

Study on the violet LED-induced fluorescence spectra of thioredoxin reductase from human brain

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The technique of fluorescence spectroscopy is applied to study thioredoxin reductase (TrxR) in the cells of human brain. Experimental results show that, by the violet light emitting diode (LED, $\lambda_{\max}=407$ nm) light irradiation, TrxR is able to emit three striking spectral bands (528–582 nm; 588–660 nm; 683–700 nm). The fluorescence intensity is linear to the concentration of TrxR. The spectrum of denatured TrxR is rather different from that of organized TrxR, which reflects the structure change between denatured TrxR and organized TrxR. Furthermore, physical and biochemical mechanisms of fluorescence production for LED light-induced TrxR spectra and its characteristics are analyzed. This paper may be useful to better understand the structure of TrxR, and to provide new spectroscopic information to improve the resolution for this kind of biology structure.

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Thioredoxin reductase (TrxR) plays an important role in the thioredoxin system and is ubiquitous in biologic cells such as hepatocyte, epithelial cell, neurocyte and different kinds of secretory cells. The so-called thioredoxin system, thioredoxin (Trx), TrxR, and NADPH, act as a disulfide reductase system and can protect cells against oxidative stress^[1]. TrxR contains a redox active disulfide and is a member of the pyridine nucleotide-disulfide oxidoreductase family of flavoenzymes that include lipoamide dehydrogenase, glutathione reductase, trypanothione reductase, mercuric reductase, and NADH peroxidase. TrxR catalyzes the reduction of the small redox protein thioredoxin by NADPH^[2]. Recent researches also find that TrxR is a selenoenzyme^[3] and one of the important proteins to antagonize nerve degenerative change, such as Parkinson's disease (PD) and Alzheimer's disease (AD)^[4]. While more and more attention is paid to research on the TrxR gene and its biological reactions and functions, the investigation of TrxR structure, especially its space structure, is rare. This hinders a good understanding of the function of TrxR. Nowadays, the methods to study protein space structure are X-ray diffraction^[5], nuclear magnetic resonance (NMR) spectroscopy^[6], Raman spectroscopy^[7], and fluorescence spectroscopy, etc.

Fluorescence spectroscopy has served as a useful and important microscopic probe to characterize various physical and chemical processes in materials for over 60 years^[8]. It has been an extremely valuable analysis tool for determining the chemical composition of biological and biomedical samples and has played an important role in elucidating structural and dynamic properties of proteins^[9–11]. In this paper, we present our fluorescence spectroscopy research on TrxR in the cells of a human brain. We show that TrxR can emit visible fluorescence at the excitation of violet LED light ($\lambda_{\max} = 407$ nm) and there exist difference in the spectral profiles for the organized TrxR and denatured TrxR.

All samples used in the experiment, TrxR in the cells of a human brain, was supplied by the department of

Biochemistry, Nanjing Medical College, Second Military Medical University, Nanjing, China. The gene of TrxR was connected with that of *Escherichia coli* (*E. coli.*) to realize protein recombination and recombinant protein which shows that TrxR gene exists in cytoplasm. When the desired protein is overproduced up to 30% of total cell protein, it is precipitated or insoluble, which is called inclusion body (IB). After concentration of IB by ultrasonication and refrigerated centrifugation, the TrxR sample is obtained by solving the IB in high concentrate denaturant and purifying it. The concentration of crude solution is 30 mg/ml. The other TrxR solutions with concentrations from 20 ml/ml to 0.2 mg/ml were all obtained by diluting crude solution with tri-distilled water. The denatured TrxR was obtained by laying organized TrxR in the room temperature and denaturing unartificially and slowly for about two months.

The experimental arrangement used to measure the spectra from TrxR solution is shown in Fig. 1. About 1 ml solution was placed in a clean, non-fluorescing quartzose vessel (QV). An about 5 mW violet LED light with the wavelength of 407 nm ($\Delta\lambda_{1/2} \approx 16$ nm) was concentrated on the front surface of the QV. The fluorescence from the QV is scanning by a grating spectrometer (1200/mm). A photomultiplier (PM) R928, made by Hamamatsu Corp. in Japan, is used to measure the fluorescence intensity. The output of the PM is connected to a computer to adjust the parameters of the apparatus and display the spectra. The collecting pace-interval, and scanning range of the grating spectrometer is 0.100 nm and 200–850 nm respectively. The emission spectrum of each sample was measured more than three times for reproducibility. The measured spectra were stable in time.

The violet LED light ($\lambda_{\max} = 407$ nm) induced spectrum of TrxR in the cells of a human brain with the concentration of 30 mg/ml is displayed in Fig. 2. Obviously, there are three fluorescence bands of 528 to 582 nm, 588 to 660 nm, and 683 to 700 nm in the spectrum with the peaks located at about 545, 612 and 692 nm.

