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In vitro study on the binding of neutral red to bovine serum albumin by molecular spectroscopy

Li Shang, Xiue Jiang, Shaojun Dong *

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, Changchun, Jilin 130022, People's Republic of China

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Abstract

In this paper, the binding of neutral red (NR) to bovine serum albumin (BSA) under physiological conditions has been studied by spectroscopy method including fluorescence, circular dichroism (CD) and Fourier transform infrared (FT-IR) spectroscopy. The Stern–Volmer fluorescence quenching constant (K_{SV}), binding constant (K_b) and the number of binding sites (n) were measured by fluorescence quenching method. Fluorescence experiments were also performed at different ionic strengths. It was found K_{SV} was ionic strength dependent, which indicated the electrostatic interactions were part of the binding forces. The distance r between donor (BSA) and acceptor (NR) was obtained according to Foster's non-radiative energy transfer theory. CD spectroscopy and FT-IR spectroscopy were used to investigate the structural information of BSA molecules on the binding of NR, and the results showed no change of BSA conformation in our experimental conditions.

Keywords: Neutral red; Bovine serum albumin; Molecular spectroscopy

1. Introduction

The interaction of small molecules especially drugs with serum albumin is of great importance because of its physiological significance [1–3]. Serum albumin as one of the most abundant carrier proteins plays an important role in the transport and disposition of endogenous and exogenous ligands present in blood. Distribution and metabolism of many biologically active compounds such as metabolites, drugs and other organic compounds in the body are correlated with their affinities towards serum albumin [4,5]. Consequently, it is important to know the affinity of ligands to serum albumin and understand the binding mechanism. For this reason, the binding of small molecules to serum albumin in vitro, considered as a model in protein chemistry to study the binding behavior of proteins, has been studied for many years [6–14]. Various techniques have been employed to study the interaction, such as fluorescence, UV–visible, circular dichroism, Fourier transform infrared (FT-IR) and resonance Raman.

BSA has been one of the most extensively studied proteins, particularly because of its structural homology with human serum albumin (HSA) [15,16]. BSA is a small protein with a single polypeptide chain, which is cross-linked by 17 disulfide bonds. It is made up of three linearly arranged, structurally distinct, homologous domains (I–III) which are divided into nine loops (L1–L9), and each domain is composed of two subdomains (A and B). BSA has two tryptophan residues that possess intrinsic fluorescence [16]: Trp-134, located on the surface of domain I, and Trp-212, located within the hydrophobic pocket of domain II.

Neutral red (NR) is a phenazine dye (the structure shown in Fig. 1), which is similar to other planar dyes in the chemical structures belonging to the acridine, thiazine, and xanthene groups. It has been used in studies of biological systems, especially as an intracellular pH indicator [17,18], a non-toxic stain [19,20], and a probe material [21–23]. Moreover, phenazine derivatives are a kind of antibiotic. Previous studies show that some phenazine dyes have antimalarial potency and selectivity, and inhibit many bacteria from growing [24]. The study on the binding of NR to the protein may provide useful information of the structural features that determine the therapeutic effectiveness of these compounds, and help us to understand

* Corresponding author. Tel.: +86 431 5262101; fax: +86 431 5689711.
E-mail address: dongsj@ns.ciac.jl.cn (S. Dong).

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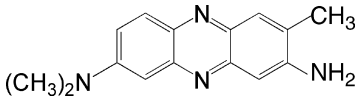

Fig. 1. Chemical structure of neutral red.

the mechanism of bioactivity of phenazine derivatives on a biomolecular level.

In one recent work [25], Salomi et al. have reported electrochemical and spectroscopic studies on the attachment of NR to BSA. However, in that study, NR was covalently coupled to the protein using a conventional coupling regent carboiimide. In this paper, the binding of NR to BSA was studied in detail for the first time under physiological conditions, by molecular spectroscopy including fluorescence, circular dichroism and Fourier transform infrared (FT-IR) spectroscopy. Utilizing the fluorescence quenching experiment results, the binding parameters were calculated. In addition, the binding mechanism was discussed.

2. Materials and methods

2.1. Reagents and materials

Bovine serum albumin (BSA, fraction V, approximately 99%), purchased from Sigma chemical company (USA), was used without further purification. Stock solution of BSA was prepared in 0.01 M phosphate buffer solution (PBS) of pH 7.40, containing 0.01 M NaCl. The concentration of BSA was calculated based on the molecular weight of 65,000 [12]. Neutral red was bought from Shanghai No. 1 chemical company (Shanghai, China), and used after recrystallization from ethanol. BSA and NR stock solutions were freshly prepared before the measurements.

