

## Bonding between fluorescence dyes and proteins

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SOME small dye molecules can bond with proteins, the spectrum of which has a characteristic of energy transferring, i. e. enhancement or quenching of fluorescence. Much information can be obtained from this phenomenon, and can be used to determine the distance between the macromolecules and some groups, such as the distance between aromatic amino acid of proteins and ligands. So they are often called "a spectroscopic ruler"<sup>[1]</sup>. We deduced the theoretical formula which the fluorescence enhancement observes in the interaction of donor and acceptor in reference [2].

In this note, we shall use these formulas to study the action of eosine, light green and rhodamine with human serum albumin, bovine serum albumin and get the association constants in different binding numbers. We will also study their effect of energy transferring and distance between acceptor and donor.

## 1 Theory

## 1.1 Bonding association constant of dye and macromolecules

When macromolecules coexist with some small dye molecules, the change of fluorescence strength can be observed. We define

$$\Delta F = F - F_D, \quad (1)$$

where  $F$  is the fluorescence strength of the system after dye's addition to the solution of macromolecules,  $F_D$  is that of free macromolecules. When macromolecules with the  $n$  ligand have only one kind of bonding site, we obtain

$$\Delta F = (1 - x)F_A + xF_b, \quad (2)$$

where  $x$  is the ratio of the concentration of the dye bonded to the macromolecules to the total concentration of the dye;  $F_A$  is fluorescence strength of free dye;  $F_b$  is the contribution of the dye bonded to the macromolecules to fluorescence intensity. Suppose that the total concentration of dye is  $M_t$ , and the concentration bonded to the macromolecules is  $M_b$ . From eq. (2) we get

$$M_b = M_t(\Delta F - F_A)/(F_b - F_A). \quad (3)$$

Assume that there are  $n$  bonding sites in macromolecule P when binding with dye M which are mutually independent and equivalent. Then for the following reaction:



where ( $K_A$  is association constant,  $K_D$  is dissociation constant), its  $K_A$  value should be expressed as

$$K_A = [nM_nP]/[M][nP]. \quad (5)$$

Assume that  $P_t$  is the total concentration of the macrobiomolecules. The concentration of product  $M_nP$  can be expressed as  $M_b/n$ , and  $M = M_t - M_b$  and  $P = P_t - (M_b/n)$ . Substituting them into eq. (5), we can obtain

$$M_t/M_b = 1/K_A(nP_t - M_b) + 1. \quad (6)$$

Combining formulas (3) and (6), we can obtain

$$(\Delta F - F_A)^{-1} = (F_b - F_A)^{-1}(1 + 1/K_A(nP_t - M_b)). \quad (7)$$

According to formulas (6) and (7),  $n$  and  $K_A$  can be determined.

## 1.2 Determination of the distance between donor and acceptor

According to Förster type dipole-dipole none-radiation-energy-transferring mechanism<sup>[3]</sup>, transferring efficiency  $E$  is related to the critical energy-transferring distance  $R_0$  and distance  $r$  between acceptor and donor:

$$E = R_0^6/(R_0^6 + r^6), \quad (8)$$

where  $R_0$  is the critical distance when energy-transferring efficiency is 50%:

$$R_0^6 = 8.8 \times 10^{-25} (K^2 n^{-4} J \varphi_D), \quad (9)$$

where  $K^2$  is the directional factor of dipole space;  $n$  is the refraction index of the medium;  $\varphi_D$  is the fluorescence quantum yield of the donor;  $J$  is the fluorescence overlap integration between the fluorescence emission spectrum of donor and the absorption spectrum of acceptor, which can be expressed as

$$J = \sum F_D(v) \epsilon_A(v) v^{-4} \Delta v / \sum F_D(v) \Delta v, \quad (10)$$

where  $F_D(v)$  is the fluorescence strength of donor at the wave number  $v$ ,  $\epsilon_A(v)$  is the mole extinction coefficient of acceptor at wave number  $v$ . Energy-transferring efficiency  $E$  can be determined by

$$E = 1 - F/F_0, \quad (11)$$

where  $F$  and  $F_0$  is the fluorescence emission strength of energy donor when energy acceptor exists or not, respectively. Obviously, if we know  $E$ ,  $K^2$ ,  $\varphi_D$  and  $n$ , and the value of overlap integration  $J$  from the experimental spectra, we can obtain  $R_0$  and  $r$ .

