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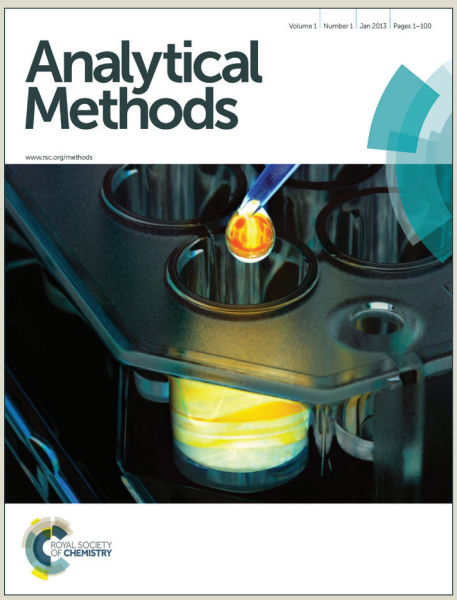

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Effects of Cu^{2+} and pH on the binding of alizarin red S to bovine serum albumin based on the analysis of protein conformation

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Characterizing the binding properties of bovine serum albumin (BSA) for alizarin red S (ARS) is necessary for the understanding of its physiological functions and developing a delivery vehicle for a range of therapeutic and diagnostic reagents. Especially, the investigations in the effects of surrounding circumstances including metal ions, pH, and protein conformation change on the interactions are very important to understand the relationship between the protein conformation and its function. Therefore, effects of Cu^{2+} and pH on the reaction between BSA and ARS were investigated using spectral and molecular docking techniques based on the conformational analysis of protein. The results show that ARS has a high affinity to the subdomain IIA of BSA through hydrogen bonds between ARS and residues ARG(193), ARG(255), LYS(220), ARG(197), and ALA(289) of BSA. In the presence of Cu^{2+}, it was firstly coordinated with ARS to form ARS-Cu complex, which then bound to the site I of BSA with the similar binding process between BSA and ARS. On the basis of the binding parameters, the presence of Cu^{2+} can not affect the binding mode of ARS to the site I of BSA, however, it can improve the binding affinity of BSA for ARS. The interactions of BSA with ARS and ARS-Cu were further explored in pH range 4.0–7.0. The binding constants (K_b) and binding number (m) at pH 4.0 are lower than those at pH 7.0 due to the unfolding of BSA conformation at low pH. Consequently, unfolded hydrophobic cavity of BSA native conformation decreases the binding affinities of BSA for ARS.

1. Introduction

Serum albumin (SA) is the most abundant protein constituents of the circulatory system (accounting for 52–60% of the total plasma protein), which facilitates the disposition and transport of various exogenous and endogenous molecules to the target tissues.[1,2] As a kind of serum albumin, bovine serum albumin (BSA) has been extensively studied due to its structural homology with human serum albumin (HSA).[3] BSA is composed by three domains I, II and III, and each domain contains two subdomains (A and B). Subdomains IIA and IIIA of BSA show specific binding properties for numerous ligands, which are named site I and site II, respectively.[2] Moreover, BSA is known to exhibit a very high conformational adaptability to a large variety of ligands.[4] Characterizing the binding properties of BSA for multitude ligands is necessary not only for the understanding of its physiological functions, but also for developing a delivery vehicle for a range of therapeutic and diagnostic reagents.[5] Therefore, the studies on this aspect become an important research field in life science, chemistry, and clinical medicine.[6]

Alizarin red S (ARS), an anthraquinone pigment, has been widely used in the textile industry and for histochemical analyses.[6,7] ARS is one representative compound of 1,2-dihydroxy-9,10-anthraquinone with a planar heterocyclic ring structure, which has been used as a ligand to determine copper, scandium, and other metal ions.[7] It was suggested that hydroxyl groups of the benzene rings and diketone moiety on ARS played crucial roles in its activities. However, as an anthraquinone dyes, ARS is not only toxic, mutagenic, and carcinogenic, but also is resistant to degradation. This is due to the fact that the complex structure of aromatic rings that affords optical stability and resistance towards light or oxidizing agents.[8] The investigation for the binding mechanism interaction between ARS and BSA is critical to understand the transportation and pathogenesis of ARS in the blood.[9,10] To date, the binding parameters such as binding constant, binding affinity, biding site, and energy transfer between ARS and BSA have been investigated by fluorescence, UV-vis spectroscopy, and linear sweep voltammetry.[10,11] We have proposed electrochemical site marker competitive method to probe the binding site and binding mode between BSA and ARS, which revealed that ARS specifically bond to the site I of BSA.[12] However, the interaction of ligand-serum albumin depends on surrounding circumstances including metal ions, pH, medium, and conformation change.[13,14]

