JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Modulation of Accessibility of Subdomain IB in the pH-Dependent Interaction of Bovine Serum Albumin with Cochineal Red A: A Combined View from Spectroscopy and Docking Simulations[#]

Priyanka Bolel, Niharendu Mahapatra, Shubhashis Datta, and Mintu Halder*

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

Supporting Information

ABSTRACT: Our recent report on the binding of Cochineal Red A, a food dye, with HSA and BSA at pH 7.4 has revealed that electrostatic forces is the principal cause of interaction. In that study issues relating to complications arising out of modulation of dye binding affinity of BSA with pH had not been explored. Here we have further explored the interaction of Cochineal Red A with BSA in pH range 4.8−7.8. Surprisingly, this system behaves differently in the texture of interaction pattern at two extremes of studied pH range, unlike HSA. Importantly, the charge on the amino acid side chains in the binding pocket is likely to play a significant role.

KEYWORDS: *differential accessibility, food dye, BSA, electrostatic interaction, molecular docking*

INTRODUCTION

Biomacromolecules such as proteins and DNA have very fundamental roles in life and are indispensable for living systems. Serum proteins, as one of the plentiful components of blood plasma, expedite the transport and delivery of various ligands (molecules) including long-chain fatty acids, drugs, and metals.[1] The absorption, distribution, metabolism, and excretion of those molecules/ligands/drugs can be greatly influenced due to binding with serum proteins.[2] Moreover, there is indication of conformational modification due to binding with small dyes and drugs, which tends to change secondary and tertiary structures.[3] Among the different types of dyes, recently, azo dyes have been used in protein−dye chromatography, which could be important with respect to protein purification.[4] Hence, the exploration of binding of azo dyes with serum proteins is useful. These azo food dyes are toxic in nature, and hence such explorations can be important from the toxicological point of view. Here, we focus on the study of interaction of an azo food dye, Cochineal Red A, with bovine serum albumin (BSA).

(4-Sulfo-1′-naphthylazo)-1-hydroxy-2-naphthalene-6,8-disulfonic acid trisodium salt (C.I. 16255), known as Cochineal Red A (Figure 1), is a synthetic organic azo food colorant that can be found in common food products such as beverages, dry mix products, candies, dairy products, sugar confectioneries, and bakery products.[5]

BSA and human serum albumin (HSA) are the major components in plasma protein for cows and humans, respectively. These albumins have different pH-dependent conformational isomers. Föster[6] classified these pH-dependent forms as "*N*" for *normal form*, "*B*" for *basic form*, "*F*" for *fast migrating form*, "*E*" for *expanded form*, and "*A*" for *aged form*. These different conformations of BSA are characterized by the change in α-helix content as given below.

$$\underset{(\alpha\text{-helix content})\rightarrow}{\text{form}}\ \underset{(35\%)}{\text{expanded}\ (E)}\ \overset{\text{pH 2.3}}{\rightleftharpoons}\ \underset{(45\%)}{\text{fast}\ (F)}\ \overset{\text{pH 4.3}}{\rightleftharpoons}\ \underset{(55\%)}{\text{normal}\ (N)}\ \overset{\text{pH 8.0}}{\rightleftharpoons}\ \underset{(48\%)}{\text{basic}\ (B)}\ \overset{\text{pH 10.0}}{\rightleftharpoons}\ \underset{(48\%)}{\text{aged}\ (A)}$$

Because conformational transitions are occurring at different pH values, the extent of interaction between protein and food dyes should depend on the solution pH. The environments of two tryptophan residues in BSA are different from each other, and thus the study of the interactions with small molecules can provide insights into the understanding of the environment-dependent molecular interactions. It would be very informative to explore how quenching of tryptophan fluorescence is affected due to modification of external factors such as pH and ionic strength (salt addition).

Therefore, we planned to study the interaction of food dye Cochineal Red A, a negatively charged azo dye, with BSA in the pH range of 4.8−7.8, within which the protein exists primarily in the normal form (*N*). The data for pH 7.4 are already reported by us elsewhere.[7] The pK_a of Cochineal Red A is 11.2,[8] for the deprotonation of phenolic OH, so in the chosen range of pH (4.8−7.8) the dye is trinegative due to three negative sulfonate groups with a phenolic OH function and binds with the protein. The choice of dye is due to several

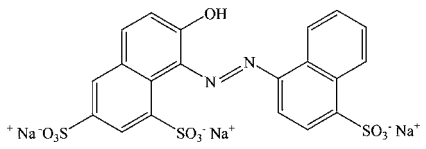

Figure 1. Molecular structure of Cochineal Red A.

Received: April 17, 2012
Revised: March 27, 2013
Accepted: April 9, 2013
Published: April 9, 2013

reasons as pointed out in our earlier paper on exploration of binding of Cochineal Red A with HSA and BSA:[7] first, it should attract the positively charged domain of protein by means of electrostatic forces; second, as Cochineal Red A is a charged dye, it should be, in principle, possible to investigate how fluorescence quenching can be modulated externally with change of pH and ionic strength (ionic atmosphere) of the medium to throw more light onto the specific nature of interaction. The present study is a comprehensive exploration of the mode of interaction of Cochineal Red A with BSA, because in our previous paper[7] we have not addressed the pH-dependent behavior.

We have studied the interaction by means of general optical spectroscopic techniques such as UV−vis, fluorescence, circular dichroism (CD) spectroscopy, and molecular docking simulation.

