Chapter III

Photoactive Yellow Protein Fluorophore Analog
in Supramolecular Assemblies
Chapter III

3.1 Introduction

The Photoactive Yellow Protein (PYP) is a small, water soluble protein that is responsible for the negative phototactility of the host organism - halophilic purple bacteria [1-3]. At the heart of its photodynamics is the PYP fluorophore, which is the phenolate form of a trans-4-hydroxy cinnamoyl moiety bound to the Cys69 residue of the protein backbone by a thioester bond. Thus, although PYP itself is soluble in water and resides in the aqueous cytosol of the cells, the PYP fluorophore is localized in the protein interior, where its local environment is characterized by a large degree of confinement, and is expected to deviate substantially from that of bulk water. This motivated us to examine the photophysics of the PYP fluorophore analogs pCT and pCT− (both introduced in Section 1.6 of Chapter I) in a medium where they are subjected to spatial confinement. Hence, we chose aqueous micelle solutions. As described in Chapter I, micelles are nano-dimensional particles comprising of supramolecular assemblies of surfactant molecules suspended in water. Each micelle has a polar, exterior surface consisting of hydrophilic head-groups of the surfactants, and a non-polar interior consisting of the hydrophobic tails of the surfactants.

In order to elucidate the photophysics of the PYP Fluorophore, extensive spectroscopic studies have been carried out on many compounds regarded as its analogs [1-17]. Computational studies have been undertaken to probe the electronic states and relaxation pathways of the PYP fluorophore and its analogs [18-21]. These analogs essentially consist of derivatives of the phenolate form of trans-4-hydroxy cinnamic acid, where the carbonyl end substituent X has been varied to produce carboxylates, esters, thioesters, ketones, amides, etc [10-13]. The electron-withdrawing capacity of the X-group was found to have a decisive role on the course of excited state dynamics. When X is relatively electron-withdrawing (like −SR or −OMe), fluorescence lifetimes become shorter, and the main radiationless relaxation channel involves intramolecular charge-transfer, associated with a single flip around the ethylenic double bond [11,13]. However, when X is weakly electron-withdrawing (like −O− or −NH2), a full-fledged stilbene-like concerted photoisomerization becomes the predominant relaxation channel [13].

The pCT− molecule, where X = -SPh, exhibits a large Stokes shift of ~5200 cm−1 in water, symptomatic of a large degree of charge-separation in the excited state [13]. Consequently, pCT− undergoes solvation dynamics in polar solvents, the solvation times increasing from <1 ps in water to ~10 ps in low alcohols like ethanol and to >100 ps in high alcohols like 1-decanol [11].
In this Chapter, we have explored the solvation dynamics of pCT\textsuperscript{−} in aqueous micelle solutions. We have chosen three different micelles, comprising of surfactants that are cationic (CTAB), anionic (SDS) and neutral (TX-100).

### 3.2 Experimental Details

#### 3.2.1 Chemicals

\( p \)-hydroxycinnamic acid was purchased from Alfa-Aesar (98\%). Other chemicals used for synthesis of pCT were of synthesis grade. Synthesis procedure and purity of synthesized pCT compound is described in details in Chapter II [Scheme 1]. Solvents used for spectroscopy were purchased from Merck, India and were of either UV or HPLC grade. They were freshly distilled before conducting spectroscopic measurement. The surfactants CTAB, SDS and TX-100 were purchased from Sigma-Aldrich.