Some metal ions are vitally essential in the function of the human organism and participate in many biochemical processes. SAs usually act as sequestration agents of metal ions and supply some specific sites to metal ions. Cu^{2+}, besides Fe^{3+} and Zn^{2+}, is the third most abundant essential transition metal ion in human body.[15] It is required in vital physiological activities such as cell respiration, biosynthesis and metabolism for all organisms. Cu^{2+}

binding to SA has been investigated extensively due to its role in copper transport and the characteristic square planar Cu^{2+} coordination to the first three amino acids of SA such as HSA, BSA, and other SAs.[16] In addition, Cu^{2+} can also react with dyes or other drug molecules to form complexes, which will influence the binding affinities of these exogenous small molecules to proteins.[17,18] Therefore, it is important to research on the effect of the Cu^{2+} on the interaction between BSA and ARS. The pH of solution is another important influence in the interaction between proteins and dyes because it is likely to change the proteins native conformation.[19] The investigations for the effects of metal ions and pH on the interaction between BSA and ARS can provide insights into understanding their environment-dependent mechanisms.

In this paper, the effects of Cu^{2+} and pH on the binding processes between BSA and ARS were investigated using spectral and molecular docking techniques based on the conformational analysis of protein. The binding mechanism was presented and the changes in the binding parameters and conformation of BSA-ARS with the effects of Cu^{2+} and pH were investigated. The obtained information can provide the interpretation of the carcinogenic mechanism of dyes and transport in the patient organism, and is expected to achieve better understanding of the relationship between the protein structure and function.

2. Experimental Section

2.1. Reagents and materials

Bovine serum albumin (BSA) (MW 67000) and alizarin red S (ARS) were obtained from Sigma and used without further purification. Phosphate buffered saline (0.1 mol L^{-1} PBS containing 0.1 mol L^{-1} KCl) at different pH was prepared from the stock solutions of 0.1 mol L^{-1} KH_2PO_4 and Na_2HPO_4 and adjusted by NaOH and H_3PO_4 solutions. Aqueous solutions were prepared at ambient temperature using ultrapure water. $CuCl_2$ and other chemicals were of analytical reagent grade.

2.2. Apparatus and measurements

Fluorescence spectra were recorded on a RF-5301PC spectrophotometer (Shimadzu) equipped with 1.0 cm quartz cells and a thermostat bath. The slit width and the excitation wavelength was 5/5 and 280 nm, respectively. The interactions between BSA and ARS or Cu^{2+} were performed by successive additions of 0.1 mmol L^{-1} ARS or Cu^{2+} solution into a 3.5 mL solution of 2.0 μmol L^{-1} BSA at various pH. UV-Vis absorption measurements were performed on a UV-3150 spectrophotometer (Shimadzu) equipped with 1.0 cm quartz cells at 298 K using the same interaction method.

Circular Dichroism (CD) measurements were performed on a J-810 spectrometer in quartz cells (Tokyo, Japan). The CD spectra of BSA, BSA-ARS, or BSA-(ARS-Cu) in 0.1 mol L^{-1} PBS at different pH were recorded over a wavelength range of 200-250 nm at scan speed of 50 nm min^{-1} and a band width of 1.0 nm. Each CD spectrum was the average of three scans.

The molecular docking methods and the docking results refer to our previous research.[12] The three-dimensional structure of BSA was constructed from the HSA template. The interaction between BSA and ARS was simulated by the program Surflex-Dock of SYBYL-X 1.1.

3. Results and Discussion

3.1. Molecular docking of ARS to BSA

The binding process between ARS and BSA has been simulated using docking technique.[12] The docking results suggest that ARS binds to the subdomain IIA in BSA (Fig. 1A) and ARS is "anchored" in the hydrophobic cavity by six intermolecular hydrogen bonds among ARS and five residues of ARG(193), ARG(255), LYS(220), ARG(197), and ALA(289), respectively. The formation of the hydrogen bonds stabilizes the BSA-ARS complex, resulting in the specific binding of ARS to site I of BSA. Especially, the phenyl of TRP(212) is vertically close to the plane of ARS with a distance of about 5.4 Å, which facilitates the intermolecular energy transfer from TRP to ARS and results in the quenching of the intrinsic fluorescence of TRP in BSA by the bonded ARS or ARS-Cu in the following investigation.