MATERIALS AND METHODS

Materials. BSA (≥98%) and HSA (∼99%, essentially fatty acid free), Cochineal Red A (mol wt 604.46), and hemin were purchased from Sigma-Aldrich. L-Tryptophan was obtained from SRL, India. Analytical grade chemicals and ultrapure water were used throughout this study. Experiments were carried out in 5 mM sodium phosphate buffer of pH 4.8, 5.5, 6.3, and 7.8. The pH of solutions was measured on a EUTECH pH 510 ion pH-meter. NaCl (analytical grade, Merck) was used to vary the salt concentration of the medium.

UV−Vis Absorbance Spectra. Vis absorbance spectra were obtained by scanning the solution on a Shimadzu UV-1601 absorption spectrophotometer against solvent blank reference in the wavelength range of 400−650 nm. Experiments were performed by keeping the Cochineal Red A concentration constant at 30 μM, and protein was varied from 0 to 60 μM.

The stoichiometry of the BSA − Cochineal Red A complex was determined by Job's method of continuous variation with increasing pH of the medium.[9,10] According to the Beer−Lambert law, if the dye and protein do not interact, the total absorbance of the mixture (A_{theor}) is equal to the sum of their individual absorbances as follows:

$$A_{theor} = \varepsilon_P C_P^0 X_P + \varepsilon_D C_D^0 X_D \quad (1)$$

ε_P and ε_D are the molar extinction coefficients of the protein and dye, respectively. C_P^0 and C_D^0 are the concentrations of the stock solutions of the protein and dye, which are equal ($C_P^0 = C_D^0 = 2 \times 10^{-5}$ M). X_P and X_D are the mole fractions of the protein and dye, respectively. The measured absorbance, A_{exp}, is the sum of absorbance of all absorbing species existing in the solution:

$$A_{exptl} = \varepsilon_P C_P + \varepsilon_D C_D + \varepsilon_C C_C \quad (2)$$

C_P and C_D are the concentrations of protein and Cochineal Red A in the mixture, respectively. ε_C and C_C are the molar extinction coefficient and concentration of the associate formed, respectively. By calculating A_{theor} and measuring A_{exptl}, the corrected absorbance can be obtained as follows:

$$\Delta A = A_{exptl} - A_{theor} \quad (3)$$

The stoichiometry of the protein−dye complex can be determined from the plot of ΔA versus X_D at a fixed wavelength (λ_{max} of dye).

Fluorescence Measurements. Steady-state corrected fluorescence spectra were recorded on a Jobin Yvon-Spex Fluorolog-3 spectrofluorometer equipped with a thermostatic cell holder using a 1 cm path length quartz cuvette. All steady-state fluorescence experiments have been carried out at 298 K. To monitor the fluorescence spectra due to tryptophan residues only, the samples were excited at 295 nm, and the emission wavelengths were recorded from 310 to 470 nm. The concentrations of BSA and HSA were kept at 1.5 and 2 μM, respectively.

Data for fluorescence quenching are plotted in terms of fluorescence ratio (F_0/F) as a function of quencher concentration, [Q] (Stern−Volmer Plot). Here, F_0 and F are observed fluorescence intensities of fluorophore in the absence and presence of quencher, respectively. All fluorescence signals are corrected for the absorption of quencher at the excitation and emission wavelengths of the fluorophore using the correction factor η as given in eq 4:[7,11]

$$\eta = \frac{A_{x_0} A_{y_0} (1 - 10^{-A_{x_i}})(1 - 10^{-A_{y_i}})}{A_{x_i} A_{y_i} (1 - 10^{-A_{x_0}})(1 - 10^{-A_{y_0}})} \quad (4)$$

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 the fluorophore and quencher (ΔA_{x_i} and ΔA_{y_i}) at the excitation and emission wavelengths, respectively. All of the corrected quenching plots are connected by smooth lines, and the binding constant (K_b) is calculated using eq 5 as follows, where n is the number of binding sites.

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_b + n \log[Q] \quad (5)$$

Circular Dichroism Spectra. Circular dichroism measurements were made on a Jasco-810 automatic recording spectropolarimeter at 298 K under constant nitrogen flush over a wavelength range of 190−260 nm, using a 0.1 cm path length cell. The spectra were recorded with a scan rate of 50 nm/min and a response time of 4 s. Three scans were accumulated for each spectrum. CD spectra were recorded as ellipticity (θ) in mdeg. For baseline correction, spectra of only the buffer solution obtained under the same condition were taken as blank and subtracted from the experimental spectra. The protein concentration was kept at 9 μM in phosphate buffer of pH 4.8 and 7.8, and the molar ratio of protein to Cochineal Red A was varied as 1:0, 1:2, and 1:6.

The mean residue ellipticity (MRE), expressed in deg cm^2 dmol^{-1}, has been calculated according to the following equation:

$$\text{MRE} = \frac{\theta_{obs}(\text{mdeg})}{10 \times n_r \times l \times c_P} \quad (6)$$

θ_{obs} is the observed ellipticity in millidegrees at 208 nm, n_r 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 percent α-helix content is then obtained from the MRE values at 208 nm using the following equation:

$$\alpha\text{-helix}\,(\%) = \left\{\frac{(-\text{MRE}_{208} - 4000)}{33000 - 4000}\right\} \times 100 \quad (7)$$

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 33000 is the MRE value of a pure α-helix at 208 nm. The value of n_r is taken as 583[12] for BSA.