Buffer solutions with different ionic strength used in the experiment were prepared by adding the crystals of sodium chloride (NaCl, analytical grade) with calculated amounts into the buffer solution. All other materials were of analytical reagent grade. Doubly purified water from Milli-Q system was used throughout experiment. All the experiments were performed at room temperature (293 K).

2.2. Apparatus and methods

2.2.1. Fluorescence measurements

Fluorescence measurements were performed on a Perkin-Elmer LS-55 Luminescence Spectrometer. The spectra were recorded in the wavelength of 320–500 nm upon excitation at 300 nm, using 10 nm/10 nm slit widths. The fluorescence quenching experiments were performed by keeping the BSA concentration constant while varying the NR concentration. All test solutions were incubated for 10 min before the spectra were obtained. A 1.00 cm path length rectangular quartz cell was used for these studies. The experiments were repeated and found to be reproducible within experimental errors.

2.2.2. CD and FT-IR experiments

Circular dichroism experiments were made on a 62A DS CD spectrometer (AVIV, USA) with a 1.0 cm path length rectangular quartz cell controlled by a thermoelectric cell holder (AVIV). CD spectra were taken in the wavelength of 200–250 nm, and the results were expressed as molar ellipticity (mdeg).

FT-IR spectra were recorded on a Nicolet 520 FT-IR spectrometer equipped with a germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector, and a KBr beam splitter. The spectra resolution used was 4 cm^{-1}, and 64 scans were co-added to ensure a good signal-to-noise ratio.

The NR-BSA solutions studied in the CD and FT-IR experiments were prepared by mixing the NR and BSA solutions to keep the final NR-BSA ratios at 0:1, 2:1 and 4:1. The BSA concentrations for CD and FT-IR experiments were 1×10^{-6}, 5×10^{-5} M, respectively.

3. Results and discussion

3.1. Fluorescence studies

Fluorescence spectroscopy is the most favorable technique in the study of molecular interactions, in particular because of its high sensitivity, and multiplicity of measurable parameters. To find the binding of NR to BSA in detail, fluorescence experiments were carried out. An excitation wavelength of 300 nm was chosen in our experiment, since it provides only excitation of tryptophan residues [26]. In order to avoid inner filter effect, very dilute solutions were used in the experiment (BSA concentration of 2×10^{-6} M and NR concentration in the range of 3.3×10^{-7}–4×10^{-6} M). Therefore the absorbance of dye–protein mixtures did not exceed 0.05 at the excitation wavelength 300 nm [27]. Because NR was prepared in ethanol, the quenching effect of ethanol was then evaluated. The result showed that the effect of ethanol on the NR interaction with BSA could be negligible in the amount used in our experiment. Also appropriate blanks corresponding to the buffer were subtracted to correct the fluorescence background.

3.1.1. Stern–Volmer analysis

Fig. 2 shows the emission spectra of BSA in the presence of various concentrations of NR at pH 7.40, characterized by a band with maximum emission at 352 nm. It could be seen from Fig. 2 that NR caused a concentration dependent quenching of the intrinsic fluorescence of BSA. The fluorescence intensity decreased gradually upon increasing the concentration of NR, without changing the position of emission maximum, indicating that NR bound to BSA without affecting the immediate environment of the tryptophan residues [28].

Fluorescence intensity data were then analyzed using Stern–Volmer equation [27]:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities at 352 nm in the absence and presence of quencher (NR), respectively, k_q the bimolecular quenching constant, τ_0 the lifetime of the flu-

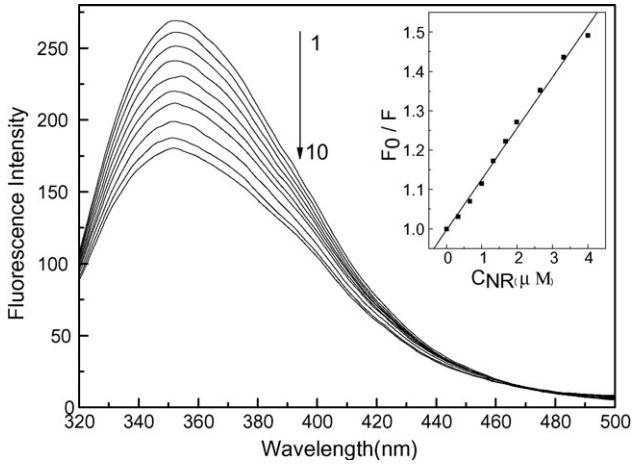

Fig. 2. Fluorescence emission spectra of 2.0×10^{-6} M BSA in the presence of different concentrations of NR at pH 7.40. The concentrations of NR (10^{-6} M): (1) 0; (2) 0.33; (3) 0.67; (4) 1.00; (5) 1.33; (6) 1.67; (7) 2.00; (8) 2.66; (9) 3.31; (10) 4.00. Inset-Stern–Volmer plots of BSA with increasing concentrations of NR.

