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ORIGINAL PAPER

Studies on the Interactions of 2, 4-Dinitrophenol and 2, 4-Dichlorphenol with Trypsin

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Abstract The interactions of 2, 4-dinitrophenol and 2, 4-dichlorphenol with trypsin were investigated by fluorescence, synchronous fluorescence, and three-dimensional fluorescence spectra techniques under physiological pH 7.40. The 2, 4-dinitrophenol and 2, 4-dichlorphenol effectively quenched the intrinsic fluorescence of trypsin via static quenching. The process of binding 2, 4-dinitrophenol and 2, 4-dichlorphenol with trypsin was a spontaneous molecular interaction procedure. The electrostatic repulsion does favor the interaction between 2, 4-DNP and trypsin. However, the interaction of 2, 4-DCP and trypsin can be explained on the basis of hydrogen bonding and van der Waals. The results of synchronous fluorescence spectroscopy and three-dimensional fluorescence spectra indicated that the structure of these trytophan and tyrosine residues environments were altered by 2, 4-DNP and 2, 4-DCP.

Keywords 2, 4-Dinitrophenol · 2, 4-Dichlorphenol · Trypsin · Fluorescence spectroscopy · Binding constant

Introduction

Nitrophenols and chlorphenols are used extensively as fungicides, herbicides, algicides, insecticides, ovicides, pharmaceuticals, and dyes, and as intermediates in chemical syntheses [1, 2]. Because of their widespread use, nitrophenols and chlorophenols are found as contaminants in industrial effluents, rivers, groundwater and pesticide treated soils [3]. They are very toxic compounds when ingested, inhaled, or absorbed through the skin. Like most nitrophenols, 2, 4-dinitrophenol (2, 4-DNP) is highly toxic to living organisms by uncoupling the oxidative phosphorylation process in mitochondria [4]. The U.S. Environmental Protection Agency has listed 2, 4-DNP as "priority pollutant" and recommends restricting its concentrations in natural water to below 0.01 $\mu g \cdot L^{-1}$[5]. 2, 4-dichlorphenol (2, 4-DCP) is cytotoxic toward freshwater and marine algae, and animal cells and tissues [6]. It has been shown to alter the activity of antioxidant enzymes (superoxide dismutase, glutathione peroxidase) [7].

Trypsin is a proteolytic enzyme that cleaves peptide bonds at the carboxylic groups of arginine, lysine, and ornithine working optimally at pH 7.5–8.5 [8]. Trypsin is a medium-sized globular protein with applications in wound healing components, in washing agents, and in biotechnology [9]. Esimbekova et al. studied the effects of redox-active compounds on trypsin activity [10]. Banerjee et al. have studied the binding of merbromine to trypsin in order to investigate that the dyes exhibit a high affinity to trypsin [11]. For a better understanding of nitrophenols and chlorphenols toxicity, the interactions of 2, 4-DNP and 2, 4-DCP with trypsin was studied in this report. The parameters such as mode of interaction, association constant and number of binding sites are important. These investigations may provide some important theoretic information for the improvement of the metabolism and distribution of 2, 4-DNP or 2, 4-DCP in life science. In this report, we provide investigations on the effect of 2, 4-DNP (or 2, 4-DCP) on the structural and optical properties of trypsin. The results may cast some light on the future study of the interaction between toxic compounds and other proteinases and have

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toxicological importance; our work should be valuable in ecotoxicology.

Materials and methods

Materials

Trypsin from bovine pancreas was purchased from Sigma Aldrich and used without further purification. The molecular mass of trypsin is approximately 23,500 Da. It consists of 223 amino acids, and its isoelectric point is 10.5. 2, 4-DNP and 2, 4-DCP were obtained from Fluka (Switzerland). The buffer Tris was purchased from Acros (Geel, Belgium), and NaCl, HCl, etc. were all of analytical purity. Trypsin solution (10.0 μM) was prepared in pH 7.40 Tris-HCl buffer solution (0.05 M Tris, 0.1 M NaCl). The 2, 4-DNP (2.5 mM) or 2, 4-DCP solution (12.5 mM) was prepared by dissolving 2, 4-DNP and 2, 4-DCP in methanol, respectively.

Equipment and spectral measurements

The UV/vis spectrum was recorded at 298 K on a SPECORD S 50 (Germany) equipped with 1.0 cm quartz cells. All fluorescence spectra were recorded on LS-50B Spectrofluorimeter (Perkin-Elmer USA) equipped with 1.0 cm quartz cells and a thermostat bath. The widths of both the excitation slit and the emission slit were set to 10.0 nm/3.0 nm for trypsin, respectively.

Procedures

A 2.5 mL solution, containing appropriate concentration of trypsin, was titrated by successive additions of 2, 4-DNP or 2, 4-DCP solution. Titrations were done manually by using trace syringes. The fluorescence spectra were then measured (excitation at 280 nm and emission wavelengths of 290~500 nm) at two temperatures (298 K, 308 K). Wherever applicable, the tryptophan fluorescence from trypsin has been corrected for inner filter effect due to the absorbance by 2, 4-DNP or 2, 4-DCP at the excitation (λ_{ex}=280 nm) and emission wavelength (λ_{em}=337 nm). The fluorescence intensity was corrected using the relationship [12]:

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

where F_{cor} and F_{obs} are the fluorescence intensity corrected and observed, respectively; A_{ex} and A_{em} are the absorbance of system at excitation and emission wavelength, respectively. Synchronous fluorescence spectra of trypsin (10.0 μM) in the presence of 2, 4-DNP (or 2, 4-DCP) were recorded at room temperature. The *D*-value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 nm or 60 nm. The three-dimensional fluorescence spectrum was performed under the following conditions: the emission wavelengths at 290–500 nm, the excitation at 200 nm, scanning number 15 and increment 10 nm with other parameters just the same as that of the fluorescence quenching spectra. The UV/vis absorbance spectra of 2, 4-DNP or 2, 4-DCP solution was recorded at 298 K.

