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Excited-state relaxation dynamics of a PYP chromophore model in solution: influence of the thioester group

Pascale Changenet-Barret *, Agathe Espagne, Nathalie Katsonis, Sandrine Charier, Jean-Bernard Baudin, Ludovic Jullien, Pascal Plaza, Monique M. Martin

Ecole Normale Supérieure, Département de Chimie, UMR-CNRS 8640 PASTEUR, 24 rue Lhomond, 75231 Paris Cedex 05, France

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Abstract

Cis–trans photoisomerization of a photoactive yellow protein chromophore model, the deprotonated *trans S*-phenyl thio-*p*-hydroxycinnamate, is studied in aqueous solution by subpicosecond transient absorption and gain spectroscopy. The excited-state deactivation is found to involve the formation, in 1.7 ps, of an intermediate state which decays in 2.8 ps. A persistent bleaching signal is observed at longer times indicating that the excited state not only relaxes to the ground state but also partly forms a stable photoproduct, possibly the *cis* isomer. This behavior is analogous to that of the native photoactive yellow protein.

1. Introduction

The photoactive yellow protein (PYP) is the blue-light photoreceptor that is thought to mediate the negative photoaxis of the halophilic purple bacteria *Ectothiorhodospira halophila* [1]. Its chromophore is a deprotonated *trans*-coumaric acid (pCA Fig. 1a) covalently linked, via a thioester bond, to the unique cystein group of the protein [2–5]. Upon photoexcitation, PYP undergoes a photocycle characterized by a series of intermediates formed on a timescale spanning from several hundred femtoseconds to seconds [6–18]. Like for rhodopsins, the *trans* to *cis* isomerization of the chromophore was shown to be the first overall step of the PYP photocycle [19]. Along this step, an early intermediate with a highly distorted geometry, involving the flipping of the thioester group and a partial isomerization of the vinyl bond, was reported to be trapped at low temperature and identified as a precursor (I_0) of the *cis* isomer (I_1) [20]. In solution, ultrafast events on the subpicosecond and picosecond

* Corresponding author. Fax: +33-1-44-32-33-25.
E-mail address: pascale.changenet@ens.fr (P. Changenet-Barret).

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timescales were assigned to the formation of I_0 [14,21].

In a first attempt to approach the intrinsic properties of the PYP chromophore, subpicosecond transient absorption spectroscopy was carried out on the fully deprotonated *trans*-coumaric acid (pCA^{2-}) in solution (Fig. 1b). *Cis*-pCA^{2-} was found to be formed with a time constant of 10 ps without any detectable intermediate [22]. This reaction is quite different from that in the native PYP but its kinetics is comparable to the excited-state decay of the denatured PYP [21]. These observations suggest that pCA^{2-} is a good model compound of the PYP chromophore, despite the absence of the thioester group. However, two major differences remain. The absorption maximum of pCA^{2-} is about 50-nm blue-shifted from that of the denatured PYP [22]. Additionally, time-resolved fluorescence experiments carried out on the denatured PYP gave evidence that a charge redistribution takes place in the excited state [21]. There was no evidence for such process in pCA^{2-} [23].

In this Letter, we report an experimental study of the excited-state relaxation dynamics of a thioester derivative of *trans*-pCA, the *trans* *S*-phenyl thio-*p*-hydroxycinnamate (pCT, sketched in Fig. 1c) in a basic aqueous solution, by subpicosecond transient absorption spectroscopy [24]. The compound has been synthesized to provide a better representation of the PYP chromophore than pCA, by including the thioester function.

2. Materials and methods

2.1. Materials

Trans-pCT was synthesized according to the following procedure [25]. Oxalyl chloride (1.5 ml, 17 mmol) was added dropwise under N_2 at 0 °C to a solution containing anhydrous dimethylformamide (0.46 ml, 6 mmol) in CH_2Cl_2 (9 ml). After gas evolution, the mixture was stirred for 1 h at 0 °C. The solvent was evaporated to give a white powder that was dissolved in tetrahydrofuran (THF, 12 ml). A mixture of pCA (885 mg, 5 mmol) and pyridine (0.49 ml, 6 mmol) in THF (9 ml) was added dropwise to preceding solution. The reaction mixture was stirred for 1 h at −30 °C. Then a solution of C_6H_5SLi 0.5 M (from C_4H_9Li 1.6 M in hexane (2.5 ml, 4 mmol) and thiophenol (0.41 ml, 4 mmol) in THF (10 ml) under N_2 at 0 °C) was added dropwise at −30 °C. The mixture was stirred for 2 h at −15 °C, then hydrolyzed and extracted with diethylether. The organic layer was washed with $NaHCO_3$ 10% up to pH 6 and dried ($MgSO_4$). The solvent was removed under high vacuum and the residue was chromatographed on a silica gel column (eluent: acetone). The solid was recrystallized from CH_2Cl_2 to give *trans*-pCT as yellow crystals (531 mg, 2 mmol, 38%). ^1H NMR (400 MHz, $CDCl_3$): $\delta(\text{ppm}) = 7.67$, (d $^3J = 15.7$ Hz, 1H), 7.45–7.55 (m, 7H), 6.87 (d, $^3J = 8.6$ Hz, 2H), 6.70 (d, $^3J = 15.7$ Hz, 1H), 5.49 (s, 1H); MS (CI, NH_3): $m/z = 257$ $[M + H^+]$, calcd. for $C_{15}H_{13}O_2S$ $m/z = 257.33$; Anal. Calcd. for

