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Reaction Mechanism Spectroscopy Studies of Protein and Orange Ⅱ

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In this paper, the interaction of Orange Ⅱ and Human Serum Albumin was studied by fluorescence spectroscopy under simulated physiological conditions. The affection of pigment and serum albumin may cause changes in the microenvironment. Fluorescence spectroscopy using synchronous fluorescence can provide a reference for the micro-environment changes in serum albumin. By calculating quenching constants at different temperatures, its quenching mechanism which is static quenching was determined. The Orange Ⅱ and HSA binding constants and binding sites were also calculated and the electrostatic force of Orange Ⅱ with HSA was determined according to the thermodynamic formula. According to the Fōrster theory, the energy transfer efficiency and biding distance of orange Ⅱ between HSA were measured and the energy transfer quenching mechanism by the energy transfer was elaborated. Further application of UV absorption spectroscopy to study the mechanism of action and the conclusion of orange Ⅱ fluorescence spectra are consistent with the HSA.

1. Introduction

Human Serum albumin (HSA) is an important carrier protein responsible for the storage and transportation in human blood (H.P.Yan, Y.Zhao.Liu, 2014), which can combine with many substance. From now on, people have been quiet familiar with the study of HSA. As early as 1975, scientists have already known that the complete primary structure of HSA is composed of 585 amino acid residue, 17 disulfide bridges and one tryptophan residue.Orange is a kind of N-containing dyestuff, which have good affinity with lactoprotein (Tan Tao, huang, ning xia, 2007).The tone of Orange changed from orange-yellow to yellow, which is suitable for the dye of food, such as cheese, meat, sugar, beverage as well as dessert. The colorant contain two types (gui-zhi li, liu ym, 2006): natural pigment and synthetic pigment. Synthetic pigment has been widely used for its brilliant colors, lower price, strong tinting strength and good stability. However, recent studies show that the intake of synthetic pigment will influence the intelligence development of children, thus causing the allergy and diarrhea and some of which may also have serious chronic toxicity and carcinogenesis. Therefore, the type and dosage of synthetic pigment must be controlled strictly.

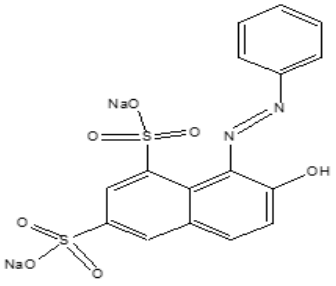

Figure 1: The orange Ⅱ structure

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At present, the study of action mechanism between protein and medicine have been widely reported, while the mechanism between protein and pigment are much rarely. The biological activity of dye molecular is embodied by its interaction with protein. Hence, the reaction mechanism of dye molecular and protein has become a common interesting task in the file of chemistry, pharmacy, clinical medicine and bioscience. In this paper, we used fluorescent and ultraviolet spectroscopy to study the reaction mechanism between Orange Ⅱ and HSA. The study may have some guiding significance in the application of synthetic pigment in food.

2. Materials and Methods

2.1. Instruments and reagents

The fluorescence photometer F-4600 was from the Hitachi Company, the ultraviolet and visible spectrophotometer UV-2550 was from SIMADZU Corporation, the digital constant temperature water bath 501A was from Shanghai Pudong Science apparatus company and the electric thermostatic bath SWQP.

The human serum albumin (HSA) (1.0×10^{-5}mol/L,>98%) was purchased from the Zhongsheng Beikong biotechnology Co. Ltd. and restored in dark condition at 4˚C. Orange Ⅱ (1.0×10^{-3}mol/L,>99%), national drug standard substance, was purchased from the Dalian Melon biotechnology Co. Ltd. A NaCl standard solution of 0.1mol/L was made to maintain the ionic strength and the Tris-HCl buffer solution (PH=7.34) was made from 0.1 mol.L^{-1}Tris and HCl.All the reagents were analytically pure and double-distilled water was used to prepare all the solutions mentioned above.

2.2. Experimental methods

2.2.1 Fluorescence spectrum

Tris-HCl buffer solution(PH=7.4), NaCl standard solution (0.1mol/L) and HSA (1.0×10^{-5}mol/L) of 1ml respectively were added into a dry colorimetric tube of 10ml. Different volume of Orange Ⅱ(1.0×10^{-5}mol/L) were added then and the volume was set to 10ml using double-distilled water. The solution was held at 298K, 303K, 308K, and 313K for 10 minutes.Set the intensity of no orange Ⅱ as F_0 and the one containing orange Ⅱ as F. Both the excitation and emission slit widths were set at 10nm. The fluorescence intensity of BSA at 340nm was recorded.