#### 3.2.2 Spectroscopy

Absorption and fluorescence spectra were measured in a HITACHI UV spectrophotometer (U-3501) and Perkin Elmer LS55B fluorimeter, respectively. For fluorescence dynamics studies, a femtosecond frequency upconversion technique was adopted. Here, the output of a femtosecond pulsed oscillator from a mode-locked Ti:sapphire laser (MaiTai-HP), centered at 750 nm and with a repetition rate of 80 MHz, was used as the gate pulse for the femtosecond fluorescence upconversion experiments. The second harmonic (375 nm) of this pulse was used as the source of excitation for the sample placed in a rotating cell. The power of the second harmonic light was restricted to 5 mW at the sample, in order to minimize photobleaching. The fluorescence emitted from the sample was upconverted in a nonlinear crystal (0.5 mm BBO, \( \theta = 38^\circ, \phi = 90^\circ \)) by mixing with the gate pulse, which consisted of a portion of the fundamental beam. The upconverted light was dispersed in a monochromator and detected using photon counting electronics. A cross-correlation function obtained using the Raman scattering from ethanol has a fwhm = 270 fs. The fluorescence decays have been recorded at the magic angle polarization with respect to the excitation pulse on FOG 100 fluorescence optically gated upconversion spectrometer from CDP Systems Corp., Moscow, Russia.
3.3 Results and Discussion

3.3.1 Steady-state spectra

Deprotonation of the phenolic –OH of pCT produces a drastic red-shift in its emission spectra recorded in aqueous micelle solutions, as is evident from Figure 1. For pCT\(^-\), a slight blue-shift (5 – 8 nm) in emission peak position is observed, especially in CTAB and TX-100 micelles, in comparison to bulk water. This blue-shift is comparable to those observed in low short-chain alcohols like methanol and ethanol [11].

![Figure 1: Comparison of steady-state emission spectral peak positions of pCT and of pCT\(^-\) in three different aq. micelle solutions (\(\lambda_{ex} = 370\) nm).](image-url)
Figure 2: Steady-state absorption, excitation and emission spectra of pCT\textsuperscript{−} in three different aq. micelle solutions. The $\lambda_{em}$ for excitation spectra and $\lambda_{ex}$ for emission spectra are indicated.
The absorption, excitation and emission spectra of pCT\textsuperscript{-} in the different aqueous micelle solutions are shown in Figure 2. We note that excitation across the absorption band does not produce any noticeable change in the emission peak position. However, a slight broadening is observed for the emission spectra of pCT\textsuperscript{-} at \( \lambda_{\text{ex}} = 370 \text{ nm} \) in some of the micelles. Conversely, the excitation spectra recorded at wavelengths spanning the emission band nearly coincide with the corresponding absorption spectra. However, even here, the excitation spectra at shorter emission wavelengths is slightly blue-shifted than those at longer emission wavelengths. This minor variation of both the emission and excitation spectra is also observed for pCT\textsuperscript{-} in water.

Keeping in mind the structure of pCT\textsuperscript{-}, with its potential H-bond donating and accepting sites, this variation is attributed to the presence of several different intermolecular H-bonded complexes between pCT\textsuperscript{-} and water. The H-bonding interaction slightly perturbs the energy levels of the ground- and excited states of pCT\textsuperscript{-}, whereby complexes absorbing predominantly at relatively shorter wavelengths tend to emit predominantly at relatively shorter wavelengths.

We note that the absorption spectrum of pCT\textsuperscript{-} in the aqueous micelle solutions does not change on prolonged exposure to excitation light, proving that it does not undergo irreversible photoisomerization into the cis-isomer. This behavior is similar to pCT\textsuperscript{-} in bulk aqueous solution.

### 3.3.2 Femtosecond Time-resolved Fluorescence Spectroscopy

The fluorescence lifetimes of pCT and pCT\textsuperscript{-} in bulk solutions are extremely short. In aqueous micelles too, the lifetimes could not be detected using a picoseconds TCSPC system with \( \sim 100 \text{ ps} \) time-resolution. Hence, a femtosecond time-resolved fluorescence instrument was used, with a \( \sim 250 \text{ fs} \) time-resolution and operating at an excitation wavelength of 375 nm, as elaborated in Section 3.2.

Femtosecond fluorescence transients of pCT\textsuperscript{-} and pCT were recorded in the aqueous solutions of CTAB and SDS micelles, and the results are shown in Figure 3. For pCT, the curves exhibit fast decay in both micelles. For pCT\textsuperscript{-} however, decays are slower, and for pCT\textsuperscript{-} in CTAB, the decay pattern shows a prominent dependence on emission wavelength, in sharp contrast to all the other cases. The curves were fitted with a poly-exponential fitting function, consisting of 3 terms:

\[
F(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + a_3 \exp(-t/\tau_3) \tag{i}
\]
Figure 3: Femtosecond fluorescence time-profiles of pCT$^-$ and pCT (inset) in aq. micelles at different emission wavelengths, as indicated ($\lambda_{ex} = 375$ nm).