orophore in the absence of quencher, K_{SV} the Stern–Volmer fluorescence quenching constant, which is a measurement of the efficiency of quenching by quencher and $[Q]$ is the quencher concentration.

The inset in Fig. 2 shows the Stern–Volmer plots, F_0/F versus [NR], according to Eq. (1). The resulted plots exhibited a good linear relationship ($R = 0.997$). As is known, a linear Stern–Volmer plot is generally indicative of a single class of fluorophore, all equally accessible to quencher [28,29]. K_{SV}, calculated by linear regression of the plots, was $1.30 \times 10^5\ M^{-1}$. From Eq. (1) we know $K_{SV} = k_q \tau_0$. For BSA, τ_0 is known to be approximately 5×10^{-9} s [27], thus $k_q = 2.60 \times 10^{13}\ M^{-1}\ s^{-1}$ was obtained. Since the maximum value of k_q for a diffusion-controlled quenching process is about $10^{10}\ M^{-1}\ s^{-1}$, the higher value (over 2000-fold) obtained here suggested that the quenching of tryptophan fluorescence occurred by a specific interaction between BSA and NR [30,31]. This also implied that the dominating quenching mechanism was not dynamic but static (formation of a complex) [32].

3.1.2. Binding parameters

Fluorescence intensity data were also used to obtain the binding constant (K_b) and the number of binding sites (n). When small molecules bind independently to a set of equivalent sites on a macromolecule, the values of K_b and n can be determined according to the method described by Liu and Wang et al. [14,32] using the equation:

$$\log \frac{F_0 - F}{F_0} = \log\ K_b + n\ \log[Q] \quad (2)$$

where K_b is the binding constant for quencher–protein interaction, n the number of binding sites per BSA, and F_0, F, $[Q]$ have the same meanings as in Eq. (1). The values of K_b and n could be measured from the intercept and slope by plotting $\log (F_0 - F)/F_0$ against $\log [Q]$.

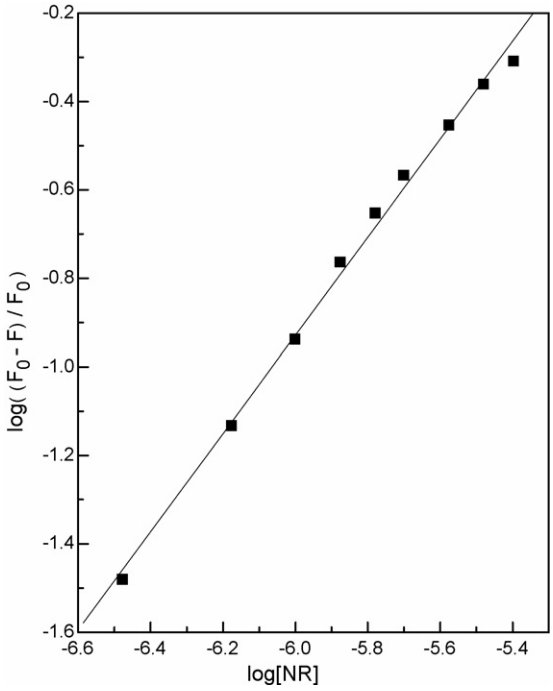

Fig. 3. Best fit of the experimental data at λ = 352 nm using Eq. (2).

By linear regression of the plots (Fig. 3), K_b, $2.36 \times 10^5\ M^{-1}$ and n, 1.10 were obtained, respectively. As compared to other small molecules with the similar structure on non-covalent binding of serum albumins previously reported, the value of K_b for NR is relatively large [26,33,34]. This may because most of its atoms are in the planar phenazine ring, so less steric hindrance would be involved in the binding process. As a result, the binding of NR to BSA became easier relatively. Also the methyl group in the ring was reported to enhance the binding of the molecule significantly [35]. Utilizing K_b, the free energy change (ΔG_0) value estimated from the relationship: $\Delta G_0 = -RT \ln K_b$ was $-30.14\ kJ\ mol^{-1}$. The negative sign for ΔG_0 indicated the spontaneity of the binding of NR to BSA.