2.2.2 Ultraviolet spectrum

Tris-HCl buffer solution(PH=7.4), NaCl standard solution (0.1mol/L) and HSA (1.0×10^{-4}mol/L) of 1ml respectively were added into a dry colorimetric tube of 10ml. Different volume of Orange Ⅱ(1.0×10^{-5}mol/L) were added then and the volume was set to 10ml using double-distilled water. By using the solution of no HSA as blank control, the solution was held at 298K for 10 minutes after shaking. Determine the ultraviolet spectrum of Orange Ⅱ interacted with HSA.

3. Results and discussion

3.1 Fluorescence quenching mechanism

Fluorescence quenching is referred to the process in which the fluorescence intensity becoming weaker for the interaction of fluorescent molecule and solvent molecule. Many interactions such as molecular rearrangement, energy transfer, formation of ground-state and collisional quenching will cause the fluorescence quenching of the excited fluorescence group. The fluorescence spectrum of Orange Ⅱ and HSA were shown in figure 2.

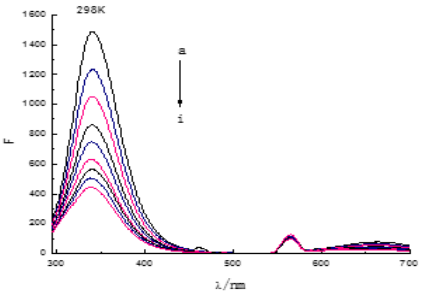

Figure 2: Quenching fluorescence spectra of HSA-Orange Ⅱ

a-i: C_{HSA}=1.0×10^{-5}mol/L, C_{Orange}= (0,0.51.0,1.5,2.0,2.5,3.0,3.5,4.0)×10^{-5} mol/L

Figure 2 shows that the fluorescence intensity of HSA decreased successively with the increasing concentration of OrangeⅡ, and the largest emission wavelength generated a slight blue-shift. The type of the fluorescence quenching can be judged by the relationship of quenching constant and temperature. The quenching constant which decreased with temperature rising is defined as static quenching, and the one increased as dynamic quenching.

The fluorescence quenching constant was substituted into Stern-Volmer equation (huang, ning xia and Gong Ping, 2008) for analysis.

$$F_0/F=1+Kq\tau_0[C]=1+Ksv[C] \quad (1)$$

In the equation, F_0 represents the fluorescence intensity without fluorescer and F with fluorescer. [C] represents the concentration of quencher and τ_0 represents the average lifetime of HSA (generally about 10^{-8}s) (C. Q. Jiang,etc, 2002). Ksv(L/mol) represents Stern-Volmer quenching constant and Kq quenching rate constant(Wang Huan,etc,2012). The results are shown in table 1.

In the static quenching action, the fluorescence intensity conform to the following equation (bao-sheng liu, etc, 2005) after adding different concentration of pigment.

$$\lg[(F_0-F)/F]=n\lg K_A+n\lg[C] \quad (2)$$

In the plot of lg[(F_0-F)/F] to lg[C], the linear graph was obtained. The value of binding constant K_A and biding-site number n of pigment and HSA were obtained(X.B.Hai,etc,2008). The results are listed in Table 1.

Table 1: Quenching reactive parameter of HSA-Orange Ⅱ at different temperature

Temperature/K	F_0/F - [c]	K_q (L/mol s)	R_a	K_A (L/mol)	R_b	n
298	0.99+7.08×10^{4}C	7.08×10^{12}	0.9982	3.55×10^4	0.9951	0.93
303	0.79+6.32×10^{4}C	6.32×10^{12}	0.9972	13.80×10^4	0.9999	1.09
308	0.88+5.80×10^{4}C	5.80×10^{12}	0.9943	27.54×10^4	0.9975	1.16
313	0.72+5.33×10^{4}C	5.33×10^{12}	0.9978	75.86×10^4	0.9984	1.28

R_a and R_b represent the linear correlation coefficient of equation F_0/F-[C] and log [(F_0-F)/F]-log[C], respectively. Table 1 shows that the value of Kq are all much greater than the largest dynamic quenching constant 2.0 ×10^{10}L ·mol^{-1} ·s^{-1} (Xu Jin hong, 2006) and are decreasing with temperature increasing, which shows that the fluorescence quenching is not a dynamic quenching from the effective collision of fluorescence molecules to quenchers, but a static quenching from the formation of a compound. The largest emission wavelength shift from 340nm to 338nm, which further illustrate the formation of a compound of OrangeⅡ and HSA. The change of binding constant are little and the biding-site number are close to 1.