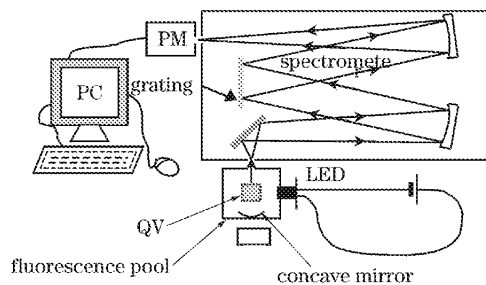


Fig. 1. Schematic diagram of the experimental setup.

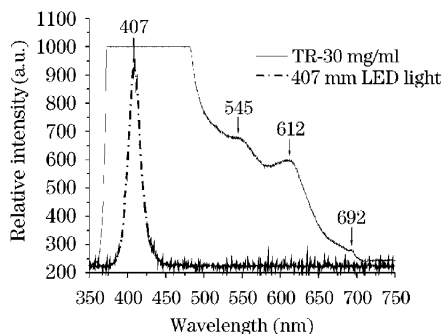


Fig. 2. The fluorescence spectrum of TrxR.

According to the experimental result, we can conclude that TrxR is able to emit fluorescence under the excitation of 407 nm LED light. Based on the principle of fluorescence^[12], there should be fluorophores containing conjugated π system in TrxR molecules, which can absorb the energy of violet LED light and emit fluorescence.

Since there are three fluorescence bands in the TrxR spectrum, based on the fluorescence knowledge and the characteristics of biomolecules in Ref. [12], we deduce an energy level diagram that contains a large of vibrational levels and three or more excited states able to emit fluorescence in the fluorophores (see Fig. 3). Generally, fluorophores are in their ground electronic state at room temperature. Upon excitation, fluorophores in ground state absorb the energy of 407 nm LED light and jump to higher vibration energy levels of the electronically excited singlet state. To regain the equilibrium or to ground state, several processes may take place. The first event is that fluorophores in the vibrational levels, above the lowest excited state, lose their excess energy (usually in the form of heat) moving to a lower vibrational state, but still in the excited electronic state. Then, from the singlet state, the fluorophores return to the electronic ground state with the emission of the fluorescence. During the process of fluorophores in the vibrational levels losing their excess energy, different fluorophores may lose unequal energy and stay in different excited levels, such as E_1 , E_2 , and E_3 . As a result, fluorophores emit fluorescence with various wavelengths when they return to the ground state (E_0). The spectrum of TrxR is broad due to the fact that molecules are in a constant state of vibration, and consequently vibrational transitions are superimposed with the electronic transitions. This means that each electronic transition consists of a large number of lines, so closely spaced that they cannot be resolved by the grating spectrometer. As a result, a fluorescence band

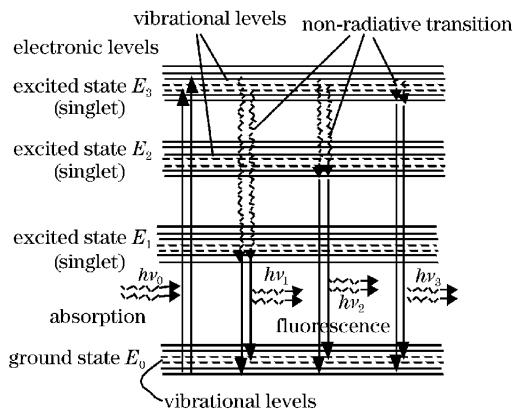


Fig. 3. Energy transitions in the fluorophores.

is formed.

In order to investigate fluorescence characteristics in detail, we measured the spectra of TrxR with different concentrations from 30 to 0.2 mg/ml (see Fig. 4). Apparently, with the decreasing TrxR concentration, the relative intensity of fluorescence is reduced. From the peak at 612 nm, we can obtain that the fluorescence intensity is linear with the TrxR concentration (see Fig. 5).

It is well known that fluorescence is the reemission of light as a result of material's absorption of light from other sources. The intensity of fluorescence has a close connection with the molar absorptivity of the solution, and the quantum efficiency of the fluorescence species in the solution^[13]. When the illuminating light has a constant wavelength and intensity, the relationship between fluorescence intensity F and molar concentration of the fluorophore C can be described as

$$F = Y_F F_0 (1 - e^{-\epsilon b C}), \quad (1)$$

where Y_F is the quantum efficiency of the fluorescence species, F_0 is the incident power of the exciting beam, ϵ is the molar absorptivity of the solution, and b is the sample cells path length.

