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Quantitative Study of State Switching in Protein using a Single Probe combined with Trilinear Decomposition

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Abstract

The present investigation attempts to separate the variation of time domain from the steady-state fluorescence and make a quantitative discussion for state-switching of α-chymotrypsin (CHT). The activity of CHT is pH dependent (inactive at low pH, like 2.2, but active at physiological pH, like 8). ANS (1-anilinonaphthalene-8-sulfonate) have two states of fluorescence corresponding to different excitation and emission processes respectively. Though a steady-state technique, the Excitation-Emission Matrix fluorescence (EEM) can record all the excitation and emission signals for the ANS-CHT complex system. The trilinear decomposition of the constructed three-way data set (using EEM data of different samples) can provide excitation and emission spectra indicating specific excitation and emission processes, respectively, and a quantitative description for the time domain processes. Besides a detailed description of the excitation-emission processes of ANS, the quantitative investigation of state-switching by CHT is also possible because the fluorescence due to $S_{1,ct}$ state of ANS is sensitive to the solvation environment, which is one of the indicators of CHT's activity. Finally, the switching output curve of ANS-CHT system in a wide pH range is obtained. This study proposes a convenient and economical protocol for investigating the state-switching in proteins.

1. Introduction

Researchers can make comprehensive observations about a biomolecule with the developments in instrumental technology and methodology. X-ray diffraction is one such technology that has proved to be invaluable to the investigation of the structure of proteins. Similarly, reported NMR methods can provide an unprecedented view of protein dynamics[1, 2]. Femtosecond-resolved spectroscopic techniques make it possible to study the ultrafast surface hydration dynamics of proteins[3-5].

The kernel of research for ultrafast surface hydration is the hydration correlation function which can provide different time components (fast and slow) indicating the dynamic behavior of the solvent shell[4-6]. Though signals in the probe-specific predetermined wavelength interval are recorded at every moment, only the maximal emission wavelength is utilized to calculate the correlation function. There seems much rise space to promote the utilization ratio for the signals acquired, because the rest of recorded wavelengths are thrown away except the maximal emission wavelength. The contents of different time-dependent components in a correlation function are compared, and a description of the motion of water molecules is achieved therefrom. Pal *et al*. have investigated the surface hydration dynamics of α-chymotrypsin (CHT) by using ANS (1-anilinonaphthalene-8-sulfonate) as a probe[6]. The results show that the contents of fast components in the correlation function rises up and the contents of slow components decrease when the pH of the solvent is switched from 3.6 to 6.7. As mentioned above, the comparisons are made specifically between different time components in the same correlation function. It was not clear if there was change (increase/decrease) in only one time-dependent process, as the same time-dependent process was not monitored when the experiment was performed under different conditions. Furthermore, variations in just one time-dependent process can lead to the same results. To gain to a more comprehensive understanding of these phenomena, researchers can look at these time-dependent processes from a much more different perspective.

Kosower's and Kanety[7, 8] found that ANS and its derivatives have two excited

states–naphthalene-excited state ($S_{1,np}$) and charge-transfer state ($S_{1,ct}$)–that can return to ground state by fluorescence emission. The fluorescence maxima and quantum yields of the $S_{1,np}$ state does not vary with changes in the solvent environment. In contrast, the $S_{1,ct}$ state is strong only in nonpolar solvents or when in solid form and its fluorescence can be quenched via charge transfer from one aromatic moiety to the other ring and solvation. The Excitation-Emission Matrix (EEM) fluorescence technique can acquire all the signals involved in the excitation and emission processes. It is possible that under certain conditions, the peaks due to emissions from the two states, when coexisting, can have a significant overlap in the EEM spectrum, making the interpretation of results for the independent states of the molecule from such investigations challenging. A technique that can separate the signals emerging from each of the states will facilitate the steady-state fluorescence investigations related to the excitation-emission process.