Results and discussion

The binding properties of 2, 4-DNP and 2, 4-DCP with trypsin

Changes in emission spectra of tryptophan are common in response to protein conformational transitions, substrate binding, or denaturizing [13]. Thus, the intrinsic fluorescence of proteins can provide considerable information about their structure and dynamics, and it often considered on the study of protein folding and association reactions. Trypsin has four tryptophans (Trp 50, Trp 141, Trp 215, and Trp 237) that can be used as intrinsic fluorophores [9]. Cysteines are strong quenchers of tryptophan fluorescence, therefore, in the native state tryptophans neighboring to cysteine residues do not significantly contribute to the overall fluorescence emission. Cys 42 and 22 are located below and above the indol ring of Trp 141. Also, Cys 168 and 220 are very close to Trp 215, and Cys 232 is located in the proximity of Trp 237. It is only Trp 51 that is relatively far from cysteine groups [9].

The emission maximum of trypsin in solution was observed at 337 nm (Fig. 1) which indicates that specific tryptophans of trypsin are partly exposed to the solvent. The effect of 2, 4-DNP and 2, 4-DCP on trypsin fluorescence intensity is shown in Fig. 1(a, b). As the data show, the fluorescence intensity of trypsin was quenched by addition of 2, 4-DNP or 2, 4-DCP, which indicated that 2, 4-DNP and 2, 4-DCP may interact with trypsin. Moreover, the blue shift of the emission maximum observed in the 2, 4-DNP-Trypsin system indicated the occurrence of conformational changes for trypsin at tertiary structure levels since the shift in the position of emission maximum reflected the changes of the polarity around the Trp residues [14].

Generally speaking, the fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule, such as excited-state reaction, molecules rearranging, energy transfer, ground state complex formation and collision quenching. It is necessary to know quenching procedure and type for researching the

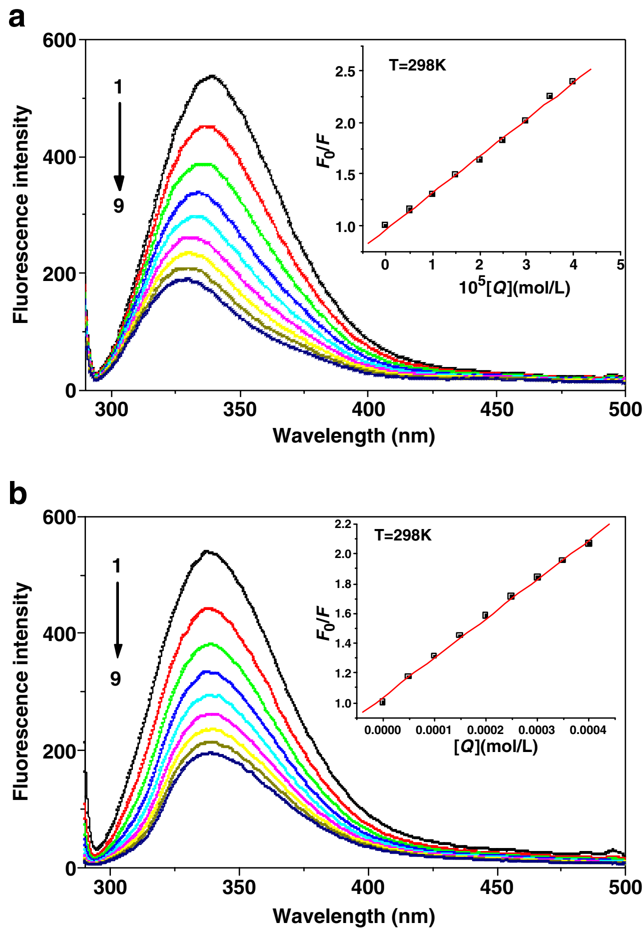

Fig. 1 Fluorescence spectra of trypsin in the presence of 2, 4-DNP (**a**) and 2, 4-DCP (**b**) at 298 K. The inset shows Stern–Volmer plots for the quenching of trypsin by 2, 4-DNP or 2, 4-DCP. Total concentration of 2, 4-DNP: c (2, 4-DNP)/ (10^{-5}M), curve (1–9): 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0, respectively. Total concentration of 2, 4-DCP: c (2, 4-DCP)/ (10^{-4}M), curve (1–9): 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0, respectively

mechanism of quenching. Quenching types often include static and dynamic quenching. In order to confirm the quenching mechanism, the fluorescence quenching data are analyzed by the well-known Stern-Volmer Eq. (2) [15] and modified Stern-Volmer Eq. (3) [16–18] to confirm the mechanism.

$$\frac{F_0}{F_{cor}} = 1 + k_q\tau_0[Q] = 1 + K_{sv}[Q] \tag{2}$$

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F_{cor}} = \frac{1}{f_0} + \frac{1}{K_a f_0}\cdot\frac{1}{[Q]} \tag{3}$$

where F_0 is the fluorescence intensities before the addition of the quencher, F_{cor} is the fluorescence intensity corrected. k_q, K_{sv}, τ_0, f_a, K_a, and $[Q]$ are the quenching rate constants of the biomolecule, the Stern-Volmer dynamic quenching constant, the average lifetime of tryptophan in trypsin system without quencher (τ_0=1.9 ns [19]), the fraction of accessible fluorescence, the effected quenching constant for the accessible fluorophores, and the concentration of the quencher, respectively. Figure 2(a, b) lists the modified Stern-Volmer curves. From Fig. 2(a, b), it is known that under certain 2, 4-DNP and 2, 4-DCP concentration, the curves of $F_0/ (F_0-F_{cor})$ versus $1/ [Q]$ were linear. All these indicated that there were obviously characters of static quenching.