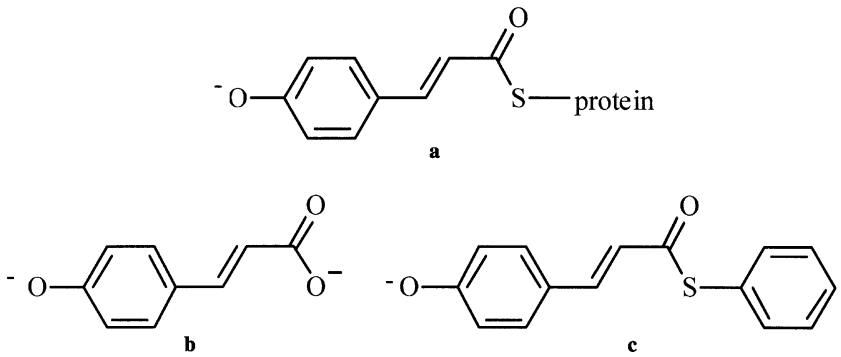

Fig. 1. Structures of (a) the native PYP chromophore, (b) *trans*-pCA and (c) *trans*-pCT.

$C_{15}H_{12}O_2S$: C, 70.29; H, 4.72; Found: C, 70.20; H, 4.82.

Samples of pCT^- were prepared in a borate Britton–Robinson buffer [26] at pH 10.6 (ionic strength 0.1 M, HPLC grade water) with an absorbance close to 1 per mm at the excitation wavelength for pump–probe experiments. The pKa of pCT was determined to be 8.85 ± 0.05 by means of a spectrometric titration. Hydrolysis of the thioester bond was found to occur at basic pH, leading to the formation of pCA^{2-}. Since the hydrolysis rate increases strongly with the pH, we fixed it at 10.6 to obtain both a complete deprotonation of pCT and a good chemical stability of the solution over a few hours.

2.2. Pump–probe measurements

Transient absorption and gain experiments were performed by the pump–probe technique using a subpicosecond homemade dye laser system described elsewhere [27]. Two intense subpicosecond beams were generated at 570 and 428 nm. Typically, 50 μJ of the 428 nm beam were focused to a diameter of 2 mm in the sample cell and used as the pump. The probe beam was a white light continuum generated in a 1-cm water cell from the 300-μJ pulses at 570 nm. The continuum was then split in two; one part was used as a reference signal, while the other part passed through the sample with a diameter close to 1 mm. The reference and signal beams were sent to the entrance slit of a polychromator (Jobin-Yvon Spex 270M, entrance slit 64 μm) through 600-μm optical fibers and were simultaneously analyzed by a 128×1024 CCD camera (Princeton Instruments). Pump and probe beams crossed in the sample, held in a 1-mm cell, at an angle of 10° and were set at the magic polarization angle. The sample solution was recirculated at room temperature. The absence of significant photodegradation was checked by measuring the absorption spectrum of the sample before and after each pump–probe experiment.

The time-resolved differential absorbance spectra (ΔA) were averaged over 500 shots and corrected from the group velocity dispersion in the probe beam. The kinetics at selected wavelengths were fitted (Levenberg–Marquardt algorithm) to a sum of exponentials convoluted with a Gaussian representing the experimental response function. The FWHM was found to be 1.5 ± 0.1 ps.

Steady-state spectra were recorded with a double-beam UV–Vis spectrophotometer (SAFAS UVmc2) and a spectrofluorimeter (Jobin Yvon Fluoromax 3).