The trilinear decomposition, also known as CANDECOMP/PARAFAC (CP) tensor decomposition[9, 10], has received significant attention in the past two decades[11-14], primarily, due to the accompanying benefit second-order advantage[15]. This decomposition methodology helps a researcher obtain qualitative and quantitative information of a system of interest even with interference in the signal from unknown analytes and therefore, provides results with chemical/physical significance. Many investigations for the quantitation of components of interest (drugs, pollutants, hormone, *etc*.) have been carried out by coupling trilinear decomposition algorithms with EEM[13, 16-20]. Most widely used algorithms to carry out such decompositions include PARAFAC (Parallel Factor Analysis), ATLD (Alternating Trilinear Decomposition)[21], SWATLD (Self-Weighted Alternating Trilinear Decomposition)[22], and APTLD (Alternating Penalty Trilinear Decomposition)[23]. The three-way data set generated by EEM of several samples is processed by the trilinear decomposition algorithm, then the pure excitation spectra, emission spectra and quantitative information of each component would be obtained at last. Phenomenon from the investigation of ultrafast hydrations show that steady-state fluorescence spectra of the probe/protein are different under different hydration conditions. The likely reason for

this phenomenon is the variations in the rate of time-dependent components of the correlation function, which correspond to diverse excitation and emission processes of the probe in different micro-environments. Though EEM is a steady-state technique, the trace of this variation in time-dependent excitation and emission processes under different conditions would appear in the EEM spectra. If the proper data set of EEM is constructed, the variation of time-dependent processes would be separated from the steady-state fluorescence by the trilinear decomposition. Once the variation of time-dependent processes has been separated from the steady-state fluorescence, it will be much more convenient to make a quantitative discussion about the hydration phenomenon. In addition, a fluorescence spectrophotometer which can provide EEM data is much more economical and convenient to use. A new way for quantitative investigation of hydration will be presented in this work.

Another objective of this work is to provide a quantitative analysis of the switch (on/off) state of a biomolecule. Some widely employed methods that have a switch output are FRET (Föster Resonance Energy Transfer) and fluorescence-quenching based methods, but the probes used in these methods are easily influenced by the environment of the solution[24]. Besides, FRET-based methods sometimes involve complicated protocols for the labeling of the substrate with the specific probe. In this study, we place a probe on the surface of CHT and detect its state-switching property based on the EEM combined with trilinear decomposition. The probe ANS is sensitive only to changes in the solvent shell around itself, so it has the potential to avoid the interference due to any changes in other parts of the solution. Moreover, any interference in the system which might occur in the EEM signals can be separated from the probe signal by trilinear decomposition. The present investigation monitors the state of a protein by using a single probe quantitatively.

2. Experimental Section

2.1 Theory of Trilinear Decomposition

An Excitation-Emission Matrix (EEM) fluorescence data which contains one fluorescence component can be represented as an outer product of normalized excitation spectrum ($\mathbf{a}$, column vector) and normalized emission spectrum ($\mathbf{b}$, column vector), then scaled by the component concentration (ρ) and its standard EEM fluorescence coefficient (ξ) as shown in eq.(1). The component ρ and ξ can be replaced by the relative content (c). The fluorescence component can be a fluorescence spectrum of a single probe, if this probe has an identical excitation-emission process under different conditions. Furthermore, the fluorescence component also can be a specific fluorescence pattern of a single probe in a corresponding condition, if this probe has multiple excitation-emission processes under different conditions. The excitation spectrum ($\mathbf{a}$) can indicate a specific excitation process which is an excitation behavior of time domain in corresponding condition. The emission spectrum ($\mathbf{b}$) can indicate a specific emission process which is an emission behavior of time domain in corresponding condition. If the concentration (ρ) is kept constant between different conditions, the variation of fluorescence coefficient (ξ) demonstrates the magnitude of the corresponding excitation-emission process.

$$\begin{aligned}\mathbf{X} &= \xi\rho\mathbf{a}\mathbf{b}^T \\ &= c\mathbf{a}\mathbf{b}^T\end{aligned} \tag{1}$$

The three-way data set $\underline{\mathbf{X}}$ can be constructed by stacking the EEM data along the third-mode according to the experimental design (Figure 1). This three-way data set has the characteristic called trilinearity. The size of $\underline{\mathbf{X}}$ is $I \times J \times K$; it contains I excitation wavelengths, J emission wavelengths and K pH variant samples. $\underline{\mathbf{X}}$ can be decomposed into three component matrices ($\mathbf{A}$, $\mathbf{B}$, and $\mathbf{C}$), one super diagonal tensor $\underline{\mathbf{I}}$, and one residual tensor $\underline{\mathbf{E}}$. The matrix $\mathbf{A}$ ($I \times N$) contains the normalized

excitation spectra of N components in the system; the matrix $\mathbf{B}$ ($J\times N$) contains the normalized emission spectra of N components in the system; and the matrix $\mathbf{C}$ ($K\times N$) contains the relative content values of N components. $\underline{\mathbf{I}}$ is a $N\times N\times N$ core tensor with ones on the super diagonal and zeros elsewhere, and it will turn into $I\times J\times K$ tensor when multiplied with $\mathbf{A}$, $\mathbf{B}^T$ and $\mathbf{C}^T$, respectively. The element wise relation of trilinear model is shown in eq.(2).