Molecular Docking Study. The available crystal structure of BSA (PDB ID: 4F5S),[13] obtained from the Protein Data Bank, has been used in docking studies. The structure of trinegative Cochineal Red A was optimized by PM3 prescription using MOPAC2009.[14] Later, Cochineal Red A was docked using AutoDock 4.2[15] into the 3D structure of BSA. AutoDock 4.2 uses the Lamarckian genetic algorithm to search for the optimum binding site of small molecules to the protein. To recognize the binding sites in serum albumins (SAs), docking was done and the grid sizes were set to 60, 60, and 60 along the X-, Y-, and Z-axes with a 0.4 Å grid spacing. The Auto Docking parameters used were as follows: GA population size, 150; maximum number of energy evaluations, 250,000. During docking, a maximum of 50 conformers were considered for each molecule, and the root-mean-square (RMS) cluster tolerance was set to 2.0 Å. Here, two Cochineal Red A molecules have been docked in BSA. The first molecule has been docked using the grid center at −1.833, 24.023, and 108.781 Å corresponding to Trp213. Then the docking of the second dye molecule was performed on the lowest energy pose of the first docked Cochineal Red A in BSA and the center of the grid was set to 14.980, 36.547, and 91.952 Å corresponding to Trp134.

dx.doi.org/10.1021/jf305395n | J. Agric. Food Chem. 2013, 61, 4606−4613

The accessible surface areas (ASA) of uncomplexed protein and their docked complexes with Cochineal Red A are calculated using Discovery Studio Visualizer 2.5 software from Accelrys Software Inc. The docked conformations with the lowest binding energy are used for analysis. A probe radius of 1.4 Å was used on structures having polar hydrogen only for calculation. The change in ASA for a residue was calculated as $\Delta ASA = ASA_{protein} - ASA_{protein-ligand}$.

Data Analysis. All data are represented as the mean ± SD. Experiments were performed in triplicates. All graphical representations and statistical analyses were done using Origin software.

RESULTS AND DISCUSSION

Absorption Spectroscopy. UV−vis absorbance measurement is a simple and effective method for detecting ground-state interaction. If there is a shift in UV−vis absorption maxima of dye in the presence of protein, it indicates ground-state complexation.[16] We have recorded absorbance spectra of Cochineal Red A in the absence and presence of BSA and observed a red shift of λ_{max} of the dye by about 5 nm in both pH 4.8 and 7.8 (see Figure S1 in the Supporting Information). Because there is a change in the environment upon switching of the dye from aqueous phase to protein pocket (less polar) due to a ground-state interaction, we have observed such a shift in the absorbance peak. The ground-state protein−dye interaction has already been reported elsewhere for HSA − Cochineal Red A at pH 7.4.[7]

Determination of Stoichiometry. As stated earlier, the stoichiometry of the BSA − Cochineal Red A complex is determined by Job's method (see Materials and Methods). The absorbance of solutions is measured with different mole ratios of Cochineal Red A and serum albumin at 507 nm, corresponding to dye spectral maxima in the visible range. The corrected absorbance of different stoichiometry (ΔA) has been plotted as a function of the corresponding mole fraction of Cochineal Red A (X_{CR}) and is displayed in Figure 2. In the case of BSA, it is found that with an increase of the pH from 4.8 to 7.8 the stoichiometry of the complex (BSA:dye) decreases from ∼1:2 to ∼1:1.2 (Table 1). For HSA it, however, remains at ∼1:1 (see Figure S2 in the Supporting Information).

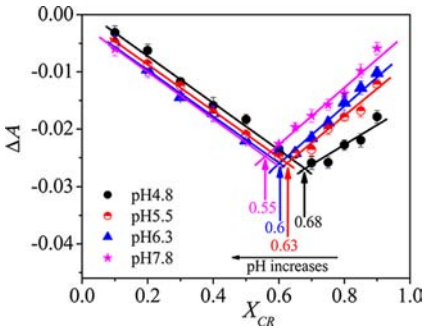

Figure 2. Job's plot for BSA − Cochineal Red A in 5 mM phosphate buffer at different pH values and 298 K. Symbols with cap include error bar.

Fluorescence Studies. Binding of Cochineal Red A with the two proteins, BSA and HSA, has been investigated by recording quenching of intrinsic tryptophan fluorescence. Fluorescence quenching can be a result of a variety of molecular phenomena such as excited-state processes, energy transfer, ground-state interaction, and collisional deactivation.[17] The effects of factors such as pH and ionic strength (salt addition) on binding affinity are also investigated. This will be of importance in determining the details of the interaction pattern. For BSA, we have carried out quenching experiments at four different pH values, namely, 4.8, 5.5, 6.3, and 7.8, and also at three different NaCl concentrations. Data for pH 7.4 can be found elsewhere.[7]

Table 1. Stoichiometry of BSA − Cochineal Red A Complex as a Function of pH of the Medium

pH	stoichiometry (BSA:Cochineal Red A)
4.8	1:2.0
5.5	1:1.7
6.3	1:1.5
7.4	1:1.3
7.8	1:1.2

Effect of pH on Quenching. The intrinsic fluorescence of BSA and HSA has been found to reduce with an increase of Cochineal Red A concentration in the entire range of studied pH. A representative set of fluorescence spectra for BSA at pH 4.8 in the presence of Cochineal Red A is shown in Figure 3.

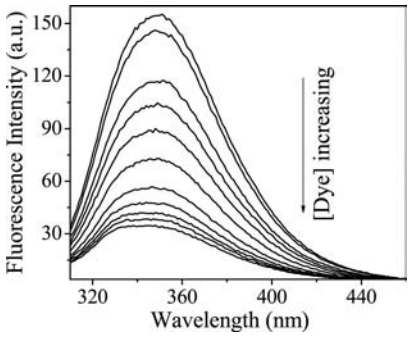

Figure 3. Emission spectra of BSA in the presence of different concentrations of Cochineal Red A at pH 4.8 and 298 K.

