence of Cochineal Red A are shown in Figure 7. Both proteins exhibit two negative bands

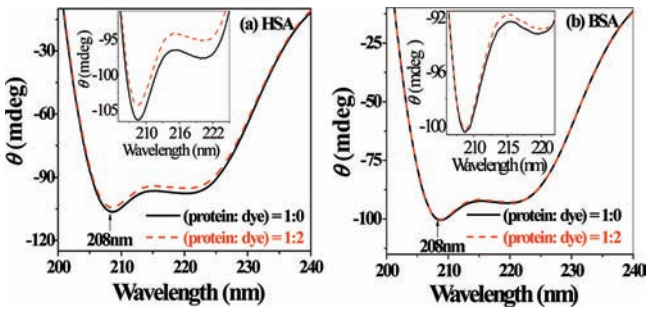

Figure 7. CD spectra of HSA and BSA free (solid line) and complexed (dotted line) with Cochineal Red A ([proteins]:[dye] = 1:2) in 5 mM phosphate buffer, pH 7.4, at 298 K. (Insets) Corresponding expanded wavelength region of CD spectra within 205–225 nm for HSA and 206–222 nm for BSA.

at 208 and 222 nm in the UV region due to the n → π^* transition for the peptide bond of α-helix.[4] The CD spectra of proteins in presence and absence of Cochineal Red A are similar in shape, indicating that their structure after dye binding is also predominantly α-helical.[12] Results obtained are analyzed in terms of mean residue ellipticity (MRE, deg·cm^2·dmol^{-1}) according to the following equation:[29]

$$\text{MRE} = \frac{\theta_{obs}}{10nlc_p} \tag{12}$$

where θ_{obs} is the observed ellipticity in millidegrees at 208 nm, n is the number of amino acid residues, l is the path length of the cell, and c_p is the molar concentration of the protein. The values of n are taken as 583 and 585 for BSA and HSA, respectively.[19] The α-helical content is then calculated from the MRE values at 208 nm:

$$\%\ \alpha\text{-helix} = \left\{\frac{(-\text{MRE}_{208} - 4000)}{33000 - 4000}\right\} \times 100 \tag{13}$$

dx.doi.org/10.1021/jf205219w | J. Agric. Food Chem. 2012, 60, 3727–3734

where MRE_{208} is the experimental MRE value of proteins at 208 nm, 4000 is the MRE value of the β form and random coil conformation at 208 nm, and 33 000 is the MRE value of a pure α-helix at 208 nm.

The observed α-helix contents of free HSA and BSA are 55.6% and 51.7%, respectively, in agreement with reported results.[29,30] Figure 7 indicates the decrease of α-helix percentage of proteins with increasing dye concentration. The helical content of are found to be 54.3% and 51.5% for HSA and BSA, respectively, at a protein:Cochineal Red A molar ratio of 1:2. From these results, it is clear that interaction of Cochineal Red A with HSA and BSA causes a conformational change of the protein with little loss of helical content.

In this article the binding of Cochineal Red A to serum albumins, HSA and BSA, at pH 7.4 has been investigated by employing different optical spectroscopic techniques. The nature of quenching curve differs with respect to the serum protein used. For HSA upward curvature is found, whereas for BSA near-saturation behavior was observed at a higher dye concentration after initial quenching. Difference in accessibility of two tryptophans (Trp 213 and Trp134) for quenching by Cochineal Red A is found to be responsible for such biphasic binding nature for the Cochineal Red A−BSA system. For HSA, the large extent of Trp214 fluorescence quenching results in the upward curvature. So the presence of an extra tryptophan residue (Trp134) in BSA as compared to HSA makes the difference in their quenching behavior. The dye binds more strongly with BSA than with HSA, which could be due to the presence of more basic amino acid side chains near the dye binding site of the former. The effect of ethanol variation on the extent of fluorescence quenching also points out that the primary binding site of Cochineal Red A is in subdomain IIA for both HSA and BSA. Absorption spectroscopy and the magnitude of bimolecular quenching constant indicate that the primary mode of binding is static. CD spectra also provides a clear indication of interaction of Cochineal Red A with serum albumins. The obtained thermodynamic parameters, that is, slight negative ΔH^0 and positive ΔS^0 values, suggest that this static quenching is electrostatic in origin, and ionic strength variation confirms that electrostatic force of attraction between protein and negatively charged dye has the lion's share in stabilizing the Cochineal Red A−protein complex. Thus the strength of dye binding is sensitive to ionic strength of the medium and the affinity of dye to proteins is decreased with increasing added salt concentration. So it may be concluded that the absorption of similar sulfonated azo food dyes by protein can, in principle, be modulated by varying the salt concentration, and this property is also useful to purify protein from such dyes by leaching with salts. This will have a tremendous therapeutic importance in that toxicity of these food dyes could be removed by increasing the salinity of the medium. Thus the present study of binding of Cochineal Red A with HSA and BSA provides a very useful insight toward characterization of dye−protein binding and its possible implication.