In Table 1, the binding constants obtained from the Stern–Volmer method are listed for 2, 4-DNP and 2, 4-DCP with trypsin. Obviously, the K_q values of protein quenching procedure initiated by 2, 4-DNP and 2, 4-DCP were all greater than 2.0×10^{10} L·moL^{-1}·s^{-1} [20], which indicated that the quenching was not initiated from dynamic collision but from the formation of a complex.

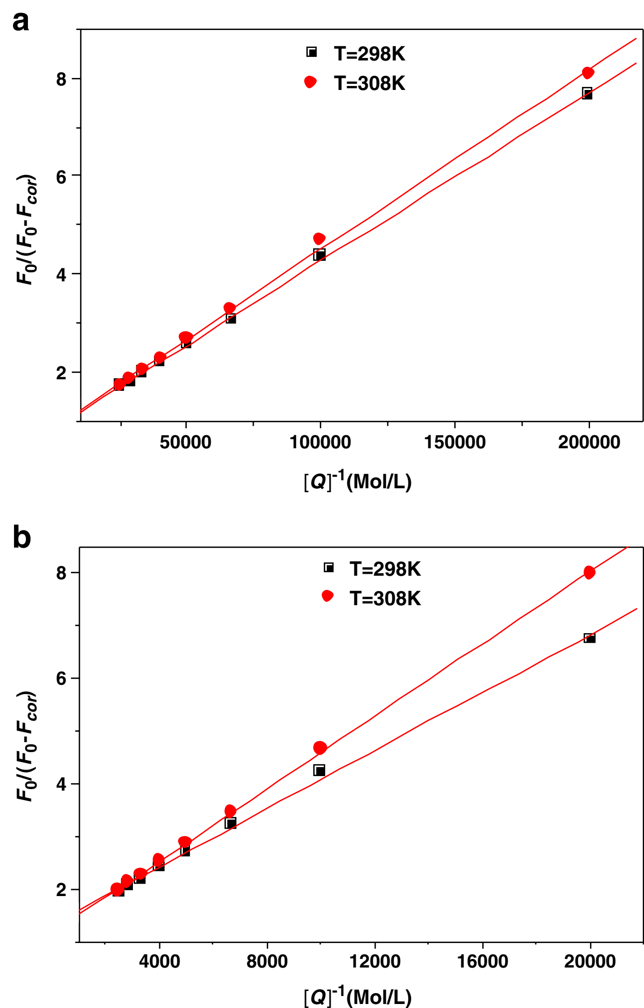

Fig. 2 The modified Sterne-Volmer curves of the interaction of 2, 4-DNP (**a**) and 2, 4-DCP (**b**) with trypsin. Tyrpsin concentration was at 10.0 μM, pH=7.40, λ_{ex}=280 nm and λ_{em}=337 nm

Table 1 Sterne-Volmer quenching constant and modified Sterne-Volmer association constant of the interaction of 2, 4-DNP-Trypsin and 2, 4-DCP with trypsin at two different temperatures

Compound	T(K)	Eq. (2)				Eq. (3)		
		K_{sv}(L·Mol^{-1})	K_q(L·Mol^{-1})	R[a]	S D[b]	K_a(L·Mol^{-1})	R[a]	S D[b]
2,4-DNP	298	3.56×10^4	1.87×10^{13}	0.9983	0.0302	2.47×10^4	0.9997	0.0544
	308	3.39×10^4	1.78×10^{13}	0.9977	0.0340	2.32×10^4	0.9993	0.0846
2,4-DCP	298	2.64×10^3	1.39×10^{12}	0.9984	0.0217	4.75×10^3	0.9988	0.0868
	308	2.54×10^3	1.34×10^{12}	0.9996	0.0103	3.33×10^3	0.9998	0.0332

[a] The correlation coefficient

[b] The standard deviation

Furthermore, the K_{sv} values decreased with an increase in temperature also indicated that the quenching was not initiated from dynamic collision but from the formation of a complex [17]. In this paper, the association constants obtained from the modified Stern-Volmer curves was applied to analyze the thermodynamic parameter and the nature of the binding forces.

Red edge excitation shift (REES) is a shift in the emission maximum toward a higher wavelength caused by a shift in the excitation wavelength toward the red edge of the absorption band [14]. The REES is due to the electronic coupling between Trp indole rings and neighboring dipoles and occurs when there are slow relaxations of solvent media. Thus, REES is particularly useful in monitoring motions around the Trp residues in the protein study [21].The Trp emission in the 2, 4-DNP-Trypsin, and 2, 4-DCP-Trypsin system was further investigated by red edge excitation shift (REES) experiments [22]. In our experiment, we chose to excite the Trp at both 295 and 305 nm to investigate the REES effect, and the results are listed in Table 2. The value of $\Delta\lambda_{em}$ max is defined as the difference of the emission maximum between that excited at 295 nm and at 305 nm. As shown, native trypsin showed a 2.0 nm REES, indicating that Trp residues in the trypsin were in a slight motionally restricted environment. In the presence of 2, 4-DNP, the values all showed an increase. The increase of $\Delta\lambda_{em}$ max meant that the introduction of 2, 4-DNP had an impact on the mobility of the Trp microenvironment and that Trp residues faced more restrictions from their surroundings in the 2, 4-DNP system [14].