At low to moderate concentrations, this function reduced to a linear relationship as

$$F = Y_F F_0 \epsilon b C. \quad (2)$$

Obviously, the function (2) is coincident with our experiment results (see Fig. 5).

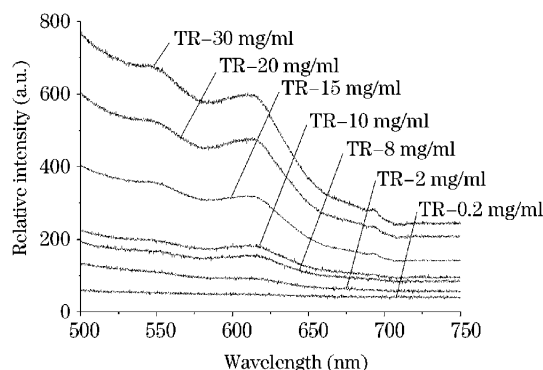


Fig. 4. The fluorescence spectrograms of TrxR with various concentration.

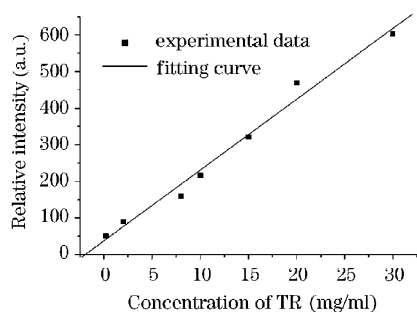


Fig. 5. The relation of fluorescence intensity and TrxR concentration ($\lambda = 612$ nm).

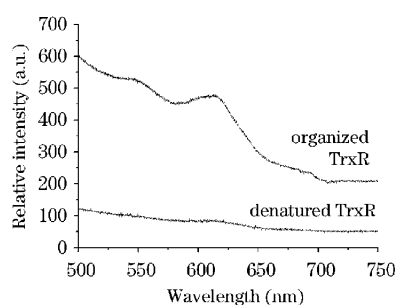


Fig. 6. Spectra of organized TrxR and denatured TrxR with the concentration of 30 mg/ml.

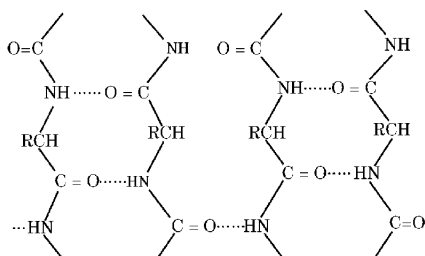


Fig. 7. Hydrogen bands in fundamental chain of a protein.

During our experiments, we also find that the fluorescence profiles of organized TrxR and denatured TrxR with concentration of 30 mg/ml are different (see Fig. 6). By comparing the two spectra, it can be easily seen that there is only one fluorescence band from 588 to 660 nm in the spectrum of denatured TrxR, while organized TrxR has two more fluorescence bands from 528 to 582 nm, and 683 to 700 nm. Besides, the fluorescence intensity of denatured TrxR is much lower than that of organized TrxR.

According to the molecular biology^[14], the highly polar nature of the C=O and N-H groups of the peptide bonds gives the C-N bond partial double bond character. This makes the peptide bond unit rigid and planar, though there is free rotation between adjacent peptide bonds. This polarity also favors hydrogen bond formation between appropriately spaced and oriented peptide bond units. Thus, polypeptide chains are able to fold into a number of regular structures which are held together by these hydrogen bonds. Figure 7 gives a diagram of hydrogen bands in fundamental chain of a protein, deduced from our measurements. Furthermore, the way in which the different sections of α -helix, β -sheet, other minor secondary structures and connecting loops fold in three

dimensions is the tertiary structure of the polypeptide. Factors of denaturation disrupt various types of noncovalent interaction between side chains, such as van der Waals forces, hydrogen bands, electrostatic salt bridges between oppositely charged groups, hydrophobic interactions and covalent disulfide bonds, and then leads to loss of secondary and tertiary structure and formation of a random coil conformation. Therefore, we can conclude that the fluorescence regions of 528 to 582 nm, and 683 to 700 nm result from the various types of noncovalent interaction and covalent disulfide bonds which hold the secondary structure and tertiary structure of TrxR, while the region of 588 to 660 nm is produced by primary structure and affected deeply by space structure of TrxR.

In summary, upon the excitation of violet LED light, TrxR is able to emit the visible fluorescence, and the emission spectra of organized TrxR and denatured TrxR are found to be substantially different, each displaying its own characteristic fluorescence bands. Currently, research on fluorescence spectroscopy of TrxR is in its embryonic stage. Further researches will be emphasized on the influence of microenvironment on TrxR fluorescence profiles and the differences between TrxR spectra in cells and in solution so that the structure and biological function of TrxR can be well understood.

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