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Thermal Effect on *Aequorea* Green Fluorescent Protein Anionic and Neutral Chromophore Forms Fluorescence

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Abstract The emission behaviour of *Aequorea* green fluorescent protein (A-GFP) chromophore, in both neutral (N) and anionic (A) form, was studied in the temperature range from 20 °C to 75 °C and at pH=7. Excitation wavelengths of 399 nm and 476 nm were applied to probe the N and A forms environment, respectively. Both forms exhibit distinct fluorescence patterns at high temperature values. The emission quenching rate, following a temperature increase, is higher for the chromophore N form as a result of the hydrogen bond network weakening. The chromophore anionic form emission maximum is red shifted, upon temperature increase, due to a charge transfer process occurring after A form excitation.

Keywords Green fluorescent protein · Chromophore · Fluorescence · Temperature

Abbreviations

BCA	Bicinchoninic acid
ESPT	Excited-state proton transfer
FPLC	Fast protein liquid chromatography
GFP	Green fluorescent protein
Gly	Glycine
His	Histidine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
Ser	Serine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Trp	Tryptophan
Tyr	Tyrosine

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Introduction

Since its discovery in the early 60s, several structural derivatives of the green fluorescent protein (GFP) have already been registered in the Protein Data Bank (http://www.pdb.org/), and their physico-chemical properties are rather well described in the literature [1–5]. The wild type A-GFP protein from the bioluminescent jellyfish *Aequorea victoria*, has 238 amino acid residues, a molecular weight (MW) of 26.9 kDa and a theoretical isoelectric point (pI) of 5.8 (from ExPASy). Its X-ray barrel-type structure was resolved at 1.85 Å (PDB entry: 1W7S; [6]), and is formed by eleven β-sheets and one α-helix, to which the GFP chromophore is connected. The latter is obtained by a post-translational cyclization reaction of the polypeptide skeleton Ser65, Tyr66 and Gly67 residues, followed by oxidation of the Tyr66 residue lateral side-chain [7–9]. Through a hydrogen bonds network involving particular polar residues and H_2O molecules, the cromophore is able to establish non-covalent interactions with the protein [10, 11]. From a structural point of view, the protein is highly regular although an exception to this overall behaviour can be observed between strands 7 and 8 of the β-barrel [12, 13], whose *termini* is formed by small sections of the α-helix that resemble "lids".

Regarding its photophysics, the wild type GFP protein is characterized by high quantum yields, ϕ_f~0.8, in contrast to what is observed for its individual cromophore in aqueous solution, ϕ_f~10^{-3} [14, 15]. In the native fold, the inhibition of molecular vibration or rotation along the chromophore double exo-metilene bond, prevents emission quenching by a rapid internal conversion process [13, 15, 16]. Inside the β-barrel, chromophore's fluorescence extinction by O_2 [17] and by hydron ions [18] is also avoid.

Most of the GFP structural events reported in the literature have been studied below room temperature. However, several applications of GFP under physiological conditions (e.g. T=20–40 °C, pH=6–8) have recently been proposed, in domains like cell biology and biotechnology. Namely, in GFP-fused proteins folding [19] and enzyme activity [20] assays, and as a biological indicator in thermal processes [21]. In face of these previous observations, it's clear that a thorough and systematic study of the photophysical properties of GFP is of the utmost relevance, and needs to address distinct medium conditions (e.g. temperature, pH, ionic force). It is the purpose of this work to contribute for the latter, performing accurate measurements of the A-GFP excitation and emission spectra in the temperature range between 20 °C and 75 °C and pH=7 (phosphate buffer), and therefore accessing the influence of temperature on A-GFP photophysical properties. The results obtained are interpreted and discussed in terms of structural features and photophysical behaviour taking into account the most recent findings on this matter.

Materials and Methods

Protein Expression and Purification

The His-tagged wild type A-GFP protein was expressed in *E. Coli* BL21 competent cells using the plasmid pRSETa containing its gene. Cells were grown at 37 °C in Luria-Bertani (LB) broth with 100 mg/ml ampicillin. The cultures were induced by addition of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for ~9 h, at 28 °C. The cells were harvested by centrifugation and resuspended in phosphate buffer at pH 8. Cells lysis was performed by sonication and the resultant crude extract centrifuged to remove cell debris. The recombinant protein with a six-His tag was purified using a Ni-NTA-agarose affinity column (from Qiagen) and Fast Protein Liquid Chromatography (FPLC; from Pharmacia). In addition, the protein was dialysed against 10 mM phosphate buffer (pH 7) and stored at −18 °C. Protein purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), from where the molecular weight MW~27 kDa, was recovered. Protein concentration was estimated by bicinchoninic acid (BCA) assay [22].