■ ASSOCIATED CONTENT

Supporting Information

Five figures showing emission spectra of HSA and BSA with increasing concentration of Cochineal Red A, effect of selected site markers on Cochineal Red A−HSA and −BSA systems, quenching plot for Cochineal Red A−HSA and −BSA systems in the presence of urea, effect of increasing amount of ethanol on the extent of fluorescence quenching for Cochineal Red A−HSA and −BSA systems, and spectral overlap of UV−vis absorption spectrum of Cochineal Red A with fluorescence spectra of HSA and BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail mintu@chem.iitkgp.ernet.in; phone +91-3222-283314; fax +91-3222-282252.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank DST-India (Fund SR/FTP/CS-97/2006), CSIR-India (Fund 01/(2177)/07 EMR-II, dated 24/10/2007), and IIT-Kharagpur (ISIRD-EEM grant) for financial support. P.B. thanks UGC-India for a fellowship. We thank Professor D. N. Nath of IACS, Kolkata, India, for single-photon counting measurements. P.B. thanks Mr. S. Datta for help in various instances during experiments.

■ ABBREVIATIONS USED

Trp, tryptophan; Lys, lysine; Arg, arginine; HSA, human serum albumin; BSA, bovine serum albumin; S−V, Stern−Volmer

■ REFERENCES

(1) Kumar, C. V.; Buranaprapuk, A. Site-specific photocleavage of proteins. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2085−2087.

(2) Colmenarejo, G. In silico prediction of drug-binding strengths to human serum albumin. *Med. Res. Rev.* **2003**, *23*, 275−301.

(3) Silva, D.; Cortez, C. M.; Louro, S. R. W. Chlorpromazine interactions to sera albumins - A study by the quenching of fluorescence. *Spectrochim. Acta, Part A: Mol. Biomol. Spectrosc.* **2004**, *60*, 1215−1223.

(4) Kamat, B. P.; Seetharamappa, J. In vitro study on the interaction of mechanism of tricyclic compounds with bovine serum albumin. *J. Pharm. Biomed. Anal.* **2004**, *35*, 655−664.

(5) Carter, D. C.; Ho, J. X. Structure of serum-albumin. *Adv. Protein Chem.* **1994**, *45*, 153−203.

(6) Peters, T. J. Serum albumin. *Adv. Protein Chem.* **1985**, *37*, 161−245.

(7) Hushcha, T. O.; Luik, A. I.; Naboka, Y. N. Conformation changes of albumin in its interaction with physiologically active compounds as studied by quasi-elastic light scattering spectroscopy and ultrasonic method. *Talanta* **2000**, *53*, 29−34.

(8) Kamat, B. P.; Seetharamappa, J. Fluorescence and circular dichroism studies on the interaction of bromocresol purple with bovine serum albumin. *Pol. J. Chem.* **2004**, *78*, 723−732.

(9) Burke, N. S.; Crawford, D. L. Use of azo dye ligand chromatography for the partial purification of a novel extracellular peroxidase from *Streptomyces viridosporus* T7A. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 523−530.

(10) Patel, A. B.; Srivastava, S.; Phadke, R. S. Interaction of 7-hydroxy-8-(phenylazo)-1,3-naphthalenedisulfonate with bovine plasma albumin: Spectroscopic studies. *J. Biol. Chem.* **1999**, *274*, 21755−21762.

(11) Sereikaite, J.; Bumelis, V. A. Interaction of serum albumin with vinyl sulfonate azo dye. *Cent. Eur. J. Chem.* **2008**, *6*, 509−512.

(12) Zhang, Y. Z.; Xiang, X.; Mei, P.; Dai, J.; Zhang, L. L.; Liu, Y. Spectroscopic studies on the interaction of Congo Red with bovine serum albumin. *Spectrochim. Acta, Part A: Mol. Biomol. Spectrosc.* **2009**, *72*, 907−914.