$$x_{ijk}=\sum_{n=1}^{N}a_{in}b_{jn}c_{kn}+e_{ijk} \qquad (2)$$
$$\text{for } i=1,2,\ldots,I;\quad j=1,2,\ldots,J;\quad k=1,2,\ldots,K$$

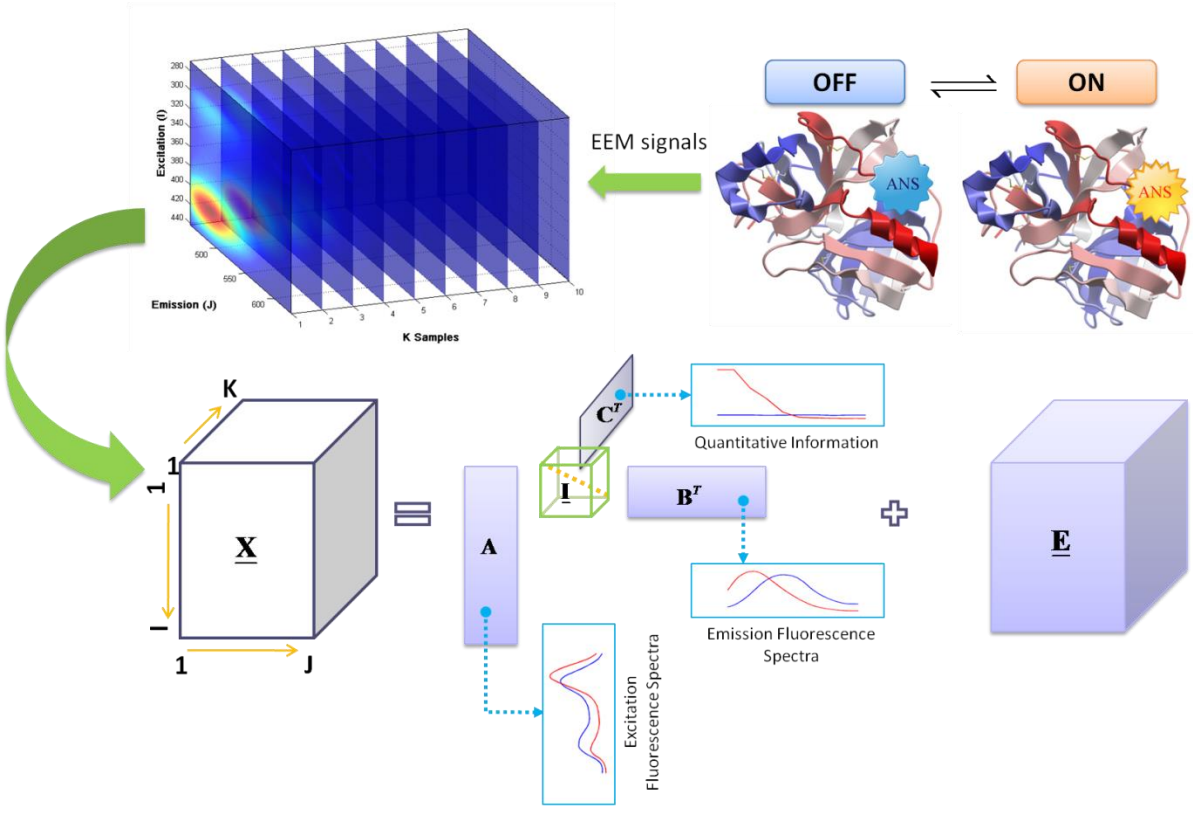

Figure 1. Scheme for trilinear decomposition.