For BSA − Cochineal Red A, positive deviation in the Stern−Volmer (S−V) plot is observed only at pH 4.8 (Figure 4), but at pH 5.5 and above, the quenching plots show negative deviation. Competitive binding studies at pH 7.4 indicate involvement of more than one binding site, which we have discussed elsewhere,[7] and also we have reported that binding of Cochineal Red A with serum albumins is predominantly (electro)static in origin. Moreover, the observed positive deviation in the quenching plot for HSA at pH 7.4 has been established to be due to the large extent of quenching.[7]

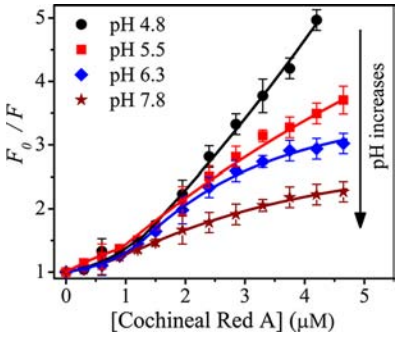

Figure 4. Stern−Volmer plot for BSA − Cochineal Red A at pH 4.8, 5.5, 6.3, and 7.8. Symbols with cap include error bar.

The upward bending of the S−V plot may arise for several reasons.[17] According to Eftink and Ghiron,[18] positive deviation in the quenching plot indicates that both tryptophan residues of BSA can be accessible to quencher and the quenching constants for each tryptophan residue are close to one another, whereas a negative deviation implies inaccessible or partially accessible tryptophan residues. Therefore, at pH 4.8 for BSA, upward curvature indicates that both tryptophans should be available for quenching by Cochineal Red A. With increase of pH, the observed downward bend (and a reduction in quenching) is suggestive of some decrease of accessibility of fluorophores.

The charge on the quencher can also have a dramatic effect on the extent of interaction.[17] For a negatively charged Cochineal Red A, quenching will depend not only on how accessible the tryptophan residues are but also on its surrounding charges.[19] The surrounding of both tryptophan residues of BSA within 15 Å radii has been examined using Visual Molecular Dynamics software (VMD 1.8.7)[20] (see Figure S4 in the Supporting Information). The dye, being negative, should be repelled by a nearby negative charge density, and hence the extent of interaction should reduce. Likewise, a nearby positive charge should attract the negative dye closer to the fluorophore tryptophan residue, resulting in the enhancement of quenching. At pH 4.8 both tryptophan residues in BSA are surrounded by positively charged side chains of histidine, lysine, and arginine amino acid residues. Therefore, the electrostatic attraction between those positively charged side chains and trinegative Cochineal Red A is responsible for a high extent of quenching of tryptophan fluorescence. At pH 5.5 the quenching plot for BSA does not show upward bending in the higher dye concentration range but shows a little negative deviation, and with further increase of pH of the medium, that is, at and above pH 6.3, the quenching curve bends down significantly (Figure 4). When the pH is higher than the isoelectric point (p*I*) of BSA, pH 4.8,[21,22] the protein moiety becomes negatively charged, and hence an overall repulsion between the macromolecule and the dye contributes to the reduction of quenching. Also, an alteration in the charge atmosphere in the dye-binding site due to pH change should contribute to the strength of electrostatic interaction. The biphasic nature of the S−V plot was also reported earlier for the quenching of intrinsic tryptophan fluorescence of BSA by iodide,[23] ascribing the differential accessibility of the two tryptophan residues of BSA as a reason. In our case with BSA at pH 4.8, both tryptophans are quenched with almost equal efficiency by Cochineal Red A, giving rise to upward bend, but with an increase of pH the observed biphasic nature of the plot indicates that both of them should not be equally accessible for quenching, as discussed elsewhere.[23] Thus, the presence of an inaccessible or partially accessible fluorophore (tryptophan) can result in biphasic quenching plots. We have monitored the quenching of HSA in the presence of equimolar L-tryptophan at pH 4.8. A downward bending is observed instead of the usual positive deviation (Figure 5), which looks similar to the BSA quenching plot at pH 7.8. It is to be noted that Cochineal Red A cannot quench the free L-tryptophan in aqueous buffer medium, which has also been confirmed in a separate run. Here, HSA-L-tryptophan quenching data have been compared with BSA quenching at pH 7.8 only to assess the relative nature of plots and to determine a possible cause of the difference in quenching behavior of BSA in two extremes of pH. It appears that the presence of any unquenchable (inaccessible/less accessible) fluorophore, in addition to a quenchable one (Trp214), results in a biphasic plot.

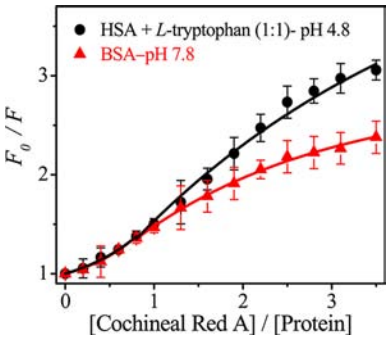

Figure 5. Quenching plot of HSA:L-tryptophan (1:1) by Cochineal Red A at pH 4.8. [HSA] = 1.5 μM. Symbols with cap include error bar.

The diminishing accessibility of tryptophan with increasing pH is also indicated by Job's plot (Figure 2). At pH 4.8 a 1:2 stoichiometry has been found for the BSA − Cochineal Red A system. Thus, the additional contribution to quenching should be from the other tryptophan (Trp134) due to binding of a second dye in its vicinity, and as a consequence positive deviation is observed. With an increase of pH, a progressive reduction in the stoichiometry from 1:2 to ∼1:1.2 is clearly indicating that the interaction of the second dye molecule becomes weak.

Site Marker Competitive Binding Study. The site marker competitive binding results are reported elsewhere.[7] It reveals that at pH 7.4 the dye binds with HSA and BSA in the warfarin binding site (site I), and in addition there is some contribution from binding in the hemin-binding site of BSA. Moreover, at pH 4.8 it is found that for BSA the value of n (obtained from eq 5) becomes >1.5 (Table 2), which is consistent with stoichiometry determinations (Table 1).

