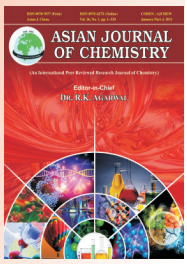
Asian Journal of Chemistry; Vol. 26, No. 24 (2014), 8423-8426

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.17844



Spectroscopic Studies on Interaction Between Enalapril Maleate and Bovine Serum Albumin

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Received: 18 April 2014; *Accepted*: 18 June 2014; *Published online*: 1 December 2014; AJC-16374

The interaction between enalapril maleate and bovine serum albumin was investigated by fluorescence and absorption spectroscopy under simulative physiological conditions. It was revealed that the quenching mechanism of bovine serum albumin by enalapril maleate is static quenching mechanism. The binding sites number and binding constant were obtained at different temperature. The thermodynamic parameters were abtained according to van't Hoff equation and the result indicates that the interaction between enalapril maleate and bovine serum albumin is driven mainly by electrostatic forces. The distance between enalapril maleate and the protein was calculated to be about 3.78 nm according to the theory of Föster energy transfer.

Keywords: Bovine serum albumin, Enalapril maleate, Fluorescence spectroscopy, Binding constant.

INTRODUCTION

It is known that the serum albumin is an important part of an organism, which plays an important role in the storage and transport of many drug molecules in the blood[1,2]. The binding ability of drug-albumin in blood stream may have a significant impact on distribution, free concentration and metabolism of drug. Therefore, researching the interaction between the small molecules of biological activity and albumin is significant for knowing the action mechanism of drugs in body[3].

In this work, bovine serum albumin is selected as our protein model because of its medical importance and structural homology with human serum albumin, which is widely used in the field of molecular and cell biology[4]. Enalapril maleate *i.e.*, N-(N-S-1-ethoxycarbonyl-3-phenylpropyl)-L-alanyl-L-proline hydrogen maleate, is a kind of white or almost white crystalline powder, odorless and slightly hygroscopic, which is an anti-hypertensive drug. It is liable to degrade to the enalaprilate in aqueous solution, which can lower blood pressure through inhibiting the angiotensin-converting enzyme, reducing levels of angiotensin-II, expanding the blood vessels and reducing peripheral resistance[5]. It can also be used to treat congestive heart failure, reduce mortality and improve hemodynamics. Because of its wide applications, many studies have been carried out to investigate the characteristics and applications of enalapril maleate[6-8], but we found that there is few investigations which have been conducted to study the interaction between enalapril maleate and bovine serum albumin, such as the binding mechanism, binding site, binding distance, *etc*.

At present, there are many methods investigating the interaction of drug small molecules and protein. For example, spectrum method, electrochemical method, capillary electrophoresis, *etc*[9-13]. Fluorescence combined with absorption spectrum is an effective technology to study the drug and protein binding mechanisms, which can provide some valuable information[14,15]. In this paper, the binding of enalapril maleate and bovine serum albumin was studied under physiological buffer solution (pH 7.4) based on the spectrum analysis technology and corresponding characteristic parameters are calculated and analyzed.

EXPERIMENTAL

Bovine serum albumin (Amresco, 96 % purity) was used without further purification. Enalapril maleate was obtained from Shanghai Modern Pharmaceutical Group Co., Ltd. (Shanghai, China). bovine serum albumin stock solution (4.8×10^{-6} mol L^{-1}) was prepared in pH 7.4 phosphate buffer solution containing 0.1 mol L^{-1} NaCl. The enalapril maleate solution (0.2×10^{-3} mol L^{-1}) was prepared in pH 7.4 phosphate buffer solution. All other chemicals were analytical reagent grade and double distilled water was used throughout.

Fluorescence measurements were performed on an RF-5301PC spectrofluorophotometer (Shimadzu, Japan) equipped with a 150-W Xenon lamp and a slit width of 5 nm, using a quartz cell of 1.0 cm path length. The absorption spectrum

was recorded with a UV-3600PC spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cell.

A solution (2.5 mL), containing appropriate concentration of bovine serum albumin (0.48×10^{-6} mol L^{-1}), was titrated by successive additions of stock solution of enalapril maleate (0.2×10^{-6} mol L^{-1}) and the final concentration of enalapril maleate is 3.10×10^{-5} mol L^{-1}. The fluorescence emission spectra were obtained at different temperatures (300 and 313 K) with exciting wavelength at 280 nm and the wavelength and intensity of maximum fluorescence peak were recorded.