Binding model

Considering the dependence of binding constant K_a on temperature, a thermodynamic process was considered to be responsible for the formation of a complex. Therefore, some thermodynamic parameters dependent on temperature such as enthalpy change ($\Delta H°$), entropy change ($\Delta S°$) and free energy change ($\Delta G°$) were used in order to further characterize the interaction between 2, 4-DNP (or 2, 4-DCP) and trypsin. Among these parameters, $\Delta G°$ reflects the possibility of reaction, and $\Delta H°$ and $\Delta S°$ are the main evidences to determine acting forces. There are essentially four types of noncovalent interactions that could play a role in ligand binding to proteins. These are hydrogen bonds, van der Waals forces, electrostatic, and hydrophobic interactions. When temperature varies in a small range, the $\Delta H°$ could be considered as a constant, then through the effected quenching constant K_a, the thermodynamic parameters are evaluated using the following equations:

$$\ln\frac{(K_a)_2}{(K_a)_1}=\frac{\Delta H^{O}}{R}\left(\frac{1}{T_1}-\frac{1}{T_2}\right) \quad (4)$$

$$\Delta G^{O}=-RT\ln K_a \quad (5)$$

$$\Delta S^{O}=\frac{\Delta H^{O}-\Delta G^{O}}{T} \quad (6)$$

From Table 3, it can be seen that the negative sign for $\Delta G°$ indicates the spontaneity of the binding of 2, 4-DNP

Table 2 Red edge excitation effects for trypsin, 2, 4-DNP-Trypsin, and 2, 4-DCP-Trypsin system

Sample		$\lambda_{em\ max}$ (nm)		$\Delta\lambda_{em\ max}$ (nm)
		λ_{ex}: 295nm	λ_{ex}: 305nm	
Trypsin		337	339	2
2, 4-DNP-Trypsin [n(2, 4-DNP) : n(Trypsin)]	4 : 1	328	335	7
2, 4-DCP-Trypsin [n(2, 4-DCP) : n(Trypsin)]	40 : 1	338	340	2

Table 3 Thermodynamic parameters of the interaction of 2, 4-DNP-Trypsin and 2, 4-DCP-trypsin system at pH 7.40

Compound	T(K)	ΔH° (KJ·mol^{-1})	ΔG° (KJ·mol^{-1})	ΔS° (J·mol^{-1}·K^{-1})	K_A (L·mol^{-1})	n	R^a	S D^b
2,4-DNP	298	−4.78	−25.06	68.05	4.09×10^4	1.02	0.9994	0.0126
	308		−25.74		3.82×10^4	1.04	0.9990	0.0162
2,4-DCP	298	−27.10	−20.97	−20.57	3.86×10^3	0.84	0.9998	0.0051
	308		−20.77		2.60×10^3	0.94	0.9994	0.0035

[a] The correlation coefficient

[b] The standard deviation

(or 2, 4-DCP) with trypsin. In 2, 4-DNP-Trypsin system, ΔH° is the negative values, and ΔS° is positive value. However, ΔH° and ΔS° are negative value in 2, 4-DCP-Trypsin system. According to the views of Ross and Subramanian [23], the positive ΔH° and ΔS° value is associated with hydrophobic interaction. The negative ΔH° and ΔS° values are associated with hydrogen bonding and van der Waals interaction in low dielectric medium. Finally very low positive or negative ΔH° and positive ΔS° values are characterized by electrostatic interactions. At pH=7.40, trypsin (isoelectric point pI=10.5) [9] bears positive charge, and 2, 4-DNP (pK_a=4.0) bears negative charge, therefore, the electrostatic repulsion does favor the interaction between 2, 4-DNP and trypsin. However, 2, 4-DCP (pK_a=7.9) doesn't bear charge, therefore, the thermodynamic parameters for the interaction of 2, 4-DCP and trypsin can be explained on the basis of hydrogen bonding and van der Waals.

Apparent binding constant and binding capacity

The apparent binding constant K_A and binding sites n can be found from equation [24, 25]:

$$\log\frac{F_0 - F_{cor}}{F_{cor}} = n\log K_A - n \times \log\left(\frac{1}{[Q_t - (F_0 - F_{cor})[P_t]/F_0]}\right) \quad (7)$$

where F_0 is the fluorescence intensities before the addition of the quencher, F_{cor} is the fluorescence intensity corrected. $[Q]$ and $[P_t]$ are the total quencher concentration and the total protein concentration, respectively. By the plot of log $(F_0{-}F_{cor})/F_{cor}$ versus log (1/ ($[Q_t]-(F_0-F_{cor})$ $[P_t]/F_0$)), the number of binding sites n and the association constant K_A can be abstain. Figure 3 was the plots of log $(F_0{-}F_{cor})/F_{cor}$ vs. log (1/ ($[Q_t]-(F_0-F_{cor})$ $[P_t]/F_0$)) for the 2, 4-DNP-Trypsin and 2, 4-DCP-Trypsin system obtained from the fluoremetric titration. In Table 3, the binding constants K_A and binding sites n were listed for 2, 4-DNP (or 2, 4-DCP) associated with trypsin. The results showed that the apparent binding constants K_A decreased with the temperature, which may indicate forming an unstable compound. The unstable compound would be partly decomposed with the rising temperature, therefore, the values of K_A decreased. The values of n approximately equal to 1 indicated the existence of just a single binding site in trypsin for 2, 4-DNP or 2, 4-DCP.