Fitting parameters for pCT$^-$ and pCT$^-$ in the micelles are shown in Tables 1 and 2, respectively, for few selected emission wavelengths. We note the occurrence of an ultra-fast $\leq 1$ ps component, a fast $2 - 20$ ps component, and a slow $\sim 55$ ps component (which had to be fixed). For pCT, the $\tau_1$ and $\tau_2$ time-constants are persistently shorter than those for pCT$^-$ at...
comparable emission wavelengths, which accounts for the faster fluorescence decay of pCT than its anion.

Table 1: Poly-exponential fitting parameters for pCT in aqueous CTAB and SDS solutions

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{em}$ (nm)</th>
<th>$\tau_1$ (ps)</th>
<th>$a_1$ (%)</th>
<th>$\tau_2$ (ps)</th>
<th>$a_2$ (%)</th>
<th>$\tau_3$ (ps)</th>
<th>$a_3$ (%)</th>
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<tr>
<td>430</td>
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<td>61</td>
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<tr>
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<td>0.52</td>
<td>56</td>
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<tr>
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<td>50</td>
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<td>43</td>
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</tr>
<tr>
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<td>0.67</td>
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<td>55</td>
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<td>4.10</td>
<td>39</td>
<td>55</td>
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We note that, for pCT\textsuperscript{−} in SDS, the slow component (~55 ps) is nearly absent, while the other two components and their relative weightage are similar to that reported earlier for pCT\textsuperscript{−} in bulk water [13]. This strongly suggest that pCT\textsuperscript{−} in SDS micelles reside in an environment very similar to bulk water, while for pCT\textsuperscript{−} in CTAB micelles, the situation is considerably different. For better insight, we constructed the time-resolved emission spectra (TRES) of pCT\textsuperscript{−} in CTAB at different time-delays, using the following procedure.

The steady-state emission intensity $I_{ss}(\lambda)$ at a given emission wavelength $\lambda$, is essentially the integral of the emission intensity $I(\lambda,t)$, between $t = 0$ and $t = \infty$:

$$I_{ss}(\lambda) = \int_{0}^{\infty} I(\lambda,t) \, dt \quad (\text{ii})$$
Table 2: Poly-exponential fitting parameters for pCT\(^-\) in aqueous CTAB and SDS solutions

<table>
<thead>
<tr>
<th></th>
<th>(\lambda_{em}) (nm)</th>
<th>(\tau_1) (ps)</th>
<th>(a_1)%</th>
<th>(\tau_2) (ps)</th>
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<td>62</td>
<td>24.50</td>
<td>37</td>
<td>55</td>
<td>01</td>
</tr>
</tbody>
</table>

But \(I(\lambda,t)\) is the time-resolved emission intensity at an emission wavelength \(\lambda\) and time-delay \(t\). It is given by:

\[
I(\lambda,t) = I(\lambda,0) \times [a_1(\lambda)\exp(-t/\tau_1) + a_2(\lambda)\exp(-t/\tau_2) + a_3(\lambda)\exp(-t/\tau_3)]
\]  

(iii)

Therefore:

\[
I_{ss}(\lambda) = \int_{0}^{\infty} I(t, \lambda) \, dt = I(\lambda,0) \times [a_1(\lambda)\tau_1 + a_2(\lambda)\tau_2 + a_3(\lambda)\tau_3]
\]

(iv)

which leads to:

\[
I(\lambda,0) = \frac{I_{ss}(\lambda)}{[a_1(\lambda)\tau_1 + a_2(\lambda)\tau_2 + a_3(\lambda)\tau_3]}
\]

(v)
Thus, once $I(\lambda, 0)$ is obtained for a given emission wavelength $\lambda$, the quantity $I(\lambda, t)$ can be calculated using Equation (iii). The set of $I(\lambda, t)$ for a given time-delay $t$ represents the time-resolved emission spectrum (TRES) at that time-delay.