The fluorescence intensities were corrected for the absorption of excitation light and re-absorption of emitted light to decrease the inner filter using the following relationship[16]:

$$F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2} \quad (1)$$

where F_{cor} and F_{obs} is the corrected and observed fluorescence intensities, respectively. A_{ex} and A_{em} are the absorbance values of enalapril maleate at excitation and emission wavelengths, respectively. The fluorescence intensity used in this paper was corrected.

RESULTS AND DISCUSSION

Fluorescence quenching of bovine serum albumin by enalapril maleate: Protein has a strong endogenous fluorescence because it contains tryptophan, tyrosine and phenylalanine residues. Fig. 1 shows the fluorescence spectra of bovine serum albumin in the presence of different concentrations of enalapril maleate at 300 K. It was observed that bovine serum albumin has a strong fluorescence emission peak at 339 nm with excitation wavelength of 280 nm. Moreover the fluorescence intensity of bovine serum albumin was decreased gradually with increasing the concentration of enalapril maleate, indicating that there was the interaction between enalapril maleate and bovine serum albumin. Furthermore, there was a slight blue shift at the maximum wavelength of bovine serum albumin (from 339 to 332 nm) after the addition of enalapril maleate, which indicates the change in conformation of bovine serum albumin.

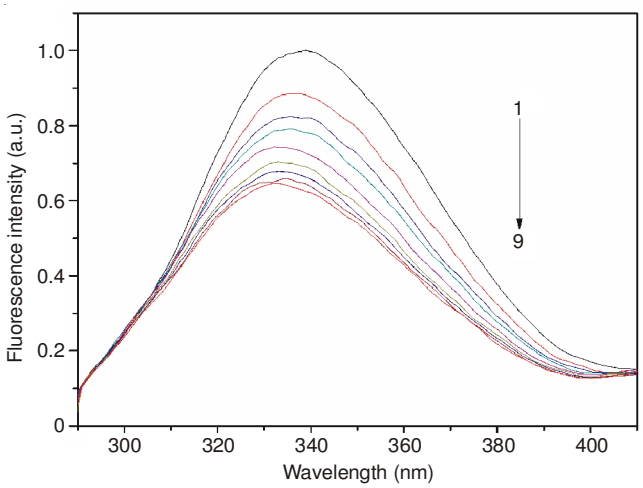

Fig. 1. Fluorescence quenching spectra of bovine serum albumin at different concentrations of enalapril maleate (the concentration of enalapril maleate corresponding to 0, 0.40, 0.78, 1.15, 1.85, 2.18, 2.49, 2.80 and 3.10×10^{-5} mol/L from curve 1 to 9; $C_{BSA} = 0.48 \times 10^{-6}$ mol/L; T = 300 K; λ_{ex} = 280 nm; pH = 7.4)

Fluorescence quenching mechanism: It is known that there are two quenching mechanisms involved in quenching process, which are usually classified as dynamic quenching and static quenching. Fluorescence quenching followed the Stern-Volmer equation[17]:

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (2)$$

$$k_q = K_{SV}/\tau \quad (3)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. k_q is the quenching rate constant and its maximum dynamic fluorescence quenching rate constant is about 2×10^{10} L mol^{-1} s^{-1} and τ_0 is the average life-time of the biomolecule without quencher (10^{-8} s)[18]. K_{SV}, [Q] are the Stern-Volmer dynamic quenching constant and the concentration of the quencher, respectively.

Fig. 2 shows the Stern-Volmer plots of the quenching of bovine serum albumin fluorescence by enalapril maleate at different temperatures (300 and 313 K) and the corresponding results are listed in Table-1. It is known that dynamic and static quenching can be distinguished by their different dependence on temperature. The quenching rate constants decrease with increasing temperature for static quenching, but the reverse effect is observed for dynamic quenching[18]. According to the result in Table-1, the values of Stern-Volmer quenching constants K_{SV} and k_q decrease with increasing temperature and the values of are much greater than the limiting diffusion rate constant of the biomolecule (2×10^{10} L mol^{-1} s^{-1}), which implied that the quenching was not initiated by dynamic collision but originated from the formation of a complex.