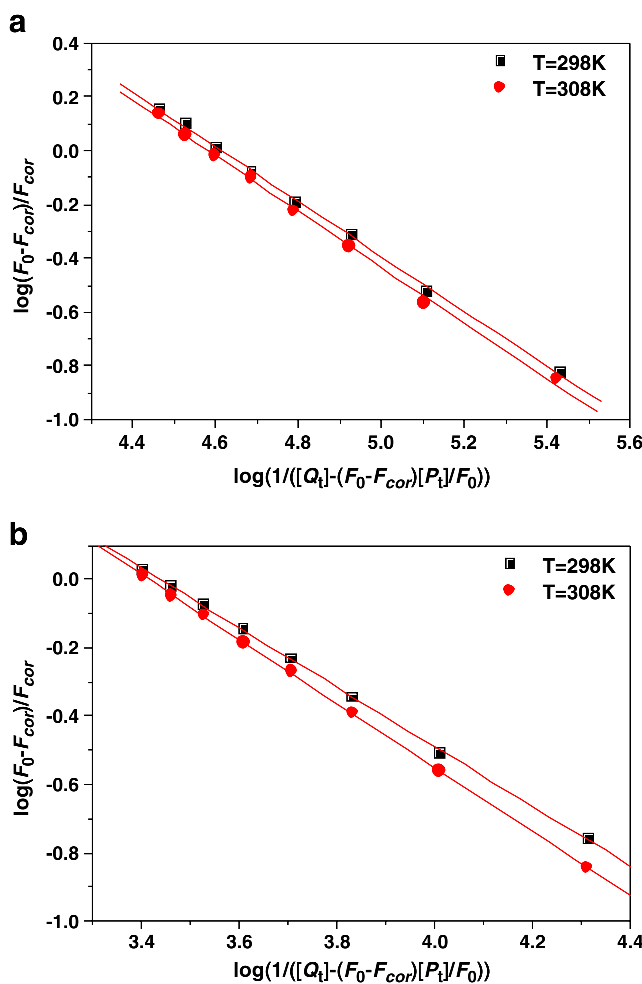

Fig. 3 The plots of log $(F_0{-}F_{cor})/F_{cor}$ vs. log (1/ ($[Q_t]-(F_0-F_{cor})$ $[P_t]/F_0$)) of 2, 4-DNP (**a**) and 2, 4-DCP (**b**) with trypsin. Tyrpsin concentration was at 10.0 μM, pH=7.40, λ_{ex}=280 nm and λ_{em}= 337 nm

Energy transfer from trypsin to 2, 4-DNP (or 2, 4-DCP)

Fluorescence resonance energy transfer (FRET) is a distance dependent interaction between the different electronic excited states of dye molecules in which excitation energy is transferred from one molecular (donor) to another molecular (acceptor) without emission of a photon from the former molecular system[25]. Here the donor and acceptor were trypsin and 2, 4-DNP (or 2, 4-DCP), respectively. There was a spectral overlap between the fluorescence emission spectrum of free trypsin (Fig. 4a) and the absorption spectra of 2, 4-DNP (Fig. 4b) and 2, 4-DCP (Fig. 4c). The spectrum ranging from 290 to 500 nm was chosen to calculate the overlapping integral.

According to Förster's theory the energy transfer efficiency E is defined as the following equation Eq. (8). Where r is the distance from the ligand to the tryptophan residue of the protein, and R_0 is the Förster critical distance, at which 50% of the excitation energy is transferred to the acceptor [26]. It can be calculated from donor emission and acceptor absorption spectra using the Förster formula Eq. (9).

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

$$R_0^6 = 8.79 \times 10^{-25} K^2 N^{-4} \Phi J \tag{9}$$

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda) d\lambda} \tag{10}$$

In Eq. (9), K^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles and K^2=2/3

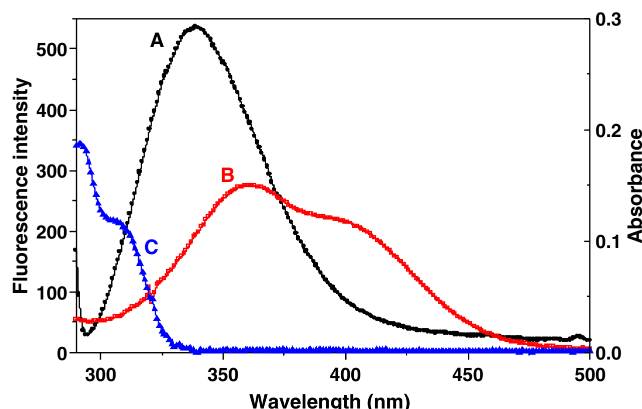

Fig. 4 Overlap of the fluorescence emission of trypsin (**a**) with the absorption spectra of 2, 4-DNP (**b**) and 2, 4-DCP (**c**). c (trypsin)= 10.0 μM, c(2, 4-DNP)=10.0 μM, c(2, 4-DCP)=100.0 μM