Fluorescence Measurements

Fluorescence spectra were recorded in a spectrofluorimeter Fluorolog F112A (from Spex) by excitation at λ_{exc}=399 nm or λ_{exc}=475 nm. Excitation spectra were obtained at λ_{em}= 506 nm. The recorded spectra were corrected for Raman scattering, and detecting system response using a correction curve previously determined for the equipment. The temperature was varied between 20 °C and 75 °C, with an estimated accuracy of ±0.5 °C, and the solutions were stirred during the measurements. All spectroscopic measurements were carried out with 0.1 μM protein dissolved in 10 mM phosphate buffer at pH=7.

Results and Discussion

The excitation spectra of A-GFP solutions (0.1 μM, pH=7) are illustrated in Fig. 1, and were obtained using an emission wavelength of λ_{em}=506 nm.

The bands visible at 400 nm and 476 nm are usually associated with the chromophore protonation states neutral (N) and anionic (A), respectively [15, 23]. The excitation band at 278 nm is due to nonspecific, more energetic electronic transitions occurring in the chromophore. At pH 7 and room temperature, the chromophore neutral form is the dominant one. As temperature is increased from 20 °C to 75 °C, both bands at 400 nm (form N) and 476 nm (form A) decrease their intensity, and this trend is enhanced for the neutral form; above 60 °C the excitation spectra become identical, and it can be concluded that beyond that threshold temperature no longer influences the chromophore photo-

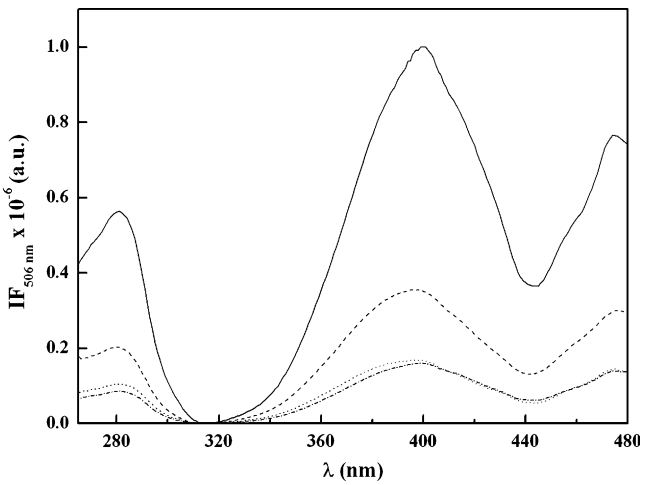

Fig. 1 Excitation spectra of A-GFP solution (0.1 μM, pH=7), λ_{em}= 506 nm: (—) 20 °C, (- -) 60 °C, (-·--) 65 °C, (····) 70 °C

physics. The blue shift occurring in the neutral form peak, from 400 nm to 396 nm, suggests the occurrence of a more energetic electronic transition as temperature is increased.

The emission spectra obtained by excitation at 399 nm and 475 nm, in the temperature range between 20 °C and 75 °C (pH=7), are recorded in Figs. 2 and 3, respectively. The chromophore neutral form selective excitation at 399 nm gives origin to the green fluorescence with maximum at 511 nm (see Fig. 2). This emission results from the excited neutral species (N*) deprotonation forming an excited intermediary (I*), as described by Chattoraj et al. [24]; the excited-state proton transfer (ESPT) should occur *via* the hydrogen-bond network from cromophore to Glu222 residue, as reported by Laino et al. [25].

When temperature is increased to 60 °C, it is very interesting to observe that the band at 511 nm markedly decreases its intensity (Fig. 2), and this fact can be mainly ascertained to the weakening of the hydrogen-bond network. Consequently, the ESPT process occurring *via* hydrogen-bond network is progressively deactivated thus quenching the emission from I*. Above 60 °C, the protein unfolds and its cromophore is released from its static environment and exposed to the solvent. The intrinsic non-radiative and dynamic events become more important, highly reducing the cromophore fluorescence intensity. Here, the most probable N* species decay channels are the weak and featureless emission from state S_1 to state S_0 at 460 nm, and the non-radiative decay through rotation along one of the ring-bridging bonds.