TABLE-1
STERN-VOLMER QUENCHING CONSTANTS OF THE INTERACTION BETWEEN BOVINE SERUM ALBUMIN AND ENALAPRIL MALEATE AT 300 AND 313 K

T(K)	K_{SV} ($Lmol^{-1}$)	k_q ($Lmol^{-1}s^{-1}$)	R
300	1.72×10^4	1.72×10^{12}	0.9984
313	1.63×10^4	1.63×10^{12}	0.9964

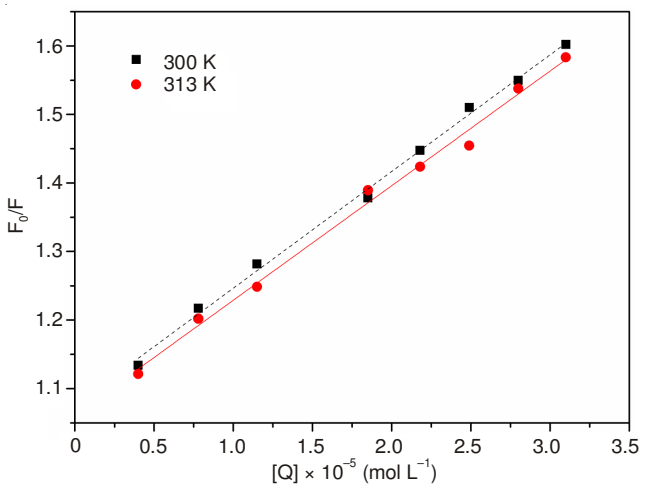

Fig. 2. Stern-Volmer plots for the quenching of bovine serum albumin by enalapril maleate at different temperatures

Binding constant and the number of binding site: According to the experimental results, we know that the fluorescence quenching type of bovine serum albumin initiated by

enalapril maleate was static quenching. For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by the binding constant formula[19]:

$$\log\left(\frac{F_0 - F}{F}\right) = n\log K_A + n\log\left([D_t] - n[P_t]\frac{F_0 - F}{F_0}\right) \quad (4)$$

where $[D_t]$ and $[P_t]$ are the total concentrations of enalapril maleate and bovine serum albumin, respectively. K_A and n are the binding constant and the number of binding sites, which were calculated and shown in Table-2. The fact that the values of n is approximately to 1 implied that just one binding site for enalapril maleate existed in bovine serum albumin.

TABLE-2
BINDING PARAMETERS, NUMBER OF BINDING SITES AND THERMODYNAMIC PARAMETERS FOR BSA–ENALAPRIL MALEATE COMPLEX AT DIFFERENT TEMPERATURES

T(K)	K_A ($mol^{-1}L$)	n	R^a	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
300	1.69×10^4	0.75	0.9991	-8.80	-24.48	51.60
313	1.46×10^4	0.73	0.9971	-8.80	-24.95	51.60

R^a is correlation coefficient

The molecular forces contributing to protein interactions with small molecular substrates may include vander Waals interactions, hydrogen bonds, electrostatic and hydrophobic interactions, *etc.*[20]. The thermodynamic parameters *e.g.*, enthalpy change (ΔH) and entropy change (ΔS) of enalapril maleate-bovine serum albumin interactions were important for confirming binding mode. Therefore, the temperature dependence of binding constant was studied. If the temperature does not vary significantly, the enthalpy change can be regarded as a constant. The thermodynamic parameters were calculated from the van't Hoff equation:

$$\ln\frac{(K_A)_2}{(K_A)_1} = \left(\frac{1}{T_1} - \frac{1}{T_2}\right)\frac{\Delta H}{R} \quad (5)$$

$$\Delta G = -RT\ln K_A \quad (6)$$

$$\Delta G = \Delta H - T\Delta S \quad (7)$$

where R is the gas constant, T is the experimental temperature K_A and is the binding constant at corresponding temperature (T). Table-2 gives the thermodynamic parameters for the interaction of enalapril maleate with bovine serum albumin. The values of ΔH and ΔG for the binding reaction between enalapril maleate and bovine serum albumin are found to be -8.8, -24.48 and -24.95 $kJmol^{-1}$ at 300 and 313 K, respectively. The negative sign for ΔG means the interaction process is spontaneous at the standard state. The negative value of ΔH and the positive value ΔS indicate that electrostatic force plays a major role in the binding of enalapril maleate to bovine serum albumin[20].