The effect of subdomain IB binder, hemin,[24] for both the proteins at pH 4.8 and 7.8 has also been explored. The partially solvent exposed Trp134 is located in subdomain IB of BSA, and quenching is found to be affected in the presence of hemin at both pH values (Figure 6). For the BSA − Cochineal Red A system the binding constants are found to be $(1.96 \pm 0.06) \times 10^9$ and $(1.5 \pm 0.1) \times 10^6$ at pH 4.8 and 7.8, respectively, but in the presence of hemin the binding constant at pH 4.8 remains unaffected, $(1.8 \pm 0.07) \times 10^9$, whereas at pH 7.8 the value reduces to $(8.9 \pm 0.08) \times 10^5$. A similar reduction in binding constant in the presence of hemin has also been observed at pH 7.4.[7] Now at pH 4.8 the experimental binding constant for hemin with BSA is found to be $(1.1 \pm 0.04) \times 10^5$, which is similar in magnitude to the literature-reported number at pH 7.4 ($\sim 10^5$).[25] However, our data show that Cochineal Red A binds much more strongly (Table 2) than hemin with BSA at pH 4.8. Thus, it may be stated that hemin competes better with Cochineal Red A at pH 7.8 than at pH 4.8, and this demonstrates a stronger binding of Cochineal Red A in the hemin-binding site at lower pH. This explains clearly that Trp134 is more accessible at pH 4.8, leading to a positive deviation of the quenching plot, but with the increase of pH accessibility reduces, which results in a downward curvature of the S−V plot. HSA − Cochineal Red A binding constants are almost identical, $(3.9 \pm 0.18) \times 10^5$ and $(4.2 \pm 0.22) \times 10^5$, in the absence and presence of hemin, respectively, at pH 7.8. This indicates the absence of competitive binding of hemin

dx.doi.org/10.1021/jf305395n | J. Agric. Food Chem. 2013, 61, 4606−4613

Table 2. Determination of Binding Constants (K_b) and Number of Binding Sites (n) of the BSA − Cochineal Red A System at Different NaCl Concentrations in the Medium of 5 mM Phosphate Buffer of pH 4.8, 5.5, 6.3, and 7.8[a]

NaCl (M)	pH 4.8		pH 5.5		pH 6.3		pH 7.8	
	K_b[b]	n	K_b[b]	n	K_b[b]	n	K_b[b]	n
0	$(1.96 \pm 0.06) \times 10^9$	1.61 ± 0.03	$(4.35 \pm 0.09) \times 10^7$	1.33 ± 0.02	$(8.99 \pm 0.36) \times 10^6$	1.24 ± 0.04	$(1.5 \pm 0.1) \times 10^6$	1.12 ± 0.03
0.06	$(7.94 \pm 0.3) \times 10^7$	1.46 ± 0.04	$(2.05 \pm 0.06) \times 10^6$	1.19 ± 0.01	$(7.13 \pm 0.22) \times 10^5$	1.13 ± 0.02	$(1.28 \pm 0.03) \times 10^5$	0.99 ± 0.02
0.2	$(8.9 \pm 0.4) \times 10^5$	1.19 ± 0.02	$(2.39 \pm 0.12) \times 10^5$	1.17 ± 0.01	$(7.74 \pm 0.38) \times 10^4$	1.12 ± 0.05	$(1.58 \pm 0.4) \times 10^4$	0.96 ± 0.03

[a]Data are presented as the mean ± SD of triplicately performed experiments. [b]Concentrations of dye and proteins are expressed as mol L^{-1}.

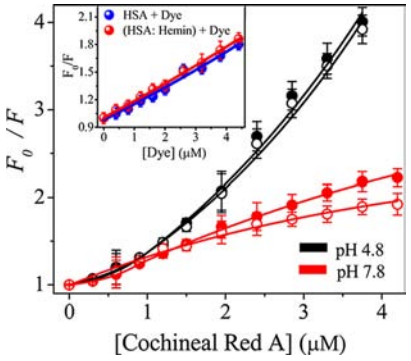

Figure 6. Effect of hemin on quenching of BSA − Cochineal Red A system in 5 mM phosphate buffer of pH 4.8 and 7.8 at 298 K. [BSA] = 1.5 μM and [protein]:[site marker] = 1:1 (solid and hollow circles represent data in the absence and presence of hemin, respectively). (Inset) Effect of hemin on quenching of HSA − Cochineal Red A system at pH 7.8. Symbols with cap include error bar.

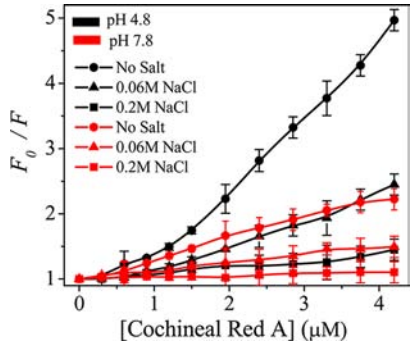

Figure 7. Effect of increasing NaCl concentration on the quenching plot of the BSA − Cochineal Red A system at pH 4.8 and 7.8. Symbols with cap include error bar.

with the dye at subdomain IB; that is, the dye is not a subdomain IB binder in HSA.