(13) Zhang, X. W.; Zhao, F. L.; Li, K. A. Studies on the reaction between ciprofloxacin and bovine serum albumin. *Chem. J. Chin. Univ.* **1999**, *20*, 1063−1067.

(14) Hu, Y. J.; Liu, Y.; Zhang, L. X.; Zhao, R. M.; Qu, S. S. Studies of interaction between colchicine and bovine serum albumin by fluorescence quenching method. *J. Mol. Struct.* **2005**, *750*, 174−178.
(15) Borissevitch, I. E. More about the inner filter effect: corrections of Stern-Volmer fluorescence quenching constants are necessary at very low optical absorption of the quencher. *J. Lumin.* **1999**, *81*, 219−224.
(16) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer: New York, 2006.
(17) Moller, M.; Denicola, A. Protein tryptophan accessibility studied by fluorescence quenching. *Biochem. Mol. Biol. Educ.* **2002**, *30*, 175−178.
(18) Feng, X. Z.; Lin, Z.; Yang, L. J.; Wang, C.; Bai, C. L. Investigation of the interaction between acridine orange and bovine serum albumin. *Talanta* **1998**, *47*, 1223−1229.
(19) Ni, Y.; Su, S.; Kokot, S. Spectrofluorimetric studies on the binding of salicylic acid to bovine serum albumin using warfarin and ibuprofen as site markers with the aid of parallel factor analysis. *Anal. Chim. Acta* **2006**, *580*, 206−215.
(20) Zunszain, P. A.; Ghuman, J.; Komatsu, T.; Tsuchida, E.; Curry, S. Crystal structural analysis of human serum albumin complexed with hemin and fatty acid. *BMC Struct. Biol.* **2003**, *3*, 6−14.
(21) Suresh, D. V.; Mahesha, H. G.; Rao, A. G. A.; Srinivasan, K. Binding of bioactive phytochemical piperine with human serum albumin: A spectrofluorometric study. *Biopolymers* **2007**, *86*, 265−275.
(22) Siemiarczuk, A.; Petersen, C. E.; Ha, C. E.; Yang, J. S.; Bhagavan, N. V. Analysis of tryptophan fluorescence lifetimes in a series of human serum albumin mutants with substitutions in subdomain IIA. *Cell Biochem. Biophys.* **2004**, *40*, 115−122.
(23) Rezaei-Tavirani, M.; Moghaddamnia, S. H.; Ranjbar, B.; Amani, M.; Marashi, S. A. Conformational study of human serum albumin in pre-denaturation temperatures by differential scanning calorimetry, circular dichroism and UV spectroscopy. *J. Biochem. Mol. Biol.* **2006**, *39*, 530−536.
(24) Ross, P. D.; Subramanian, S. Thermodynamics of protein association reactions: forces contributing to stability. *Biochemistry* **1981**, *20*, 3096−3102.
(25) Liang, J. G.; Cheng, Y. P.; Han, H. Y. Study on the interaction between bovine serum albumin and CdTe quantum dots with spectroscopic techniques. *J. Mol. Struct.* **2008**, *892*, 116−120.
(26) Maruthamuthu, M.; Selvakumar, G. Selective quenching of tryptophanyl fluorescence in bovine serum-albumin by the iodide ion. *Proc. Indian Acad. Sci. Chem. Sci.* **1995**, *107*, 79−86.
(27) Michnik, A.; Drzazga, Z. Effect of ethanol on the thermal stability of human serum albumin. *J. Therm. Anal. Calor.* **2007**, *88*, 449−454.
(28) Sahoo, D.; Bhattacharya, P.; Chakravorti, S. Reverse micelle induced flipping of binding site and efficiency of albumin protein with an ionic styryl dye. *J. Phys. Chem. B* **2010**, *114*, 10442−10450.
(29) Varlan, A.; Hillebrand, M. Spectral study of coumarin-3-carboxylic acid interaction with human and bovine serum albumins. *Cent. Eur. J. Chem.* **2011**, *9*, 624−634.
(30) Li, Y.; He, W.-Y.; Tian, J.; Tang, J.; Hu, Z.; Chen, X. The effect of Berberine on the secondary structure of human serum albumin. *J. Mol. Struct.* **2005**, *743*, 79−84.