## 2 Experimental

### 2.1 Major reagents and instruments

0.05 mol/L Tris-HCl and 0.2 mol/L NaCl solutions were prepared and kept in the system with pH = 7.4 and a certain ionic strength. Solutions of human serum albumin (Sigma Company), bovine serum albumin (Institute of Biochemistry, Chinese Academy of Sciences) and  $\gamma$ -globulin (muscle injection, made in Spain), eosine (AR), light green (AR) and rhodamine (AR) were adjusted to adequate concentrations. Spectrofluorophotometer was RF-540 of Shimadzu made in Japan, and UV-visible recording spectrophotometer was UV-265 of Shimadzu made in Japan.

### 2.2 Experimental

(i) Dye solution (2 mL) and Tris-HCl NaCl solution (2 mL) were put to two quartz cells; protein solution of adequate concentration was added to them time by time. Then their fluorescence emission spectra were determined. (ii) With fixed emission wavelength, the fluorescence spectra of the protein was determined. (iii) The UV-absorption spectra of the dye

were determined when its ratio to protein molecule was 1:1.

### 3 Results

#### 3.1 Association constant with different bonding numbers of protein and dye

We subtract the fluorescence strength  $F_{Dn}$  obtained after dropwise addition of protein to Tris-HCl-NaCl solution from fluorescence strength  $F_n$  obtained after dropwise addition of protein to dye solution (the first point is  $F_A$ ; the highest point is  $F_b$ ).  $M_b$  can be evaluated in different  $F_n$  values from formula (4) and dye concentration  $M_t$ .  $P_t$  value is evaluated after the addition of different amounts of protein solution (suppose that the volume of protein solution added can be ignored. The total volume of the solution is 2 mL). According to formula (8), making a plot of  $(\Delta F - F_A)^{-1}$  against the  $(nP_t - M_b)^{-1}$ , and supporting  $n = 2$  and 4, we can obtain good linear correlation coefficient  $C$  as table 1. Slope of the line is  $(F_b - F_A)^{-1} \times K_A^{-1}$ . We can calculate the association constants  $K_A$  of dye and protein as shown in table 1.

Table 1 Association constant ( $K_A$ ), bonding site number ( $n$ ), linear interrelation coefficient  $C$  and interaction distance  $r$  between some dyes and some proteins

Dye	Protein	$n=2$ $K_A$	$C$	$n=4$ $K_A$	$C$	$K_A^{(2)}/K_A^{(4)}$	$J \times 10^{-14}$	$R_0/\text{nm}$	$E$	$r/\text{nm}$
Eosine	Human	$2.16 \times 10^5$	0.986 4	$0.98 \times 10^5$	0.988 3	2.20	1.30	2.64	0.263	3.11
	bovine	$2.53 \times 10^5$	0.992 9	$1.12 \times 10^5$	0.994 0	2.26	3.67	3.13	0.257	3.74
	$\gamma$ -globulin	$3.80 \times 10^5$	0.987 2	$1.61 \times 10^5$	0.991 1	2.36	3.20	3.06	0.045	5.1
Rhodamine	Human	$2.85 \times 10^5$	0.995 6	$1.25 \times 10^5$	0.996 5	2.28	1.51	2.70	0.040	4.59
	bovine	$3.94 \times 10^5$	0.997 5	$1.63 \times 10^5$	0.999 8	2.42	1.44	2.68	0.045	4.46
	$\gamma$ -globulin	$5.94 \times 10^5$	0.995 0	$2.30 \times 10^5$	0.996 9	2.58	1.51	2.7	0.015	5.43
Light green	Human	$3.35 \times 10^5$	0.995 7	$1.43 \times 10^5$	0.997 0	2.34	5.20	3.32	0.073	5.07
	bovine	$4.20 \times 10^5$	0.998 7	$1.74 \times 10^5$	0.999 1	2.41	6.30	3.44	0.120	4.78
	$\gamma$ -globulin	$5.20 \times 10^5$	0.976 2	$2.05 \times 10^5$	0.984 9	2.54	6.05	3.41	0.030	6.08

#### 3.2 Distance between fluorescence donor and acceptor

Fixing emission wavelength, we determined the excitation spectra of association substance when the ratio of the three kinds of proteins to three kinds of dyes is 1:1 (mole ratio), respectively. The result shows that the excitation wavelength does not change after addition of dyes. We also observe the change of the fluorescence strength fixing emission peak of dyes after addition of protein, and have found that fluorescence enhancement effect appears after addition of protein. So there is energy-transferring between tryptophane residue in protein and dye molecules.