3.2 The acting force of Orange and HSA

The main acting forces of the pigment and biomolecule are hydrophobic force, hydrogen bonds, van der Waals force, electrostatic attraction, and so forth. The interaction of Orange and HSA at four temperatures 298,303,308 and 313K were determined and the thermodynamic parameters can be obtained by the Vant-Hoff equation(Sun H.W.,etc.2010).

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$$\ln K=-\frac{\Delta H}{RT}+\frac{\Delta S}{R} \quad (3)$$

$$\Delta G=\Delta H-T\Delta S \quad (4)$$

K referred to the biding constant under the corresponding temperature, R is the gas constant. The results are shown in table 2.

Table 2. Thermodynamic parameters of the interaction between Orange Ⅱ and HSA at different temperatures

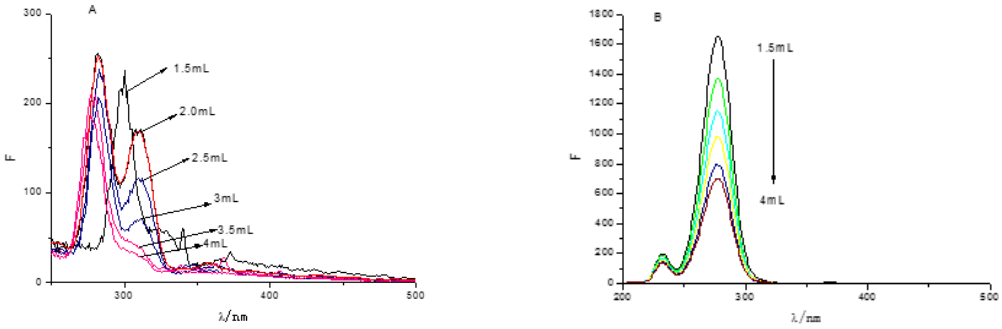
T (K)	K_A ($L.mol^{-1}$)	ΔH ($J.\cdot mol^{-1}$)	ΔS ($J .mol^{-1}.K^{-1}$)	ΔG ($kJ.\cdot mol^{-1}$)	r
298	3.55×10^4	0.3	0.0326	-9.41	0.9936
308	13.80×10^4			-9.48	
313	27.54×10^4			-9.90	
318	75.86×10^4			-10.07	

As shown in table 2, ΔH >0、ΔS >0、ΔG <0, which states that the interaction of OrangeⅡ and HSA is an endothermic and spontaneous combination process (D.P. Ross and S. Sabramanian, 1981). ΔH = 0.3J·mol^{-1} suggests electrostatic effect because the ΔH in it is small and even close to zero(Liu B.S.,etc,2011).

3.3 The effect of conformation from Orange to protein

Synchronous fluorescence spectrum is a simple and effective method for the determination of fluorescence quenching. It can provide the information of the polarity variation of the microenvironment of the chromophore. At the condition of $\Delta\lambda$ =15 nm, the synchronous fluorescence property is from tyrosine residues (Tyr); and when $\Delta\lambda$ =15 nm, from tryptophan residues (Trp) (Q.L. Guo, etc, 2009).

The synchronous fluorescence spectra of Tyr and Trp of HSA after adding OrangeⅡ are shown in figure 3.



A: The synchronous fluorescence spectrum at $\Delta\lambda$ 15 nm

B: The synchronous fluorescence spectrum at $\Delta\lambda$ 60 nm

Figure 3: The synchronous fluorescence spectra of HSA and OrangeⅡ

Figure 3 shows that the fluorescence intensity decreased regularly with the increasing dosage of OrangeⅡ.The largest emission wavelength at $\Delta\lambda$ 15 nm in figure A has a slight red shift and the wavelength at $\Delta\lambda$ 60 nm in figure B has no obvious shift, which illustrate that the existence of OrangeⅡ leads to a weak change of HSA conformation. At the same time, the surrounding microenvironment of the Tyr during the combination also changed, seen from the figure.