Effect of Added NaCl. The thermodynamic data as previously reported by us reveal that binding is primarily electrostatic in origin.[7] The insignificant change of tryptophan lifetime of both serum proteins in the presence of Cochineal Red A[7] rules out the possibility of FRET.[26,27] We have carried out quenching experiments in buffer media containing various added NaCl to see the effect of ionic atmosphere on the affinity in order to further establish the electrostatic mode in different pH conditions. The increase in salt concentration (ionic strength) should not in principle affect the strength of hydrophobic interaction, but electrostatic attraction should be disfavored. To prepare solutions of various ionic strengths, the required weighed amount of NaCl (analytical grade, Merck) has been added to 5 mM phosphate buffer, and this NaCl-containing buffer has been used to make various protein−dye compositions in salt variation studies.

Figure 7 indicates that for BSA the extent of quenching decreases significantly with the increase of NaCl concentration (increase in ionic strength), and it is also reflected in the decrease of binding constants (Table 2). Moreover, the effect of NaCl is greater at pH 4.8 than at pH 7.8. That is, electrostatic interaction is stronger at pH 4.8. The binding constant in high salt has been found to be quite low (Figure 7). This is a clear indication that electrostatic interaction is the primary mode of binding of Cochineal Red A in the studied pH range. Other studies have also confirmed that the electrostatic attraction is significant between the negative sulfonate group and the positively charged amino acid side chains of serum albumin.[28,29] Thus, Cochineal Red A, having three sulfonate groups, is likely to attract the positively charged side chain. Recently, we have reported that another sulfonated azo food dye, tartrazine, binds with SAs by electrostatic mode, and it has been shown that interionic attraction theory can be successfully applied to such protein−ligand association.[30]

Conformational Investigation: Circular Dichroism Spectroscopy. CD spectra of BSA in the absence and presence of Cochineal Red A are shown in Figure 8 at two pH values (4.8 and 7.8). They exhibit two negative bands at 208 and 222 nm in the UV region, characteristic of typical α-helix structure.[31] The CD spectra of proteins in the presence and absence of Cochineal Red A are similar in shape, indicating a predominantly α-helical[32] structure even after dye association.

The observed α-helix contents of free BSA at pH 4.8 and 7.8 are 51.2 and 48.8%, respectively. After complexation with Cochineal Red A, the α-helix contents become 50.4 and 50.0% at pH 4.8 and 48.48 and 48.46% at pH 7.8, for protein:dye ratios of 1:2 and 1:6, respectively. A similar change in α-helix content in the presence of Cochineal Red A (at 1:2 ratio) has also been observed at pH 7.4.[7] The percentage of α-helical structure of BSA has been found to reduce more at pH 4.8 than at pH 7.8.

Docking Studies and Accessible Surface Area (ASA) Calculation. Docking of the ligand with macromolecules provides insight into the preferred binding location and can be exploited to corroborate experimental observations to a large extent. The trinegative Cochineal Red A has been docked into the 3D structure (PDB ID: 4F5S) of BSA using AutoDock Tools.[15] The principal ligand binding sites in BSA are located in hydrophobic cavities of subdomains IIA and IIIA. Competitive site marker binding experiments indicate more than one binding site of Cochineal Red A in BSA.[7] Keeping this in mind, we have attempted to dock two Cochineal Red A molecules into BSA as mentioned under Materials and Methods. The docked pose of Cochineal Red A with BSA (2:1) is given in Figure 9a. Accessible surface areas (ASA) have been calculated (see Table S2 in the Supporting Information). If we combine

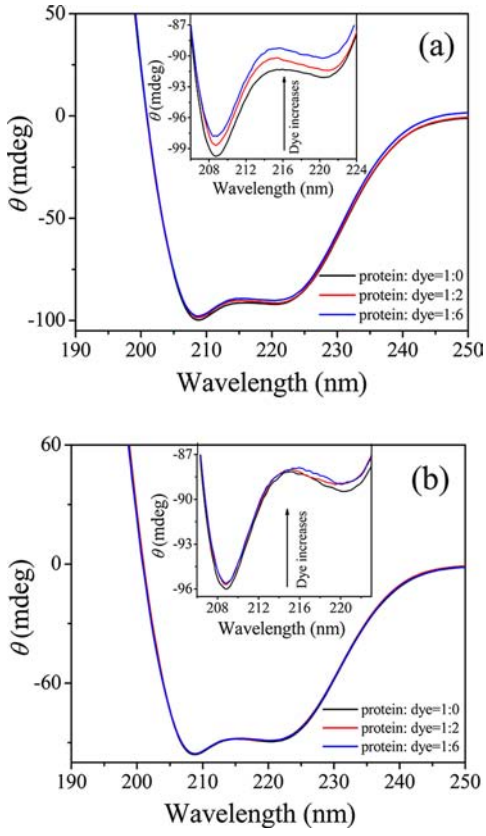

Figure 8. CD spectra of free BSA and BSA complexed with Cochineal Red A in 5 mM phosphate buffer of (a) pH 4.8 and (b) pH 7.8 at 298 K. [Proteins]:[dye] ratios are 1:0, 1:2, and 1:6. (Insets) Corresponding expanded wavelength region of CD spectra within 205−225 nm.