3.1.3. The effect of ionic strength on the binding

In order to determine the influence of the ionic strength on the binding process [36], fluorescence experiments were carried out in the presence of different concentrations of NaCl (10, 100 and 200 mM). For NaCl, which is not an anionic quencher, its influence on the BSA fluorescence intensity only comes from the ionic strength [37].

Fig. 4 shows the Stern–Volmer plots in the solution with different ionic strength. With increasing the ionic strength of the solution, the decrease of the BSA fluorescence intensity on the addition of the same amount of NR became smaller gradually, which meant a decrease of quenching efficiency in the binding. The slope of Stern–Volmer plots is actually related to the accessibility (degree of exposure) of the tryptophans to the aqueous quencher (NR). As can be seen in Fig. 4, at higher concentration of NaCl, the slope was smaller, implying decreased accessibility of NR molecules to BSA binding sites. As a result, the fluorescence intensity of the system decreased tardily at

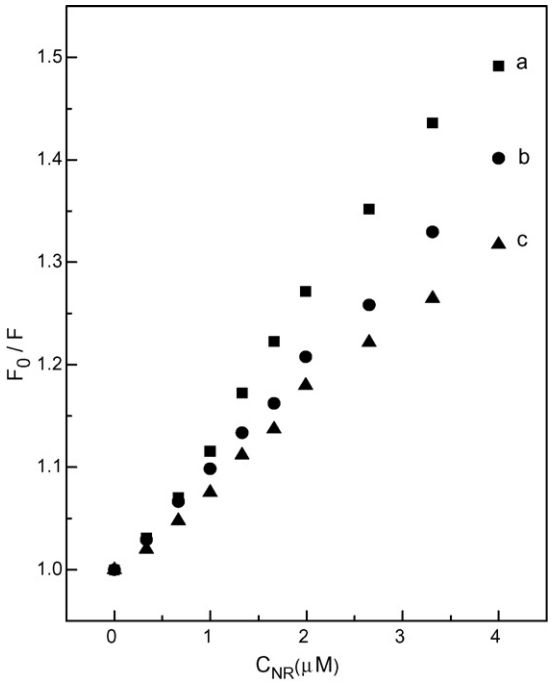

Fig. 4. Stern–Volmer plots of BSA in the presence of NR at different NaCl concentrations: (a) 10 mM; (b) 100 mM; (c) 200 mM.

higher concentration of NR which indicated the beginning of saturation of the BSA binding sites [38]. The Stern–Volmer constant obtained was decreased from 1.30×10^5 to 8.16×10^4 M^{-1} when concentration of NaCl increasing from 10 to 200 mM. The fact that the quenching was not favored by the increase of the ionic strength, also leads to the idea that the electrostatic interactions should be part of the binding forces. Whether there are other forces (hydrophobic force, van der Waals interaction or hydrogen bonds, etc.) involved in the binding process is unclear at present due to the lack of thermodynamic information, and needs further study.

3.1.4. Energy transfer between BSA and NR

The fluorescence studies proved that BSA formed a complex with NR, and the distance between tryptophan residues and bound NR molecules can be determined according to Föster's non-radiative energy transfer theory [39]. According to this theory, the efficiency of energy transfer between the donor (BSA) and acceptor (NR), E, can be calculated using the Eq. (3):

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{3}$$

where r is the distance between the donor and acceptor, and R_0 is the critical distance when the transfer efficiency is 50%, which can be calculated by Eq. (4):

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \tag{4}$$

where k^2 is the spatial orientation factor of the dipole, N the refractive index of medium, Φ the quantum yield of the donor in the absence of acceptor and J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption

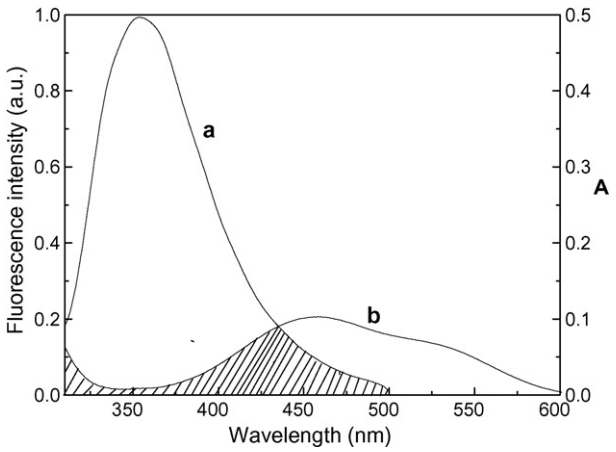

Fig. 5. Spectral overlap of BSA fluorescence emission (curve a) with NR absorption (curve b). $C_{NR} = C_{BSA} = 1.0 \times 10^{-5}$ M.

spectrum of the acceptor. J can be obtained by the Eq. (5):

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \tag{5}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength λ; and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. Fig. 5 shows the spectral overlap of BSA fluorescence emission (curve a) with NR absorption (curve b).