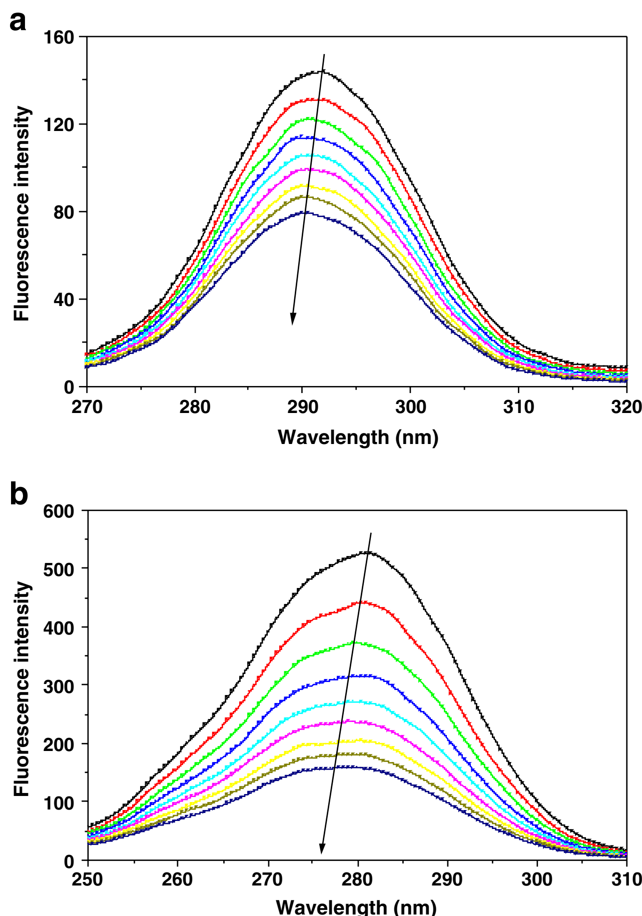

Fig. 5 Synchronous fluorescence spectrum of trypsin in the presence of 2, 4-DNP at 298 K. Total concentration of 2, 4-DNP: c (2, 4-DNP)/ (10^{-5}M), curve (from *up* to *down*): 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0, respectively. (**a**) Δλ=15 nm and (**b**) Δλ=60 nm

for random orientation as in fluid solution; N is the average refractive index of medium in the wavelength range where spectral overlap is significant; Φ is the fluorescence quantum yield of the donor; J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor (Fig. 4), which could be calculated by Eq. (10), where, $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to $\lambda+\Delta\lambda$; ε (λ) is the extinction coefficient of the acceptor at λ. In the present case, N=1.36, Φ=0.146[27], according to Eqs. (8)–(10), we could calculate that $J=1.66\times10^{-14}$cm^3·L·mol^{-1}, E=0.284, R_0= 3.396 nm, r=3.960 nm for 2, 4-DNP; $J=1.72\times10^{-16}$cm^3·L·mol^{-1}, E=0.036, R_0=1.58 nm, r=2.73 nm for 2, 4-DCP. The average distance between a donor fluorophore and acceptor fluorophore was on the 2–8 nm scale, which indicated that the energy transfer from trypsin to 2, 4-DNP (or 2, 4-DCP) occurred with high probability [28, 29], while r was bigger than R_0 in the present study also

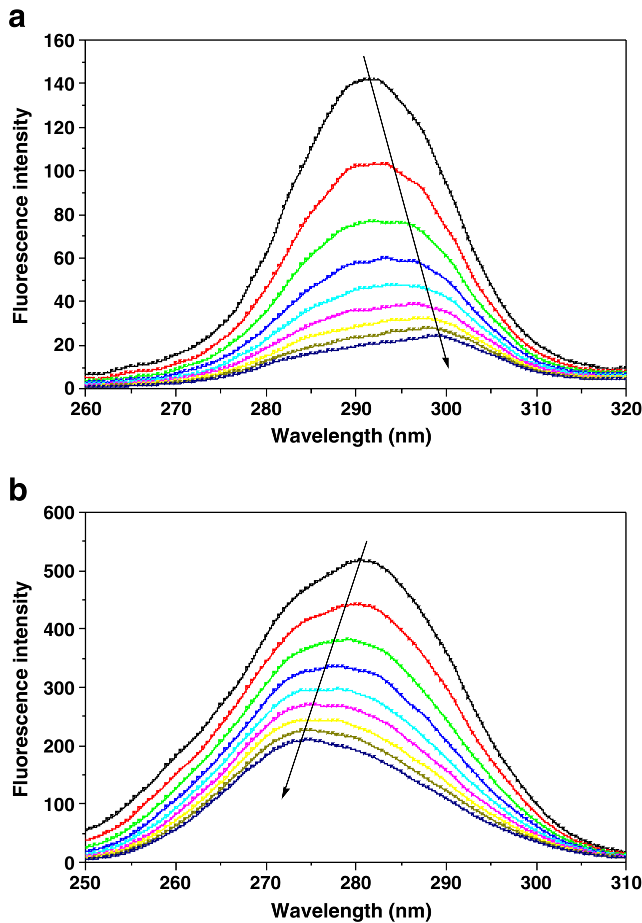

Fig. 6 Synchronous fluorescence spectrum of trypsin in the presence of 2, 4-DCP at 298 K. Total concentration of 2, 4-DCP: c (2, 4-DCP)/(10^{-4}M), curve (from *up* to *down*): 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0, respectively. (**a**) Δλ=15 nm and (**b**) Δλ=60 nm

revealed that 2, 4-DNP and 2, 4-DCP could strongly quench the intrinsic fluorescence of trypsin by static quenching.

Conformation investigation

The synchronous fluorescence spectroscopy gives information about the molecular environment in a vicinity of the chromospheres molecules and has several advantages, such as sensitivity, spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects [30]. Vekshin et al. [31] suggested a useful method to study the environment of amino acid residues by measuring the possible shift in wavelength emission maximum λ_{max}, which corresponds to the changes of the polarity around the chromophore molecule. When the *D*-value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 nm or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine residues or tryptophan residues [32]. The effect of 2, 4-DNP and 2, 4-DCP on trypsin synchronous fluorescence spectroscopy was shown in Fig. 5 and 6.