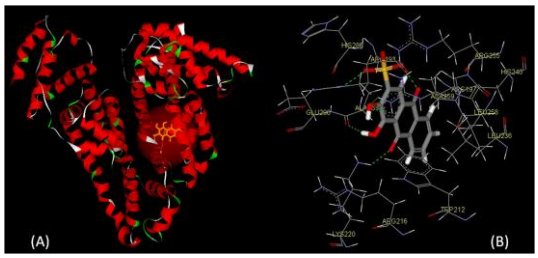

Fig. 1 Molecular modeling of the BSA-ARS complex. (A) ARS is docked in the subdomain IIA of BSA. ARS is highlighted with red spheroid. (B) Stereoview of the docking models of the BSA-ARS complex. The amino acid residues are represented using lines and ARS is represented using sticks, and hydrogen bonds are indicated by the green dashed lines.

3.2. Specific interaction between ARS and BSA

The absorption spectra of ARS are often used to monitor the interaction of ARS with proteins. As shown in Fig. 2A, ARS shows an obvious absorption peak at 425 nm in 0.1 mol L^{-1} PBS (pH 4.8), which is attributed to the absorption of anthraquinone chromophore. With the addition of BSA into ARS solution, a dramatic decrease of absorbance occurred and a new increasing absorption peak at 532 nm appeared. A distinct isosbestic point was observed at 460 nm. The results indicate that a new complex of BSA-ARS forms in solution. Similar interacting processes between ARS and Cu^2 were obtained with decreasing absorption at 425 nm and an isosbestic point at 449 nm. Furthermore, the maximum absorption of ARS-Cu complex at 508 nm did not shift with the addition of Cu^{2+} (Fig. 2B). However, the addition of BSA into ARS-Cu (1:1) solution improves the absorption with an obvious red shift of absorption maximum from 508 to 526 nm, which is different from those of ARS-Cu and BSA-ARS systems (Fig. 2C). The results suggest that a new ternary complex of BSA-(ARS-Cu) forms in solution.

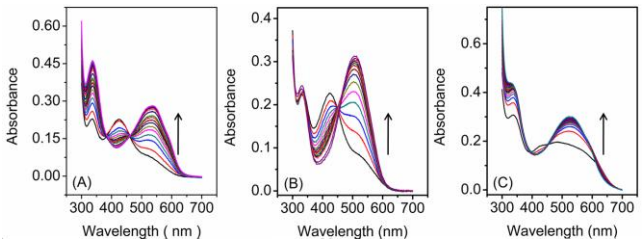

Fig. 2 UV-vis absorption spectra of ARS with the additions of BSA (A) and Cu^{2+} (B), and ARS-Cu with the additions of BSA. (c(ARS)=60.0 μmol L^{-1}; c(ARS-Cu)=60.0 μmol L^{-1}; c(BSA)= 2.0~68.0 μmol L^{-1}; c(Cu^{2+})= 10.0~160.0 μmol L^{-1}; 0.1 mol L^{-1} PBS at pH 4.8).

It was reported that the ARS-Cu complex was formed by the coordination among copper atom and oxygen atoms of carbonyl and hydroxyl groups with the stoichiometric ratio of 2:1 at pH 5.2,[20] which resulted in the decrease of the absorption of ARS and a new absorption at 508 nm. However, with the addition of BSA into ARS-Cu system, the absorptions of ARS and BSA-ARS did not appear at 425 nm and 532 nm, respectively, suggesting no competitive reaction among them. Moreover, ARS-Cu system as a complex binds to site I of BSA with the similar binding process between BSA and ARS (Fig. 3). Therefore, Cu^{2+} can not affect the binding mode of ARS to the site I of BSA.

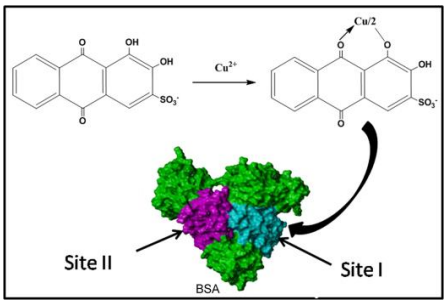

Fig. 3 Schematic diagram for the interaction of ARS with Cu^{2+} and the binding process of ARS-Cu to site I of BSA.