Energy transfer from bovine serum albumin to enalapril maleate: According to Föster theory, the efficiency of fluorescence resonance energy transfer (FRET) depends mainly on the following factors: (1) the extent of overlap between the donor emission and the acceptor absorption, (2) the orientation of the transition dipole of donor and acceptor, (3) the distance between the donor and acceptor (below 7 nm)[14]. The spectral overlap between the fluorescence spectra of bovine serum albumin and the absorption spectrum of enalapril maleate is shown in Fig. 3. The energy transfer efficiency is defined as the following equation:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (8)$$

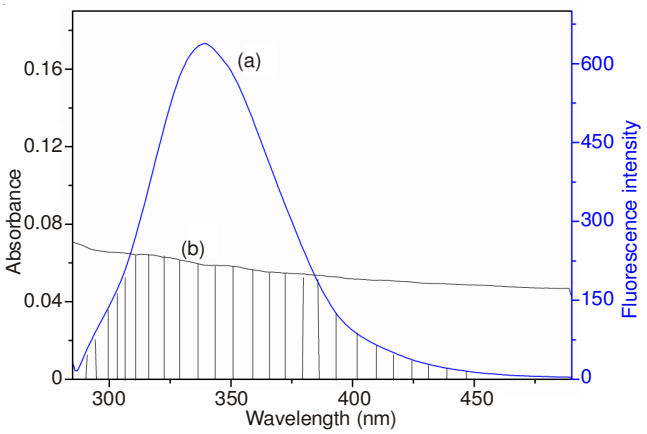

Fig. 3. Overlap of the fluorescence spectrum of bovine serum albumin (a) and the absorption spectrum of enalapril maleate (b) ($C_{BSA} = C_{enalapril\ maleate} = 4.8 \times 10^{-6}$ mol/L)

where r is the distance between acceptor and donor and R_0 is the critical distance when the transfer efficiency is 50 %[21]. R_0 is given by the following equation:

$$R_0^6 = 8.79 \times 10^{-25} k^2 N^{-4} \varphi J \quad (9)$$

where k^2 is the spatial orientation factor of the dipole, N is the refractive index of medium, φ is the quantum yield of the donor and J is the overlap integral of the fluorescence emission spectrum of the donor (bovine serum albumin) with the absorption spectrum of the acceptor (enalapril maleate), which can be calculated by the following equation:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (10)$$

where F(λ) is the fluorescence intensity of the donor in the wavelength range λ to λ + Δλ, ε(λ), is the molar absorption coefficient of the acceptor at λ. In the present case[22-24], k^2 = 2/3, N = 1.336 and φ =0.118. From eqns. 8 to 10, we could calculate that J = 1.84×10^{-14} cm^3 L mol^{-1}, R_0 = 2.71 nm, E = 0.12, r = 3.78 nm. It is seen that the donor-to-acceptor distance is much smaller than 7 nm, which implies a high probability of the energy transfer from bovine serum albumin to enalapril maleate. It is further confirmed that the fluorescence quenching is static quenching between enalapril maleate and bovine serum albumin.

Conclusion

The interaction between enalapril maleate and bovine serum albumin in physiological buffer solution was studied by fluorescence and absorption spectroscopy. The results showed that the fluorescence of bovine serum albumin was quenched by enalapril maleate through a static quenching process. The binding constant and the number of binding site were obtained at 300 and 313 K, respectively. According to the thermodynamic parameters calculated from van't Hoff

equation, it is confirmed that the electrostatic force plays an important role in stabilizing the complex. The average binding distance between the donor (bovine serum albumin) and the acceptor (enalapril maleate) is about 3.78 nm.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from the National Natural Science Foundation of China (10722043), the ministry of education of China Doctor Station Fund (20093218110024), Funding for Outstanding Doctoral Dissertation in NUAA (BCXJ12-10), Funding of Jiangsu Innovation Program for Graduate Education (CXLX12_0143), Fundamental Research Funds for the Central Universities, Huaian Science and Technology Support Program (HASZ2013007) and the Young Teachers of Huaiyin Institute of Technology (HGC1318).

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