There are several widely employed algorithms, such as PARAFAC, ATLD, SWATLD and APTLD, that can implement the trilinear decomposition process. We preferred ATLD, previously designed by us, to decompose the experimental data set, because the ATLD is convenient to use, insensitive to the estimated component number (N), and always converges fast. Likewise, when the data was decomposed using other algorithms, the same results, as obtained from ATLD decomposition, were

observed. The results obtained from the ATLD decomposition are discussed in this paper as a demonstration for the trilinear decomposition algorithms. The fitting efficiency can be demonstrated as the lack-of-fit (LOF)[25] described in eq.(3).

$$LOF = \frac{\left\|\underline{\mathbf{E}}\right\|_F^2}{\left\|\underline{\mathbf{X}}\right\|_F^2} = \frac{\sum_{k=1}^{K}\sum_{j=1}^{J}\sum_{i=1}^{I} e_{ijk}^2}{\sum_{k=1}^{K}\sum_{j=1}^{J}\sum_{i=1}^{I} x_{ijk}^2} \quad (3)$$

2.2 Materials, Measurements and Data Processing

Fluorescence measurements were recorded using 1.00 cm quartz cell in an F-4500 fluorescence spectrophotometer (HITACHI) fitted with a xenon lamp. The excitation wavelength varied from 270 to 470 nm (2.0 nm steps) and the emission wavelength varied from 430 to 630 nm (2.0 nm steps). The excitation and emission monochromator slit widths were 5.0 and 5.0 nm, respectively. The scanning rate was 2400 nm $\cdot$min^{-1}.

All computer programs were written in Matlab and all calculations were carried out on a PC with the Windows Vista operating system.

ANS (ammonium salt, 95% pure) was purchased from Sigma. CHT was obtained from Aladdin chemical company. The concentrations of ANS and CHT in experimental samples were kept constant (ANS: 0.5 μmol/ml; CHT: 37.5 μg/ml). The pH of the experimental sample was adjusted by the addition of Na_2HPO_4/citric acid buffer solutions. The pHs of the various aqueous solutions were 2.2, 2.6, 3, 3.2, 3.6, 4, 5, 6, 7, and 8.

3 Results and Discussion

There are two excitation-emission pathways for ANS[7, 8]. In the first process, ANS is excited from ground state (S_0) to $S_{1,np}$, which is then followed by a fluorescence emission ($S_0 \rightarrow S_{1,np} \rightarrow h\nu$). The second process involves a longer sequence of events wherein after the initial formation of $S_{1,np}$, an intramolecular electron transfer reaction results in the formation of the charge-transfer state, $S_{1,ct}$, which finally returns to the

ground state with a fluorescence emission ($S_0 \rightarrow S_{1,np} \rightarrow S_{1,ct} \rightarrow h\nu$). The execution of second process consumes much more time[26]. The $S_{1,ct}$ state can be quenched without fluorescent emission *via* electron-transfer reaction[27]. If the solvation is greater at $S_{1,ct}$, the screening will be greater, and the electron-transfer reaction will be faster. If the solvation of water molecules could be inhibited, the fluorescent emission from $S_{1,ct}$ would be possible. This is the reason why ANS and its derivatives serve as an effective and simple biological probe to analyze the hydration conditions. There is merely a single site on the surface of CHT near the Cys-1-122 disulfide bond where the ANS binds to[28]. It was reported that the binding site is quite polar with ionized groups on one side of the ANS and water on the other. However, the ANS-CHT complex has fluorescent emission from $S_{1,ct}$ state at low pH, which was peculiar.

Under the experimental conditions, the EEM profiles of ANS dissolved in water is not influenced by the variations in pH. However, the EEM profiles of ANS-CHT vary based on the pH of the solution. In Johnson's research[29], it suggested that the binding constants of ANS-CHT complex are about the same at different pHs, and the difference of the calculated results may be caused by fluorescence quenching. The EEM profiles of ANS-CHT complex in a buffered solution with a pH of 8 is exactly the same as that of ANS in water, which indicates that the mobility of the hydrated layer on the CHT surface is really fast (bulk-type[4, 6]). The EEM profiles of the complex at two extreme pHs (2.2 and 8) are shown in figure 2. Two distinct fluorescence patterns, which represent two individual excitation-emission processes , can be observed. In the pH range from 3.2 to 6, there is an overlap in these two features in the EEM.