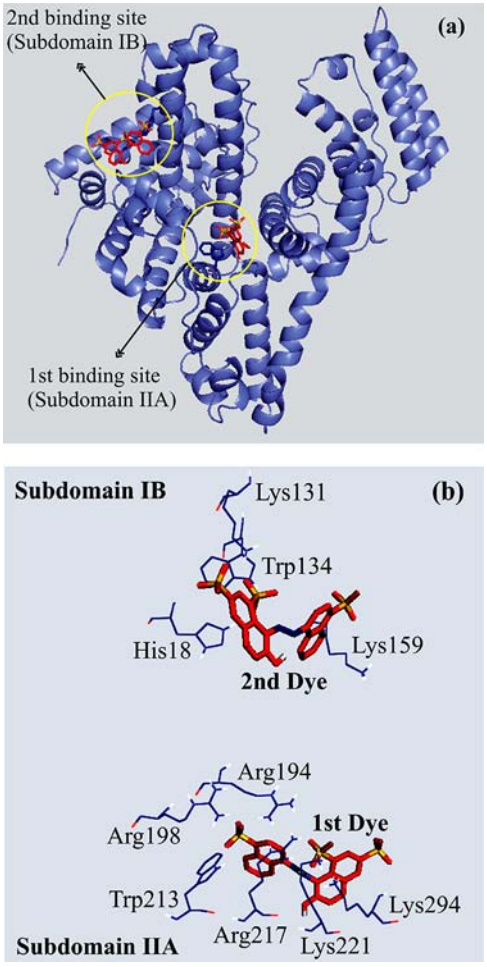

Figure 9. (a) Docked poses of Cochineal Red A in BSA; (b) interacting amino acid residues around the binding sites of Cochineal Red A at subdomains IIA and IB.

the results obtained from our experiments and docking studies, we have the following points to make:

(a) In the BSA − Cochineal Red A system the first dye molecule binds in subdomain IIA, that is, site I. Trp213 of BSA is a part of subdomain IIA. A considerable loss of ASA of Trp213 upon docking with Cochineal Red A (first molecule) indicates binding near that tryptophan residues (see Table S2 in the Supporting Information). As subdomain IIA corresponds to the warfarin-binding site, the presence of warfarin decreases the extent of interaction of Cochineal Red A as discussed elsewhere.[7] However, with hemin, which binds near Trp134 in subdomain IB,[24] we get the reduction in binding constant only with BSA. When the docking of a second Cochineal Red A molecule on the first docked pose is investigated, it is found that the second dye docks near Trp134 of BSA in subdomain IB (Figure 9). The distance between the first docked Cochineal Red A and Trp213 is 2.5 Å and that for the second docked Cochineal Red A and Trp134 is 5.2 Å. Loss of ASA of Trp134 in the BSA − Cochineal Red docked complex (see Table S2 in the Supporting Information) also suggests the dye binding near Trp134.

(b) More than one binding site (n) of Cochineal Red A is prominent at pH 4.8 for BSA (see Table 2). It is also demonstrated from molecular docking that two dye molecules bind in two different sites (Figure 9a). Stoichiometry data (Table 1) also support this.

(c) If we have a closer look into the dye-binding sites (Figure 9b), the following positively charged amino acid residues, namely, His18, Lys131, and Lys159 in subdomain IB and Lys221, Lys294, Arg194, Arg198, and Arg217 in subdomain IIA of BSA, are in close vicinity of docked Cochineal Red A, and the electrostatic interaction between those positively charged amino acid side chains with Cochineal Red A is an obvious consequence. When the pH of the medium increases, some of these side chains (e.g., His) may be deprotonated to solvent and the consequent lesser strength of interaction due to decrease in the net positive charge of the pocket results in a reduction of the extent of quenching. Hence, a downward bending in the S−V plot is observed at higher pH. Therefore, molecular docking study can smartly account for the fluorescence quenching pattern of BSA by Cochineal Red A.

In this paper we have provided a comprehensive understanding of the interaction of a negatively charged azo food dye, Cochineal Red A, with BSA, in particular within the 4.8−7.8 pH range by employing different optical spectroscopic techniques and molecular docking simulations. In a previous paper, we had explored the electrostatic binding of Cochineal Red A with BSA and HSA only at pH 7.4.[7] In that case we have not provided the pH dependence of such binding in particular. It is found that the texture of the quenching curve depends very much on the pH of the medium in the case of BSA. The quenching plot is upward at pH 4.8 as both tryptophan residues are available for quenching but a biphasic nature has been observed due to differential accessibility of the two tryptophans at higher pH.

dx.doi.org/10.1021/jf305395n | J. Agric. Food Chem. 2013, 61, 4606−4613

On the other hand, upward curvature is always observed for HSA at all pH values where differential accessibility issue is absent (see Figure S3 in the Supporting Information). Fluorescence studies, site marker competitive binding, stoichiometry, and molecular docking confirmed that the primary binding site of Cochineal Red A is in subdomains IIA and IB for BSA and only in subdomain IIA for HSA.

There are plenty of instances in the literature that have reported negative deviation in the S−V plot for protein−ligand interaction due to the selective quenching of solvent-exposed tryptophan residues and buried ones being inaccessible.[19,33,34] For BSA, it has also been reported that the inaccessibility of the buried Trp213 residue toward water-soluble quencher is the reason for the observed negative deviation quenching plot[28,35] as the quencher cannot penetrate into the hydrophobic core of the protein. From our experimental findings with Cochineal Red A, we conclude that with an increase of pH the decreasing accessibility of the solvent-exposed Trp134 for quenching is responsible for the downward nature of the S−V plot, so the current study complements our previous paper and combining these two completes the understanding of binding of Cochineal Red A with serum proteins. This is important as the exploitation of pH-dependent interaction of azo dyes with BSA could be useful in protein−dye chromatography and pH-mediated detoxification of azo colorants.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures and tables. 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.

Funding

We thank DST-India (Fund SR/FTP/CS-97/2006), CSIR-India (Fund 01/(2177)/07 EMR-II, dated Oct 24, 2007), and IIT−Kharagpur (ISIRD-EEM grant) for financial support. P.B. thanks UGC−India for a fellowship. N.M. thanks CSIR and S.D. thanks IIT−Kharagpur for individual fellowships.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the anonymous reviewers for their critical comments and suggestions.

■ DEDICATION

#Dedicated to Professor Mihir Chowdhury on his 75th Birthday.