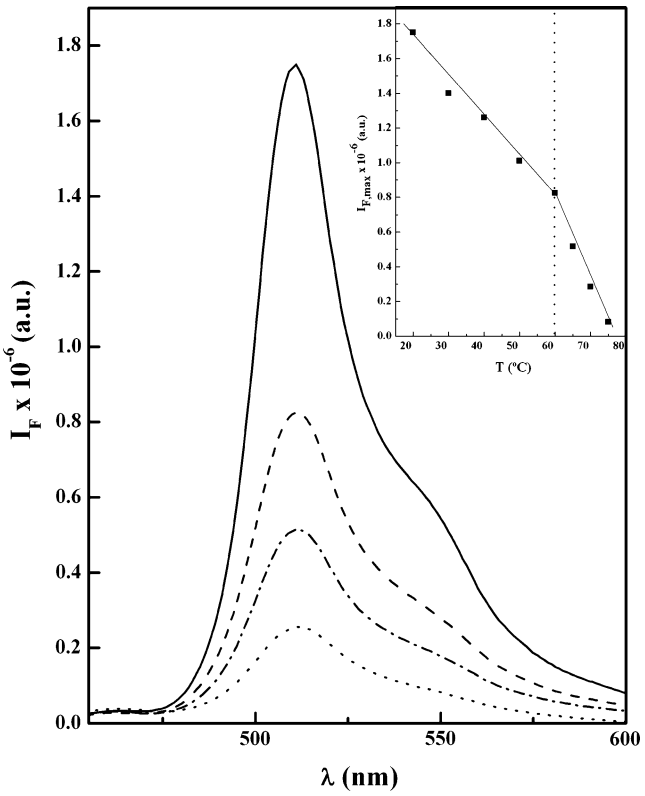

Fig. 2 Emission spectra of A-GFP solution (0.1 μM, pH=7), λ_{exc}= 399 nm: (—) 20 °C, (- -) 60 °C, (-·-·) 65 °C, (····) 70 °C. Insert: Emission maxima variation with temperature increase

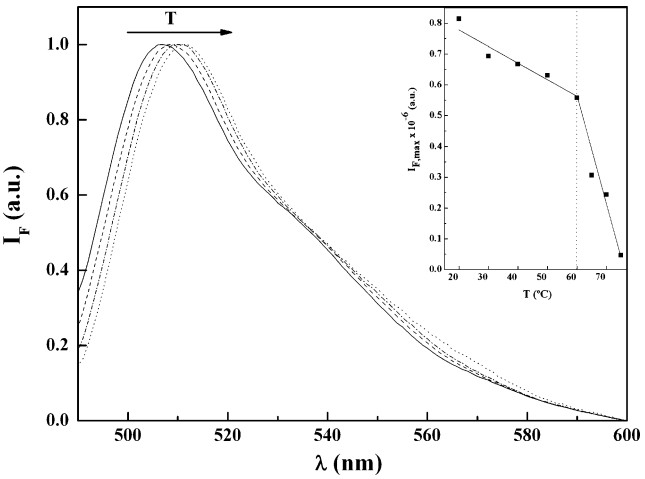

Fig. 3 Normalized emission spectra of A-GFP solution (0.1 μM, pH=7), λ_{exc}=475 nm: (—) 20 °C, (- -) 40 °C, (-·-·) 60 °C, (····) 70 °C. Insert: Emission maxima variation with temperature increase

The cromophore anionic form emission spectrum, obtained by excitation at 475 nm (Fig. 3), exhibits a blue shifted emission band with maximum at 507 nm, when compared with the corresponding neutral form emission spectrum.

It can also be observed in Fig. 3 that, upon temperature increase the anionic form emission maximum is shifted to the red by roughly 6 nm, resulting from cromophore solvent exposure during protein unfolding. Upon excitation, the anionic species dipolar moment changes by $\Delta\mu \approx 6.8$ D. The dipolar moment variation, not observed in the neutral form, results from a charge density transfer occurring between the close neighbours (~9 Å) oxygen atoms located on the phenolic and imidazolinone rings [24, 26]. This dipolar change is responsible for the solvent molecules reorganization around the anionic form electronic excited state decreasing its energy. It should be noted that this phenomenon is more visible for solvent exposed chromophore molecules and leads to the observed red shift. The emission intensity decrease upon temperature increase is due to the enhancement of dynamic quenching processes, but also to the prevalence of non-radiative decay through a *hula-twist* rotation above 60 °C.

The A-GFP chromophore emission is highly quenched by temperature increase and this effect is twice more pronounced on its neutral form before unfolding (see inserts on Figs. 2 and 3). Opposite to the A* decay, the N* decay occurs mainly *via* an ESPT process involving the hydrogen-bond network. Following a temperature increase this mechanism is progressively deactivated due to the

network breakage, causing the accentuated decrease on the chromophore neutral form emission.

The emission maximum break, observed at 60 °C for both N and A forms (see inserts on Figs. 2 and 3), reflects the temperature induced protein unfolding. The melting temperature value determined here of 60 °C is smaller than $T \approx 78$ °C, observed for the A-GFP (at pH=7) by Ward et al. [27]. Above 60 °C the A-GFP structure collapses and its emission rapidly decreases due to the accentuation of intrinsic non-radiative and dynamic quenching events.

Conclusions

The hydrogen bond network weakening, caused by a temperature increase up to 75 °C, is responsible for the ESPT process deactivation at higher temperature values. This event is particularly relevant in the excited neutral form decay *via* deprotonation and conversion to the intermediary I*, leading to a pronounced fluorescence quenching of the later. Regarding the anionic form, due to the occurrence of charge density transfer after excitation, its fluorescence maximum is progressively red shifted upon temperature increase. Above 60 °C the protein unfolds, exposing its chromophore to the solvent. Consequentially, the intrinsic non-radiative and dynamic quenching decay events become predominant, highly reducing chromophore both forms fluorescence intensity.

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