The TRES thus constructed were plotted against the emission wavenumber axis in Figure 4. Each TRES could be fitted satisfactorily with the lognormal function [22-25].

![Figure 4: TRES and S(t) decay for pCT$^-$ in CTAB micelles.](image)

It is immediately apparent from the lognormal fits that the TRES undergo a time-dependent Stokes shift in the time-scale of ~50 ps. The peak emission wavenumber $\nu_{\text{max}}(t)$ for each TRES were determined. Using the $\nu_{\text{max}}(t)$ values for different time-delays $t$, the relaxation function $S(t)$ was constructed as:

$$S(t) = \frac{[\nu_{\text{max}}(t) - \nu_{\text{max}}(t = \infty)]}{[\nu_{\text{max}}(t = 0) - \nu_{\text{max}}(t = \infty)]} \quad (\text{vi})$$

where $\nu_{\text{max}}(t)$ is the peak emission wavenumber of the TRES at time-delay $t = t$. The quantity $\nu_{\text{max}}(t=\infty)$, i.e., the peak wavenumber at $t = \infty$, is taken to be the peak wavenumber of the steady-state emission spectrum.
The $S(t)$ function thus serves to quantify the time-dependent Stokes shift. When plotted against time in Figure 4, it shows a monotonic decay. It could be fitted with a triple-exponential decay, yielding time-constants of 300 fs (24%), 2.5 ps (37%) and 11 ps (39%).

Using the similar procedure, Zewail et al. had earlier attributed the decay of $S(t)$ to solvation dynamics, which was associated with time-constants from < 1 ps in bulk water to ~15 ps in bulk ethanol [11,13]. Following the same line of reasoning, we propose that pCT$^-$ in CTAB micelles is distributed over a range of environments, from bulk water-like (solvation time constant ~ 0.2 ps) to bulk alcohol-like. The latter probably represents the polar surface of the CTAB micelles, which may accommodate the pCT$^-$ molecules due to favorable electrostatic attraction between the cationic surfactant head-groups and anionic pCT$^-$ molecule.

3.4 Conclusion

pCT$^-$ has widely been regarded as an authentic fluorescent analog of the PYP Fluorophore. Inside the PYP biomolecule, the fluorophore resides in a local environment characterized by severe steric constraint, as it is confined within a nano-dimensional space and restricted by intermolecular H-bonding with several neighboring amino acid residues. As such, the photophysics of the fluorophore bound to PYP is primarily governed by the environment prevailing within the protein nanospace, which plays a critical role in deciding the radiationless decay coordinates [26 – 32]. In consequence, there has been little in common between the photophysics of the original PYP Fluorophore its analogs – most notably pCT$^-$ - taken in bulk aqueous solution, a point emphasized by Zewail et al. [13]. The question therefore remained as to how the PYP Fluorophore analogs would respond when embedded in a medium that offers a degree of spatial constraint, but is not an exact replica of the protein nanospace of PYP.

In this work, we demonstrate that the pCT$^-$ molecule in an aqueous CTAB micelles undergoes a time-dependent Stokes shift which could be explained in terms of solvation dynamics as in bulk polar solutions. The electrostatic attraction between the cationic CTAB head-groups and the anionic pCT$^-$ molecule drives a substantial proportion of the latter to occupy the polar surface of the CTAB micelle. Here, the pCT$^-$ molecules are surrounded by water molecules at the micelle-water interface, which are characterized by a slower solvent relaxation time than water in bulk aqueous solutions. This shows up in the time-constants of $S(t)$ decay, which includes a substantial proportion of a ~10 ps component never observed in bulk
aqueous solutions. Thus, the photophysics of PYP Fluorophore analogs in supramolecular assemblies like CTAB micelles is primarily governed by solvation dynamics of interfacial water molecules. Moreover, unlike the PYP Fluorophore inside the protein nanospace, we found no evidence of any serious perturbation of the radiationless decay coordinates of pCT$^{-}$ in micelles.

3.5 References

Chapter III