The effects of pH in solution on binding affinity of BSA for ARS are also investigated by UV-vis Spectroscopy. When the pH is lower than the isoelectric point (pI 4.8) of BSA,[19] BSA carries positive charge because of some protonated amino acid residues including histidine, lysine, and arginine. In contrast, BSA becomes negatively charged when pH is higher than pI. Moreover, the conformation of BSA can be unfolded in high acidic environment, resulting in a partial loss of the native conformation of BSA. As shown in Fig. 4A and B, the absorption spectra of the interactions between BSA and ARS (or ARS-Cu) at pH 4.0 show the same trend with those in pH 4.8. However, the binding capacity obviously decreased in pH 4.0, judging from the variations in the spectral behavior. Fig. 4C and D show that the absorption spectra of ARS and ARS-Cu with their protein complex overlap in partial, but the absorptions of new formed BSA-ARS or BSA-(ARS-Cu) complex dramatically increase to the highest degree. The results are ascribed to that the native conformation of BSA in pH 7.0 is benefit for the hydrophobic interaction, however, unfolded hydrophobic cavity of BSA decreases the binding capacity of ARS to BSA even through it is partially unfolded.

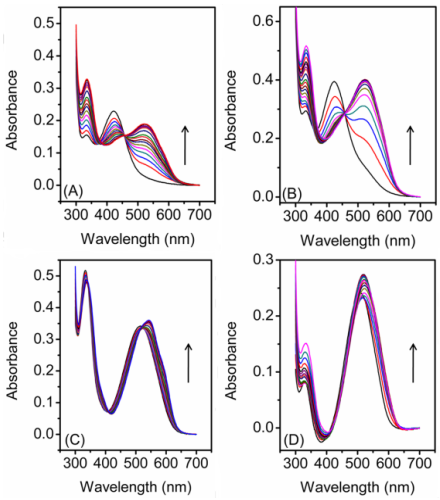

Fig. 4 UV-vis absorption spectra of ARS (A, B) and ARS-Cu (C, D) with the additions of BSA at pH 4.0 and pH 7.0, respectively. (c(ARS)=60.0 μmol L^{-1}; c(ARS-Cu)=60.0 μmol L^{-1}; c(BSA)=2.0~68.0 μmol L^{-1}; c(Cu^{2+})= 10.0~160.0 μmol L^{-1}).

3.3. CD spectra and Conformation of BSA

CD spectroscopy is a sensitive technology to monitor the secondary structure alteration of protein. Fig. 5 shows the CD spectra of BSA in the presence of ARS and ARS-Cu at pH 4.0 and 7.0. Two negative peaks at 223 nm and 209 nm were obtained, which represent the transition of n–π^* and π–π^* of α-helix structure of BSA.[21] The negative peaks changed slightly with the addition of ARS or ARS-Cu (Fig. 5A and B) at constant pH, suggesting that the secondary structure of BSA was not greatly affected by the interactions. Moreover, the presence of Cu^{2+} had a little effect on the conformation of BSA. However, as shown in Fig. 5C, decreased negative peaks at pH 4.0 shows that there is an obvious conformational change at low pH. Therefore, the change in conformation of BSA causes the unfolding of hydrophobic cavity in subdomain IIA of BSA at low pH, which decreases the binding affinity of BSA for ARS and ARS-Cu.

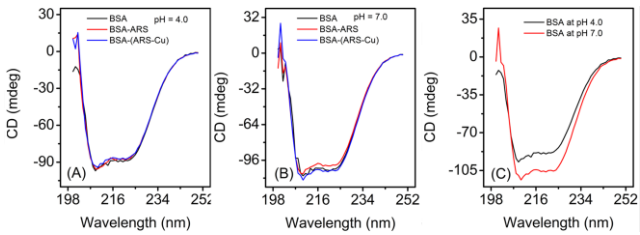

Fig. 5 CD spectra of 1.0 μmol L^{-1} BSA in the presence of 1.0 μmol L^{-1} ARS or ARS-Cu complex in 0.1 mol L^{-1} PBS at pH 4.0 (A) and pH 4.8 (B); (C) CD spectra of 1.0 μmol L^{-1} BSA at different pH.

3.4. Investigation of the binding mechanism and binding mode

In order to further understand the effects of Cu^{2+} and pH, the binding parameters of BSA with ARS and ARS-Cu were investigated by recording quenching of intrinsic tryptophan (TRP) fluorescence. The fluorescence of BSA is mainly due to the TRP residues, TRP(133) and TRP(212), which are easily quenched by

bound ligands.[3,22] Fig. 6 shows the emission spectra of BSA, along with the additions of ARS, ARS-Cu, Cu^{2+} at a temperature of 293 K. As shown in Fig. 6A and B, the fluorescence intensity of BSA dramatically decreased at 342 nm, indicating the effective quenching of BSA fluorescence by ARS and ARS-Cu. Moreover, no obvious shift occurred in the maximum emission wavelength with increasing concentrations of ARS and ARS-Cu, which suggests that the tertiary structure of BSA remained unaltered during this process. Fig. 6C shows that there is no obvious changes in the fluorescence of BSA with the addition of Cu^{2+}, which suggest the weak interaction between them .