It was apparent from Fig. 5 that the emission maximum of tyrosine and tryptophan residues did significant blue shift, which indicated that the conformation of trypsin was changed by 2, 4-DNP, the polarity around the tyrosine and tryptophan residues was decreased and the hydrophobicity was increased. In Fig. 6, the emission maximum of tyrosine residues did significant red shift, the tryptophan residues did significant blue shift, which indicated that the conformation of trypsin was also changed by 2, 4-DCP, the polarity around the tyrosine residues was increased, while the hydrophobicity around tryptophan residues was increased [24]. It has been also shown in Fig. 7(a) that the slope was higher when $\Delta\lambda$ was 60 nm indicating that a significant contribution of tryptophan residues in the fluorescence of trypsin, 2, 4-DNP was closer to trytophan residues compared to tyrosine residues. However, the slope was higher when $\Delta\lambda$ was 15 nm in Fig. 7(b) indicating that 2, 4-DCP was closer to tyrosine residues compared to tryptophan residues. The structure of these trytophan and tyrosine residues environments were altered by 2, 4-DNP and 2, 4-DCP.

The three-dimensional fluorescence contour maps are a rising fluorescence analysis technique in recent years. The excitation wavelength, the emission wavelength and the

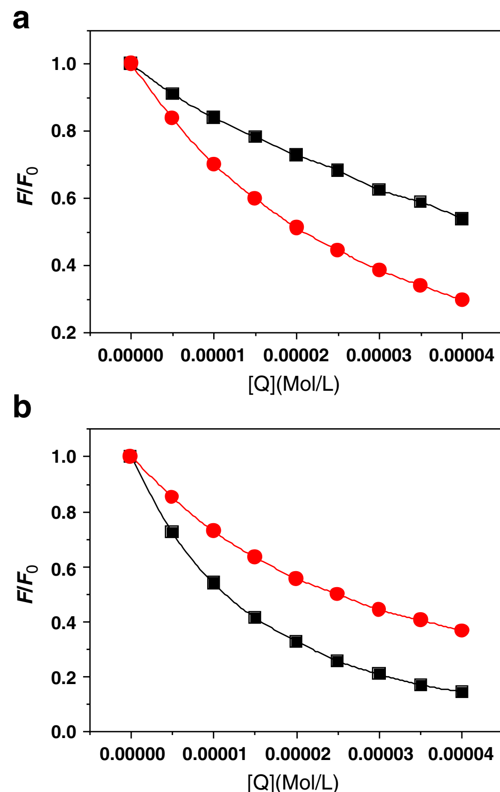

Fig. 7 The quenching of trypsin synchronous fluorescence by 2, 4-DNP (**a**) and 2, 4-DCP (**b**). (■) Δλ=15 nm and (●) Δλ=60 nm