■ ABBREVIATIONS USED

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

■ REFERENCES

(1) Peters, T. J. Serum albumin. *Adv. Prot. Chem.* **1985**, *37*, 161−245.
(2) Flarakos, J.; Morand, K. L.; Vouros, P. High-throughput solution-based medicinal library screening against human serum albumin. *Anal. Chem.* **2005**, *77*, 1345−1353.
(3) 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.
(4) 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.
(5) Sayar, S.; Ozdemir, Y. First-derivative spectrophotometric determination of ponceau 4R, sunset yellow and tartrazine in confectionery products. *Food Chem.* **1998**, *61*, 367−372.
(6) Föster, J. F. Some aspects of the structure and conformational properties of serum albumin. In *Albumin Structure, Function and Uses*; Rosenoer, V. M., Oratz, M., Rothschild, M. A., Eds.; Pergamon Press: New York, 1977; pp 53−84.
(7) Bolel, P.; Mahapatra, N.; Halder, M. Optical spectroscopic exploration of binding of cochineal red A with two homologous serum albumins. *J. Agric. Food Chem.* **2012**, *60*, 3727−3734.
(8) Dossi, N.; Toniolo, R.; Susmel, S.; Pizzariello, A.; Bontempelli, G. Simultaneous RP-LC determination of additives in soft drinks. *Chromatographia* **2006**, *63*, 557−562.
(9) Shahir, A. A.; Javadian, S.; Razavizadeh, B. B. M.; Gharibi, H. Comprehensive study of tartrazine/cationic surfactant interaction. *J. Phys. Chem. B* **2011**, *115*, 14435−14444.
(10) Forte-Tavcer, P. Interactions between some anionic dyes and cationic surfactants with different alkyl chain length studied by the method of continuous variations. *Dyes Pigm.* **2004**, *63*, 181−189.
(11) 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.
(12) 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.
(13) Bujacz, A. Structures of bovine, equine and leporine serum albumin. *Acta Crystallogr., Sect. D* **2012**, *68*, 1278−1289.
(14) Stewart, J. J. P. *MOPAC2009*; Stewart Computational Chemistry: Colorado Springs, CO, 2008.
(15) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. Automated docking using a lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* **1998**, *19*, 1639−1662.
(16) Zhang, X. W.; Zhao, F. L.; Li, K. A. Studies on the reaction between ciprofloxacin and bovine serum albumin. *Chem. J. Chinese Univ.* **1999**, *20*, 1063−1067.
(17) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer: Berlin, Germany, 2006; pp 277−318.
(18) Eftink, M. R.; Ghiron, C. A. Fluorescence quenching studies with proteins. *Anal. Biochem.* **1981**, *114*, 199−227.
(19) Lehrer, S. S. Solute perturbation of protein fluorescence − quenching of tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry* **1971**, *10*, 3254−3263.
(20) Humphrey, W.; Dalke, A.; Schulten, K. VMD: visual molecular dynamics. *J. Mol. Graphics* **1996**, *14*, 33−38.
(21) Elgersma, A. V.; Zsom, R. L. J.; Norde, W.; Lyklema, J. The adsorption of bovine serum albumin on positively and negatively charged polystyrene latices. *J. Colloid Interface Sci.* **1990**, *138*, 145−156.
(22) Chun, K. Y.; Stroeve, P. Protein transport in nanoporous membranes modified with self-assembled monolayers of functionalized thiols. *Langmuir* **2002**, *18*, 4653−4658.
(23) Moller, M.; Denicola, A. Protein tryptophan accessibility studied by fluorescence quenching. *Biochem. Mol. Biol. Educ.* **2002**, *30*, 175−178.
(24) 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.

(25) Rus', O. B.; Puchkaev, A. V.; Ivanov, A. I.; Metelitsa, D. I. Interaction of albumins and hemin with aromatic antioxidants: a spectrophotometric and fluorometric study. *Appl. Biochem. Microbiol.* **2000**, *36*, 36−45.
(26) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer: Berlin, Germany, 2006; pp 13−14.
(27) Valeur, B. *Moleculer Fluorescence: Principles and Applications*; Wiley-VCH: Weinheim, Germany, 2001; pp 113−114.
(28) 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.
(29) Sereikaite, J.; Bumelis, V. A. Interaction of serum albumin with vinyl sulfonate azo dye. *Cent. Eur. J. Chem.* **2008**, *6*, 509−512.
(30) Bolel, P.; Datta, S.; Mahapatra, N.; Halder, M. Spectroscopic investigation of the effect of salt on binding of tartrazine with two homologous serum albumins: quantification by use of the Debye−Hückel limiting law and observation of enthalpy−entropy compensation. *J. Phys. Chem. B* **2012**, *116*, 10195−10204.
(31) 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.
(32) Hu, Y. J.; Liu, Y.; Shen, X. S.; Fang, X. Y.; Qu, S. S. Studies on the interaction between 1-hexylcarbamoyl-5-fluorouracil and bovine serum albumin. *J. Mol. Str.* **2005**, *738*, 143−147.
(33) Midoux, P.; Wahl, P.; Auchet, J. C.; Monsigny, M. Fluorescence quenching of tryptophan by trifluoroacetamide. *Biochim. Biophys. Acta* **1984**, *801*, 16−25.
(34) Xing, D. X.; Dorr, R.; Cunningham, R. P.; Scholes, C. P. Endonuclease-III interactions with DNA substrates. 2. The DNA-repair enzyme endonuclease-III binds differently to intact DNA and to apyrimidinic apurinic DNA substrates as shown by tryptophan fluorescence quenching. *Biochemistry* **1995**, *34*, 2537−2544.
(35) 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.