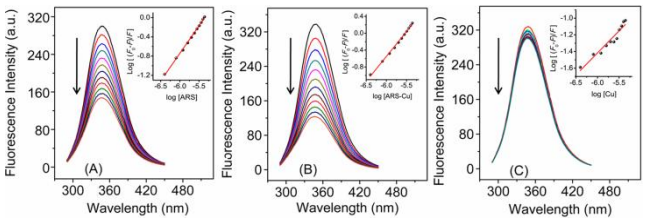

Fig. 6 The fluorescence spectra of 2.0 μmol L^{-1} BSA in 0.1 M PBS with the additions of ARS (A), ARS-Cu (B), and Cu^{2+} (C). (*c*(ARS) and *c*(ARS-Cu) from top to bottom: 0.0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.4 μmol L^{-1}; insets: plots of log [(F_0-F)/F)] *vs.* log [ARS] for the corresponding systems; pH = 4.8, T = 293 K, λ_{ex} = 280 nm).

We have proved that the fluorescence quenching process of BSA by ARS was mainly governed by a static quenching mechanism arising from the formation of the BSA-*m*ARS complex.[12] According to above results of the UV-vis spectroscopy, ARS-Cu reacted with BSA as the interaction between BSA and ARS, and thus the quenching in BSA fluorescence was resulted from the formation of BSA-*m*(ARS-Cu) ternary complex.

According to Eq.(1):[23]

$$\log\frac{F_0-F}{F}=\log K_b+m\log[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities of BSA in the absence and presence of the quenching agent (ARS or ARS-Cu), m is the average binding number of ARS molecules per protein molecule, K_b is binding equilibrium constant, and Q is the quenching agents of ARS and ARS-Cu. The binding parameters of BSA-mARS and BSA-*m*(ARS-Cu) at various pH were obtained from the linear relationships between log[(F_0-F)/F] and log[ARS] or log[ARS-Cu] (data at pH 4.0 and 7.0 were not shown), which are summarized in Table 1.

As shown in table 1, under the certain conditions of pH, increasing K_b and m show that ARS binds much more strongly with BSA in the presence of Cu^{2+}, which suggest that there is no competitive reaction among them while Cu^{2+} can improve the affinity of ARS to BSA. For BSA-*m*ARS and BSA-*m*(ARS-Cu) systems, K_b and m at pH 7.0 are found to be about 93 and 67 times higher than those at pH 4.0, respectively. The results illustrate that the affinities of ARS and ARS-Cu to BSA are decreased by the partial unfolded conformation of BSA. The interactions between BSA and Cu^{2+} are also improved by increased pH even though they are weak, which is due to the enhanced electrostatic forces between negatively charged BSA and Cu^{2+}. ARS and BSA are both negative charge carriers at pH 7.0, however, the interactions between them are not affected by the electrostatic repulsion but slightly increases. It can be inferred that hydrogen bonds predominate in the acting forces between them and the effects of electrostatic forces are relatively weak. The conclusions are in agreement with that of molecular docking.

Table 1. Various binding parameters of BSA with ARS and ARS-Cu at three different pH.

	BSA-*m*ARS		BSA-*m*(ARS-Cu)		BSA-Cu	
	logK_b	m	logK_b	m	logK_b	m
pH=4.0	4.53	0.84	5.46	0.97	0.90	0.33
pH=4.8	6.13	1.14	6.54	1.18	1.76	0.53
pH=7.0	6.50	1.14	7.29	1.29	3.18	0.70

4. Conclusions

In this paper, the effects of Cu^{2+} and pH on the binding process between ARS and BSA were investigated by UV-vis spectroscopy, CD spectroscopy, fluorescence, and molecular docking techniques. The binding mechanism, binding parameters, conformation of protein were investigated at various pH and in the presence of Cu^{2+}. ARS-Cu complex and ARS exhibit the similar binding behavior that they bind to site I of BSA. The presence of Cu^{2+} and native conformation of BSA at pH 7.0 improve the affinities of ARS to BSA. The research can provide important insights into the interactions of serum proteins with anthraquinone dyes and supply useful information about the effects of environment on their binding processes.

Acknowledgments

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Notes and references

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