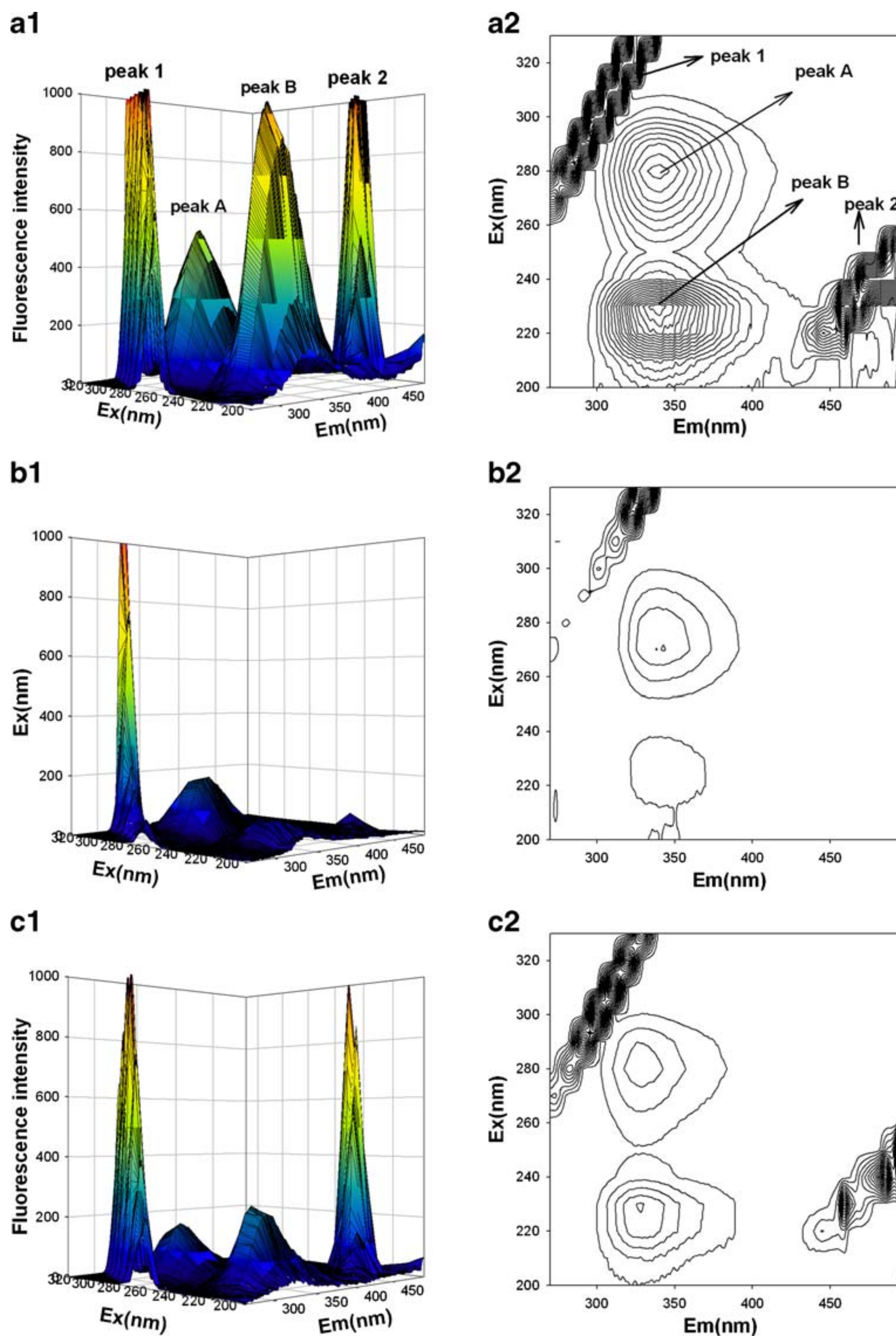

Fig. 8 The three-dimensional projections and the corresponding contour spectra of trypsin (**a**), 2, 4-DCP-Trypsin (**b**), and 2, 4-DNP-Trypsin (**c**), [the concentration of trypsin: A 10.0 μM, B 10.0 μM, C 10.0 μM; the concentration of 2, 4-DCP: A 0 μM, B 400.0 μM; the concentration of 2, 4-DNP: A 0 μM, C 40.0 μM]

fluorescence intensity can be used as the axes in order to investigate the synthetically information of the samples, and the contour spectra can also provide a lot of important information [33]. Figure 8 presented the three-dimensional fluorescence spectrum of trypsin (a), 2, 4-DCP-Trypsin (b), and 2, 4-DNP-Trypsin (c), respectively. As shown in Fig. 8, peak 1 and peak 2 are the rayleigh scattering peak ($\lambda_{ex}=\lambda_{em}$) and the second-ordered scattering peak ($\lambda_{em}=2\lambda_{ex}$) [17, 18], respectively. With the addition of 2, 4-DNP or 2, 4-DCP, the fluorescence intensity of peak 1 decreased. The production of resonance light scattering spectra (RLS) is correlated with the formation of certain aggregate and the RLS intensity is dominated primarily by the particle dimension of the formed aggregate in solution [34]. Bearing these points in mind, it is inferred from the results that the added 2, 4-DNP or 2, 4-DCP may interact with

Table 4 Three-dimensional fluorescence spectral characteristics of trypsin, 2, 4-DNP-Trypsin, and 2, 4-DCP-Trypsin system

System		Peak A ($\lambda_{ex}/\lambda_{em}$)	Δλ (nm)	Intensity	Intensity ratio	Peak B ($\lambda_{ex}/\lambda_{em}$)	Δλ (nm)	Intensity
Trypsin		280/337	57	527.56	0.56 : 1	230/340	110	940.11
2, 4-DCP-Trypsin [n(DCP) : n(Trypsin)]	10 : 1	280/340	60	373.90	0.87 : 1	230/335	105	429.99
	25 : 1	275/337	62	256.82	1.42 : 1	225/337	112	180.49
	40 : 1	270/342	72	202.52	2.24 : 1	225/337	112	90.10
2, 4-DNP-Trypsin [n(DNP) : n(Trypsin)]	1 : 1	280/335	55	378.31	0.60 : 1	230/337	107	627.88
	2.5 : 1	280/331	51	256.18	0.68 : 1	230/331	101	375.30
	4 : 1	280/327	47	185.13	0.72 : 1	230/328	98	257.37

trypsin in solution, forming a new complex that could be expected to be an aggregate. The size of complex particles may be smaller than that of trypsin, and thus the decreased light-scattering signal occurred under the given conditions. In Fig. 8(a), two typical fluorescence peaks could be easily observed in three-dimensional fluorescence contour map of trypsin. We think that peak A mainly reveals the spectral characteristic of tryptophan and tyrosine residues. The reason is that when trypsin is excited at 280 nm, it mainly reveals the intrinsic fluorescence of tryptophan and tyrosine residues. The tryptophan, and tyrosine in the binding cavity of protein have conjugated π-electrons and easy to form charge transfer complex with other π-electrons system. Besides peak A, there is another new strong fluorescence peak B. And the excitation wavelength of this peak is 230 nm, which can provide some clues for us to investigate the characteristic of this peak which is mainly caused by the transition of $n \rightarrow \pi^*$ of trypsin's characteristic polypeptide backbone structure C=O [35]. Analyzing from the intensity changes of peak A and peak B, they decreased obviously but to different degree (Table 4). We can conclude that the interaction of 2, 4-DCP or 2, 4-DNP with trypsin induced the slight unfolding of the polypeptides of protein, which resulted in a conformational change of the protein [18]. The above phenomena and the analysis of the fluorescence characteristic of the peaks revealed that the binding of 2, 4-DNP and 2, 4-DCP with trypsin induced some micro-environmental and conformational changes in trypsin.

Conclusion

This work examined the effect of toxic ligand binding on trypsin. The intrinsic tryptophan fluorescence of trypsin proves to be an important tool for a better understanding regarding the crucial but specific changes occurring in the molecule. The results obtained gave preliminary information on the binding of 2, 4-DNP and 2, 4-DCP to trypsin. The experimental results showed that the fluorescence quenching of trypsin by 2, 4-DNP and 2, 4-DCP was a result of the formation of complex; static quenching was confirmed to result in the fluorescence quenching. The results revealed the presence of a single class of binding site on trypsin. The electrostatic repulsion does favor the interaction between 2, 4-DNP and trypsin. However, the interaction of 2, 4-DCP and trypsin can be explained on the basis of hydrogen bonding and van der Waals. The results of synchronous fluorescence spectroscopy and three-dimensional fluorescence spectra indicated that the structure of these trytophan and tyrosine residues environments were altered by 2, 4-DNP and 2, 4-DCP. The binding study of 2, 4-DNP and 2, 4-DCP with trypsin has toxicological importance. This study is expected to provide important insight into the interactions of the physiologically important proteinases with nitrophenols and chlorphenols.

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