3.4 The energy transfer between Orange and HSA

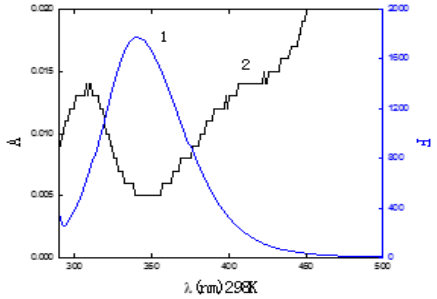


Figure 4: Orange Ⅱ UV absorption（2） with HSA fluoresence（1）

$C_{HSA}=C_{Orange}=1\times10^{-6}\ mol.L^{-1}$

According to the Föster dipole-dipole non-radiation energy transfer theory, the critical energy transfer distance R_0 of HSA and Orange was calculated by the integration of the overlap section using equation(Zhang Q.L.,etc,2011).

According to the energy transfer efficiency E of the theoretic donor (HSA) and the receptor (OrangeⅡ), the results were calculated by formula (5).

$$E = R0^6/(R0^6+r^6) \quad (5)$$

In the formula, r is referred to the combination distance of donor and receptor; R_0 is the critical distance with the transfer efficiency E setting at 50%, calculated by the formula (6) (Q.L. Guo, etc, 2009).

$$R06=8.8\times10^{25}k_2\cdot n^{-4}\cdot\Phi\ D\cdot J \quad (6)$$

k_2 refers to the dipole space orientation factor; n refers to the refraction index of the medium;*Φ D* refers to the fluorescence quantum yield of donor without receptor; *J* refers to the overlap integration of the fluorescence emission spectrum of donor and the absorption spectrum of receptor, calculated by the formula (7) (L.S. L

iu, etc, 2005).

$$J=\Sigma F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda/\Sigma F(\lambda)\Delta\lambda \quad (7)$$

F(λ) refers to the fluorescence intensity at the corresponding wavelength λ of the donor. ε（λ） refers to the molar absorption coefficient of the receptor. The value of k_2, *n*, Φ*D* is 2/3, 1.336, 0.15 respectively(Sun H.W.,etc.2010).

The energy transfer efficient E can be calculated by the formula (8).

$$E=1-F/F_0 \quad (8)$$

F, F_0 refer to the intensity of HSA with Orange and without Orange, respectively.

The results are listed in table 3.

Table 3: The energy transfer efficiency E and other parameters of OrangeⅡ and HSA

Temperature/K	E %	$J/(10^{-16}\ cm^3\cdot L\cdot mol^{-1})$	R_0/nm	r/nm
298	40.34	9.33	1.65	1.76
303	31.14	7.80	1.60	1.83
308	29.44	7.74	1.60	1.85
313	20.73	7.17	1.60	2.00

Obviously, the value of r is less than 7nm (Li Fu,etc, 2014), which indicates that the energy transfer of HSA and OrangeⅡ may be non-radiation energy transfer. It also states that the fluorescence quenching of HSA occurred by the combination of OrangeⅡ and HSA and their energy transfer. The action of them is static quenching effect.

3.5 Ultraviolet absorption spectrum

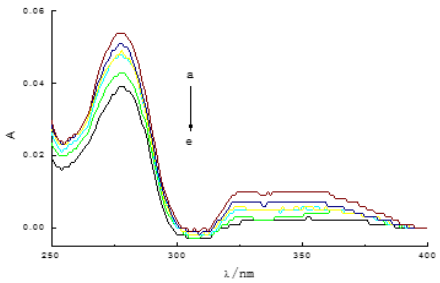


Figure 5: The UV absorption spectrum of HSA and OrangeⅡ

a-e:C_{HSA}=1.0×10^{-5} mol/L, C_{Orange}=(0,0.5,1.0,1.5,2.0)×10^{-5} mol/L

Figure 5 shows that the UV absorbance decreased gradually with the increasing OrangeⅡ, which further indicates the formation of HSA with OrangeⅡ, thus making the less free concentration of protein and lower absorbance.

4. Conclusion

In this paper, we studied the interaction between HSA and OrangeⅡ using the spectrum technology. The results show that the quenching type of HSA and OrangeⅡ is static quenching and the binding force is electrostatic force. The binding effect of them are strong. The binding constant has little changes with the temperature variation and the binding site is about 1.Synchronous fluorescence spectra indicate that the existence of OrangeⅡ leads to a weak change of HSA's conformation. At the same time, the surrounding microenvironment of the Tyr during the combination also changed. The study provides some theoretical direction in the application of pigment in the food addition.

Acknowledgment

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