In the present case, $k^2 = 2/3$, $N = 1.36$, and $\Phi = 0.15$ [40]. According to the above Eqs. (3)–(5), we found $J = 1.64 \times 10^{-14}$ cm^3 L mol^{-1}, $R_0 = 2.35$ nm, and $r = 2.25$ nm. Obviously, the acceptor–donor distance is less than 8nm, which indicated the energy transfer from BSA to NR occurred with high possibility. It also suggested that the binding of NR to BSA was through energy transfer, which quenched the fluorescence of BSA [41,42].

3.2. CD and FT-IR studies

To investigate further whether any structural changes of BSA molecules occurred on the binding of NR, CD experiments at pH 7.40 were carried out. The spectra were recorded in the absence and presence of NR. The CD spectra of BSA (curve a in Fig. 6) showed a strong negative ellipticity at 209 and 222 nm, which is characteristic of an α-helical structure of protein [43]. However, the addition of NR caused no change of the spectrum in either the position or the intensity of the bands in our experiments (curve b in Fig. 6). This revealed that no change of BSA conformation had occurred on the binding of NR.

FT-IR is another powerful technique for structural characterization of proteins besides CD spectroscopy. The protein amide bands have a relationship with the secondary structure of protein. If there had been a change of BSA conformation, an obvious shift of the peak corresponding to the N–H residual amide would have been observed [44]. The results showed that no changes occurred in the spectra of BSA, either (data not shown). This result was in agreement with the result of CD experiments that

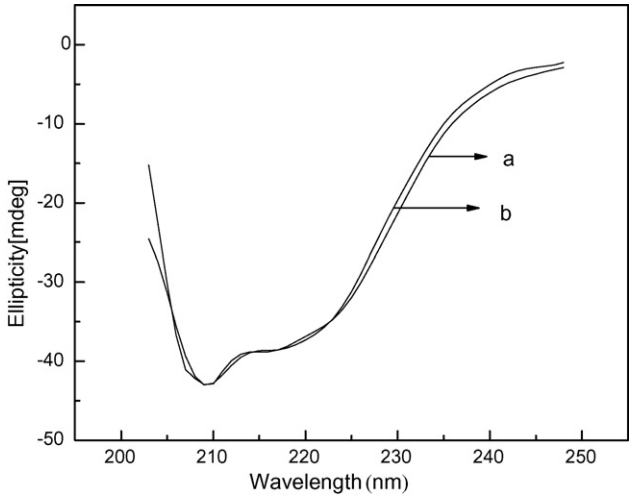

Fig. 6. Circular dichroism spectra of 1.0×10^{-6} M BSA in the absence (curve a) and presence of 4×10^{-6} M NR (curve b) at pH 7.40.

interaction with NR had not changed the secondary structure of BSA.

Previous studies on the interaction between BSA and anesthetic chloroform found that retention of protein conformation was important for ligands binding [45]. This may also be the case of the interaction between BSA and NR. Also occupancy of the tryptophan sites by the binding ligands could stabilize the native conformation of protein. Frazier and co-workers [28] studied the interaction of flavonoids with BSA and observed that flavonoids could bind tightly to the protein without affecting its secondary structure, which agrees with our results.

4. Conclusions

In this paper, the binding of neutral red to bovine serum albumin was investigated by molecular spectroscopy including fluorescence, CD and FT-IR spectroscopy. The study shows that NR binds to BSA with high affinity, and quenches the intrinsic fluorescence of BSA efficiently. According to fluorescence quenching method, binding constant (K_b), $2.36 \times 10^5\ M^{-1}$ and the number of binding sites (n), 1.10 are obtained. And electrostatic interactions are found to be part of the binding forces in the quenching process. The distance between donor and acceptor (r), 2.25 nm, is also obtained according to Föster's non-radiative energy transfer theory. In addition, the results of both CD and FT-IR indicate no change of BSA conformation on the binding of NR in our experimental conditions.

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