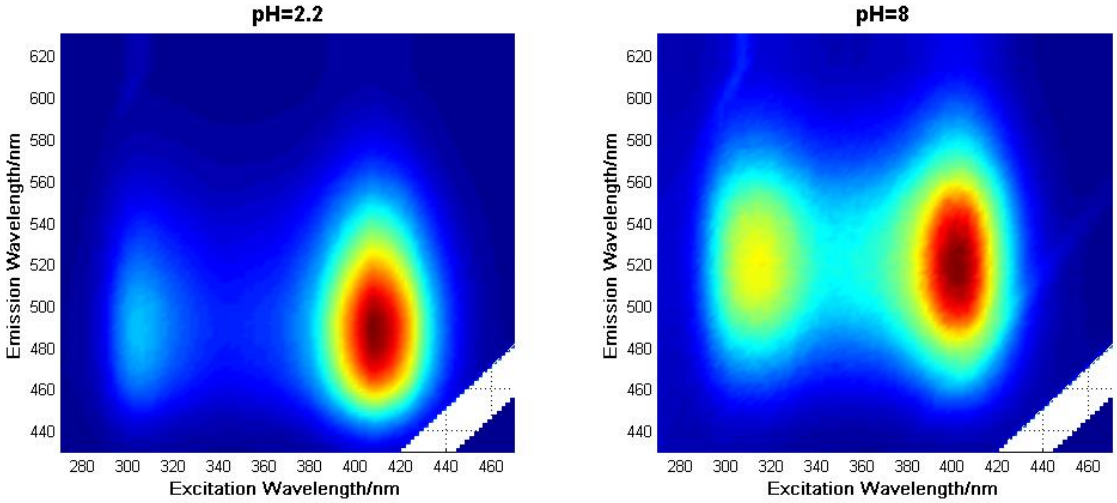

Figure 2. The EEM profiles measured for ANS-CHT complex at two extreme pH. The Rayleigh Scattering had been hollowed in order to make the response of the object obvious.

In order to avoid the Rayleigh Scattering, which lacks trilinearity, truncated EEM data (EX=[270 nm, 442 nm], EM=[454 nm, 630 nm]) in each sample is considered for the construction of the three-way data set. The ATLD algorithm is utilized to implement the trilinear decomposition because it is not sensitive to the number of components. The ergodic test, evaluated for 2~4 components, suggests that there are two fluorescent components in the system. Because when the components number is larger than 2, the spectra of rest components look like noise and have almost zero response in matrix **C** which means little contribution to fitness. Other widely used algorithms provide almost the same results as ATLD, when the component number is set to 2. A series of assistant methods have been developed to overcome the problem how to estimate the chemical component number (Kruskal *et al.*[30], Xie *et al.*[31], Bro *et al.*[32], Chen *et al.*[22], Xia *et al.*[25]), researcher can use appropriate methods for correct estimation. The decomposition results are shown in figure 3. The assignment of the excitation and emission processes is made based on the resolved emission spectra, because the $S_{1,ct}$ has a shorter maximal emission wavelength (486 nm)[7, 8]. The excitation spectra obtained after the decomposition of EEM reveal more details pertaining to the excitation process. The two peaks in excitation spectrum of $S_{1,ct}$ are separated by a slightly larger extent than the two peaks in the excitation spectrum of $S_{1,np}$. Excitation at

longer wavelength (410 nm) is more suitable for ANS to transfer into $S_{1,ct}$ state when compared with the excitation at shorter wavelength. The qualitative description for the excitation and emission processes is made possible because trilinear decomposition can separate the overlapping fluorescent signals.