Using the above fluorescence emission spectra of albumin and UV-absorption spectra of dye, combining them with formula (4), evaluating the sum after cutting the overlapping part of spectrum into very small rectangle area, we obtained the overlapping integration  $J$  value when the three proteins correspond to the three dyes as shown in table 1.

Taking tryptophane as a standard substance ( $\varphi = 0.14^{[4]}$ ), with the same excitation wavelength and instrument parameter, the quantum yield of tryptophan in the three albumen  $\varphi = 0.118$  is determined by relative method. Index of refraction  $n$  is the average value 1.336 of water and organism. Directional factor is the average value  $K^2 = 2/3$  of donor-acceptor in every direction randomly. By substituting the above values into eq. (9), we can obtain the energy-transfer distance  $R_0$  between the three proteins and the three dyes respectively as shown in table 1. Using the above determined fluorescence strength of the association substance when

the mole ratio of protein to dye is 1:1, according to eq. (11), we obtained energy-transferring efficiency value  $E$  as shown in table 1. So from  $R_0$  and  $E$  according to eq. (8) we obtained the distances  $r$  between the 214 th tryptophan residue of albumin and dye molecule as shown in table 1.

#### 4 Discussion

Human serum albumin has one tryptophan residue; bovine serum albumin has two. Bonding and energy-transferring can occur between them and dye molecules<sup>[5]</sup>.  $\gamma$ -globulin consists of 4 peptide chains which are joined together by S—S bond, where there are two heavy chains and two same light chains, which are Y-type symmetric structure. They have two antigen bonding sites and two complement bonding sites<sup>[6]</sup>. These sites can combine dye molecules and energy transferring can occur.

According to the fluorescence spectra of the three dyes to the three proteins, they have analogous effects. Their association constants are all in  $10^5$  degree. And when bonding number  $n = 2$  and  $n = 4$ , the value  $K_{\Lambda}$  shows obviously that  $K_{\Lambda}^{(2)}$  is larger than  $K_{\Lambda}^{(4)}$ , and that the ratio of  $K_{\Lambda}^{(2)}$  to  $K_{\Lambda}^{(4)}$  is between 2.20 and 2.60. This result conforms to the general rule of multistage association dissociation. Under the above 9 kinds of conditions the linear interrelation coefficient  $C$  is obtained. When  $n = 4$ ,  $C$  is between 0.984 9 and 9.999 7; when  $n = 2$ ,  $C$  is between 0.976 2 and 9.998 7. Though when  $n = 4$ ,  $C$  is better; when  $n = 2$ ,  $C$  still satisfies eq. (7). This shows that to dye the bonding sites of protein are non-specific.

According to association constant,  $K_{\Lambda}^{(2)}$  and  $K_{\Lambda}^{(4)}$  of  $\gamma$ -globulin and the three kinds of dye are all larger than those of the corresponding serum albumin, but according to the overlapping integration of specific fluorescence emission spectra and UV-visible absorption spectra of the three kinds of dye to the three kinds of protein, energy-transferring efficiency  $E$  and distance  $r$  between acceptor and donor which is calculated from eqs. (8) and (10), the  $r$  and  $E$  of human and bovine serum albumin approximate to those of the three kinds of dye. But energy transferring efficiency  $E$  of  $\gamma$ -globulin is much lower than that of serum albumin, and the distance  $r$  between acceptor and donor is all larger than those of serum albumin. This shows that the energy transferring between antigen bonding sites and complement bonding sites of  $\gamma$ -globulin is less effective than tryptophan residue. So we can see that the combining stability is not proportional to energy transferring.

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