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# Laser induced autofluorescence in the monitoring of β-mercaptoethanol mediated photo induced proton coupled electron transfer in proteins

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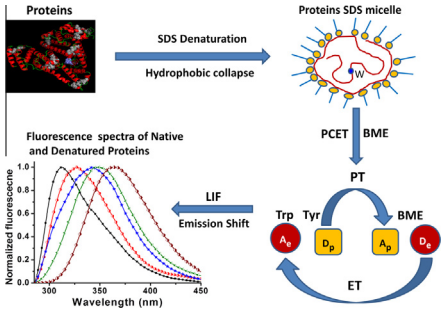
## HIGHLIGHTS

- SDS induces hydrophobic collapse to tryptophan residues in BSA and HSA.
- Photo-induced radical anion formation results in rapid autofluorescence quenching.
- Proton transfer from hydroxyl group of tyrosine results in unusual red shift.
- LIF spectra gives a clear evidence of BME induced PCET in BSA and HSA.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Photo induced proton coupled electron transfer (PCET) is an important process that many organisms use for progression of catalytic reactions leading to energy conversion. In the present study, the influence of SDS and BME on the redox properties of tyrosine and tryptophan for five different globular proteins, BSA, HSA, RNase-A, trypsin and lysozyme were studied using laser induced autofluorescence. The proteins were subjected to denaturation under SDS, SDS plus heat and SDS plus β-mercaptoethanol (BME) plus heat and the corresponding fluorescence were recorded. The influence of BME on the autofluorescence properties of the proteins were evaluated upon tris-2-corboxy-ethyl phosphine (TCEP) denaturation. The BSA and HSA when exposed to SDS alone, exhibited hydrophobic collapse around their tryptophan moieties. However, these proteins when treated with SDS plus BME plus heat, an unusual red shift in the emission was observed, may be due to proton transfer from hydroxyl group of the excited tyrosine residues to the local microenvironments. The observation was further confirmed with similar proton transfer in absence of tryptophan in RNase-A showing involvement of tyrosine in the process. A drastic quenching of fluorescence in all of the proteins under study were also observed, may be due to photo-induced electron transfer (PET) from BME to the intrinsic fluorophores resulting in radical ions formation, evaluated upon DCFDA measurements.



## Introduction

The proteins in native states demonstrate numerous biological functions and many of them such as, water splitting and photosystem II related reactions, nitrogen fixation and ribonucleotide reductase reactions etc. are governed by proton coupled electron

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transfer (PCET) reactions [1]. The PCET is an important reaction mechanism involved in catalytic reactions and knowing more about it is helpful for understanding of their roles in living systems. Further, proteins are vital molecules involved in regulation of biological functions and PCET reactions with the help of various amino acids including tyrosine and tryptophan. These aromatic amino acids along with phenylalanine are responsible for fluorescence in proteins providing a scope to access PCET reactions using this property. Further, the fluorescence yield of tryptophan is more as compared to tyrosine and phenylalanine in proteins [2]. Many studies have also demonstrated that unfolding of proteins result in exposing of their tryptophan moieties to the outer aqueous environment and thereby shifting of corresponding emission towards red due to the solvent relaxations [2–4]. The extent of shift at physiological pH depends on the level of unfolding which further relies upon the bonds stabilizing the structures in the proteins and the type of denaturants used in the process [2–6]. The tyrosine on the other hand is least sensitive to the solvent exposures at physiological pH in contrast to high pH where spectral property gets altered upon solvent exposures [2,7]. At high alkaline pH, protonation from the hydroxyl group of the excited tyrosine reported to induce red shift in its emission [2,7–9].

There are several methodologies available for denaturing proteins and among them SDS and thermal mediated denaturation are the most studied ones. In SDS induced denaturation, it interacts with the hydrophobic groups of the proteins to bring about the observed variations in them [10–12]. There are several models to predict SDS–protein interactions during denaturation and among them the necklace and bead model where micelles like clusters are formed is the most satisfying model [11,12]. Further, beta-mercaptoethanol (BME) is a well-known disulphide braking agent used along with the SDS for protein denaturation. The thermal denaturation of the proteins in presence of SDS and BME is one of the most extensively studied methods of protein denaturation. Further, the BME is also found useful in redox systems to maintain the photo stability of fluorophores in proteins [13]. In the present study, the unfolding and redox properties of the five different globular proteins (BSA, HSA, lysozyme, ribonuclease A and trypsin) showing PCET reactions was evaluated using laser induced fluorescence. Further, influence of BME on the spectral properties of tyrosine and tryptophan was analyzed using tris-2-corboxy-ethyl phosphine (TCEP) denaturation and 2–7 dichlorofluoresceindiacetate (DCFDA) assay.