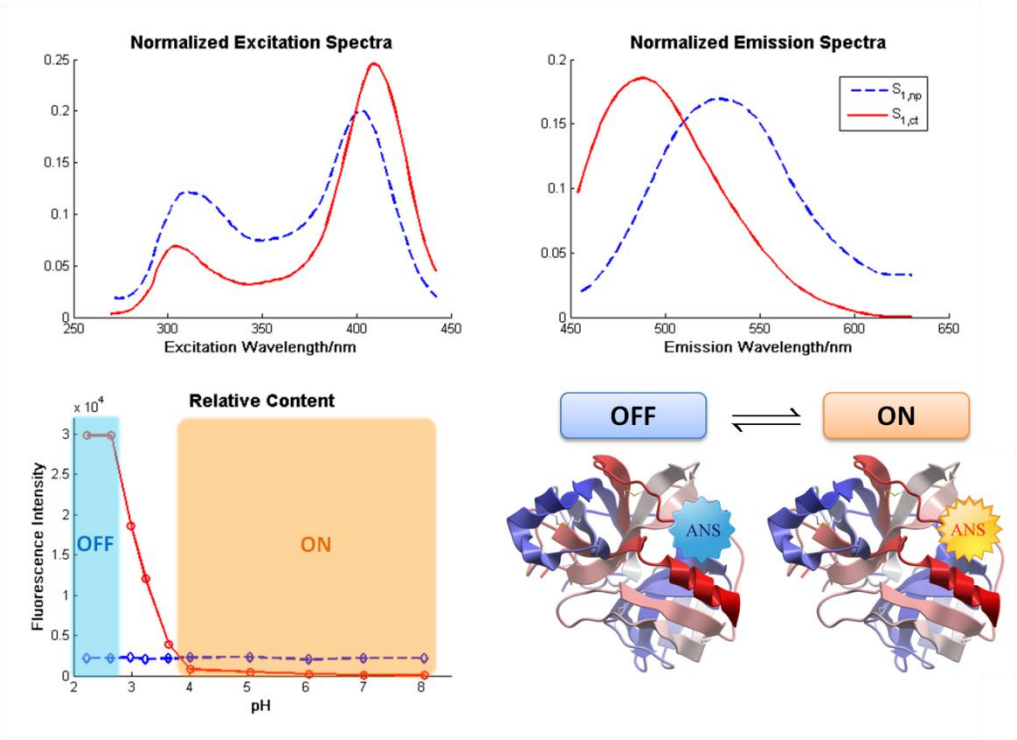

Figure 3. The decomposition results of ATLD. The LOF for the present EEM data is 0.81%, which demonstrates the good fitness.

A significant advantage of the trilinear decomposition is that it provides a quantitative description for each excitation-emission process in time domain. It can be found out that fluorescence intensity of emission from the $S_{1,np}$ state does not change as the pH varies (figure 3), which had been proven in the previous researches. Because the binding constant of the ANS-CHT complex does not change over the experimental pH range, significant changes observed in the intensity of emitted fluorescence from the $S_{1,ct}$ state is caused predominantly by the variations in the solvation environment around the ANS. The electric field generated by the ionized groups of CHT in aqueous solution with low pH≤2.6 constrains the mobility of water molecules on the protein surface. The weaker solvation of biological water on the surface of CHT make the fluorescent emission from $S_{1,ct}$ state of ANS to be possible. The horizontal line of $S_{1,ct}$ fluorescent intensity, where pH≤2.6, indicated the

saturation state of the ionized groups and the switch-off state of CHT. The water molecules on the surface of CHT are rigid to a certain extent[6, 33-35]. When the pH is higher than 3, the mobility of the water molecules on the surface of CHT is significantly enhanced. The less rigid water molecules on the surface of CHT make the active recognition dynamically favorable, which implies a switch-on state for CHT. Almost all CHT molecules (>0.97%) are in switch-on state at pH values greater than 4. This complete change in state is accomplished in a narrow interval of ~1 pH. The curve of $S_{1,ct}$ fluorescence gives a quantitative on-off description for CHT, and the amount of the CHT in 'on' state can be represented as a percentage of the total protein or concentration which is calculated from the curve by using the response of of $S_{1,ct}$ at pH=2.2 as a reference point.

4 Conclusion

When a fluorescent probe is sensitive to short range interactions, its excitation and emission spectra is likely to vary with change in its environment. A typical example is the tryptophan residue in proteins[36-38]. The multi-state fluorescence provides rich information about the interactions in the system when each spectrum is quantitatively separated. The fluorescence properties of ANS is determined by the solvation and polarity in its microenvironment, which makes ANS suitable for use in reporting on the hydration on the surface of the protein. The EEM combined with trilinear decomposition can quantitatively investigate the excitation-emission processes in time domain even when their EEM profiles overlap significantly. This is because the EEM records all the excitation and emission signals, though it is a steady-state technique, and the trilinear decomposition has ability to achieve results full of chemical/physical meaning. At last, the state-switching of CHT has been described quantitative by the $S_{1,ct}$ fluorescence of ANS which is separated by trilinear decomposition. This work provides a convenient and economical vehicle for quantitative analysis of the protein state. Researchers can use a fluorescent probe with appropriate multi-state fluorescence (may be sensitive to solvation, polar, hydrogen bond, *etc*.) in the objective position of protein, and the

selectivity in data analysis can be guaranteed by trilinear decomposition coupled with EEM.

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