## Materials and methods

### *SDS and SDS plus BME induced denaturation*

The stock solutions for each of the standard proteins (100 μM) and denaturation buffers for SDS alone (8% or 0.26 M) and SDS (8%) + BME (1.41 M) under study were prepared using 0.1 M tris buffer of pH 7.0. In case of trypsin, it was prepared fresh just before the experiment and temperature was strictly maintained at 0–4 °C. Various concentrations of working standards (denaturant-protein mixtures: 0.125%, 0.25%, 0.5%, 1.0%, 1.5% and 2.0%) for SDS, SDS + heat (95 °C [14,15], using dry bath model-QBD4, Grant instruments, UK) and SDS + BME + heat denaturation were prepared by mixing different volumes of SDS denaturant stock and SDS + BME denaturant stock solutions with 100 μM protein stock solutions. The BME concentrations in the corresponding working standards were 21, 42, 84, 168, 252 and 336 mM respectively. The final concentration of the proteins in each of the protein-denaturant working standards was maintained at 10 μM.

The protein working standard is the final reaction mixture which in context of the current study is, Protein + SDS or Protein + SDS + BME or Protein + SDS + TCEP. These working standards are prepared by mixing different stock standards (stock standards are the higher concentrated solutions for each of the reactants mentioned above, which upon dilution provide required lower concentrations for actual use) in which fluorescence recordings were performed. The fluorescence recordings were done in the spectral region 300–500 nm at 281 nm excitations.

### *SDS plus TCEP induced denaturation*

The TCEP stock solution (300 mM) was prepared by mixing 0.86 g of TCEP in 0.1 m tris buffer of pH 7.0. The working standards for SDS + TCEP buffer was prepared by mixing 8% SDS stock buffer with 100 μM protein stock solutions maintaining TCEP concentrations of 5, 10, 20, 30, 40 and 50 mM respectively.

### *DCFDA (2–7 dichlorofluoresceindiacetate) assay*

The stock solution for DCFDA (100 μM) assay was prepared in DMSO and de-esterified with basic pH in dark at room temperature for 30 min. This solution was then neutralized with tris buffer of pH7.0 and kept in dark until used [16,17]. Various concentrations (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μM) of the stock solutions were added to the reaction tubes containing only 0.1 M tris buffer, 0.1 M tris buffer plus 10 μM BSA, 0.1 M tris buffer plus 42 mM BME and 0.1 M tris buffer plus 10 μM BSA plus 42 mM BME respectively. Solutions were mixed by gentle vortex followed by incubation in dark for 2 min. The fluorescence intensity for all of the samples at an emission maximum of 525 nm was recorded at 488 nm excitation using spectrofluorometer (Varioskan Flash, Thermo scientific).

### *Measurements of autofluorescence*

The fluorescence of the protein working standards were recorded in the spectral region 300–500 nm at 281 nm pulsed laser excitations from Nd-YAG (*Model LM1278 LPY 707G-10, Litron lasers, UK*) second harmonic (532 nm) pumped frequency doubled dye laser (*Model-PULSARE Pro, FINE ADJUSTMENTS, Germany*) system with Rhodamine 6G dye at 10 Hz repetition. The laser beam was allowed to incident normally into the cuvette containing samples under study and the corresponding fluorescence were collected at 90° to the excitation using an optical fiber probe of core diameter 200 μm, numerical aperture 0.22. Subsequently, the fluorescence was coupled to the spectrograph (*Model-Shamrock, ANDOR, Ireland*) ICCD (Intensified charge couple device, *Model-iStar, ANDOR, Ireland*) system through 300 nm sharp edge cut-off filter (*Shamrock*) for dispersion and detection as shown in Fig. 1. The fluorescence spectra recorded were subjected to spectral pre-processing using GRAMS/AI 8.0 spectroscopy software for baseline corrections, smoothening and normalization. The spectra were normalized with respect to the highest peak intensity and plotted using origin 8.0.

## Results

### *SDS induced denaturation*

When all the five proteins were treated with different concentrations of SDS alone, the BSA and HSA showed almost similar fluorescence patterns, displaying a blue shift of about 20 and 34 nm at the initial concentration (0.125%) as compared to their native forms (Fig. 2A and B). However, as the concentrations of SDS was increased, the shift in BSA was revert back slowly displaying 6 nm red shift at the final concentration of 2% SDS (Fig. 2A) whereas the HSA maintained constant shift throughout (Fig. 2B).