3. Results and discussion

3.1. Steady-state spectroscopy

Absorption and spontaneous emission spectra of *trans*-pCT^- in a buffer solution at pH 10.6 are shown in Fig. 2. The absorption maximum at 395 nm ($\varepsilon = 29,000\ \mathrm{l\ mol^{-1}\ cm^{-1}}$) is comparable to that of the denatured protein ($\lambda_{max} \sim 400$ nm) [21] but 60-nm red-shifted from the pCA^{2-} absorption peak ($\lambda_{max} = 333$ nm) [22]. Car Parrinello ab initio molecular dynamics on pCT^- shows that the substitution of the oxygen atom by a sulfur atom induces a red-shift of the electronic transition and that the π electron delocalization does not extend to the phenyl ring attached to the sulfur atom [28]. Thus, the thioester group most significantly contributes to the red-shift of the pCT^- absorption. For comparison, the native PYP absorption spectrum undergoes an additional 50-nm red-shift (PYP, $\lambda_{max} = 446$ nm), which should be

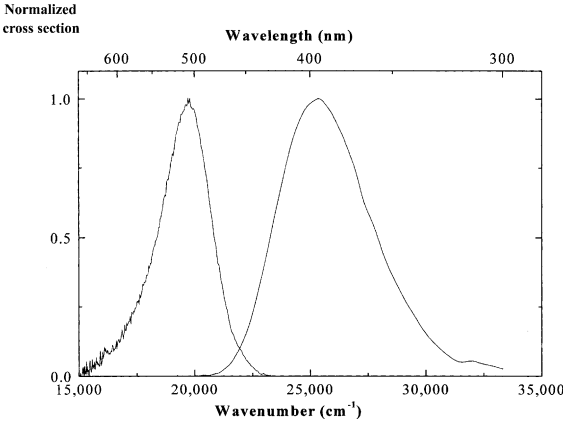

Fig. 2. Absorption and emission spectra of deprotonated *trans*-pCT in a borate buffer at pH 10.6, at room temperature.

attributed to the specific interaction with the protein environment.

The fluorescence spectrum of *trans*-pCT$^-$ in water at pH 10.6 is centered around 500 nm. For comparison the fluorescence spectra of the denatured and native PYP are located in the same region, respectively, at 496 and 494 nm [21]. The pCT$^-$ fluorescence spectrum exhibits positive solvatochromism. In a low polar solvent such as decanol ($\varepsilon = 7.2$), the fluorescence spectrum is 20-nm blue-shifted from that measured in water ($\varepsilon = 78.3$) indicating that an important charge redistribution occurs in the excited state. Such a polarity effect was not observed for pCA^{2-} [23].

On the other hand, it should be noted that the fluorescence spectrum of pCT$^-$ in water is not the mirror image of the absorption spectrum, the latter being about 2000 cm^{-1} broader. It is worth mentioning that pCA^{2-} and its amide analog, *p*-hydroxycinnamide (pCM$^-$), in water exhibit the same feature, the absorption spectrum being 2800 cm^{-1} broader than the emission spectrum in both cases. The absorption band of pCA^{2-} and pCM$^-$ in water have a noticeable shoulder on its blue side and in dimethylformamide pCM$^-$ exhibits two fully separated bands [23]. This is a strong indication that two electronic bands lie under the main absorption band of the PYP chromophore analogs in water. The presence of two electronic excited states lying under the main absorption band has also been discussed for the native PYP [14,16,18].

3.2. Steady-state photolysis

Unlike for pCA^{2-} [22], in a basic aqueous solution steady-state photolysis experiments do not show the formation of a stable *cis* isomer of pCT$^-$. The absorption spectrum recorded after a few hours irradiation at 430 nm, remains almost unchanged. One might think that an efficient back reaction to the *trans* isomer occurs, possibly due to the high energy of the *cis* isomer. We nevertheless found that the steady-state photolysis of the amide analog of pCT$^-$, *N*-phenyl *p*-hydroxycinnamide (pCMPh$^-$) leads to a stable *cis* isomer [23]. This rules out the steric hindrance of the phenyl ring attached to the thioester group as the cause for a possible destabilization of the *cis* isomer in pCT$^-$. Finally, it is worth noting that quantum chemical calculations showed that the *cis* configuration (I_1) of the chromophore in the native PYP is 25 kcal/mol higher in energy than that of the *trans* configuration [28]. In contrast, the *cis* configuration of pCA^{2-} is only 5 kcal/mol higher in energy than the *trans*. This difference was attributed to the local constraints of the protein environment that destabilize the *cis* configuration. It comes out from our steady-state photolysis of pCT$^-$ that the presence of the thioester group itself might be, in part, responsible of the destabilization of the *cis* isomer.

3.3. Time-resolved transient absorption spectra

Fig. 3 shows the time-resolved differential absorbance spectra (ΔA) of *trans*-pCT$^-$ in water at pH 10.6, after excitation at 428 nm (resolution: 1.5

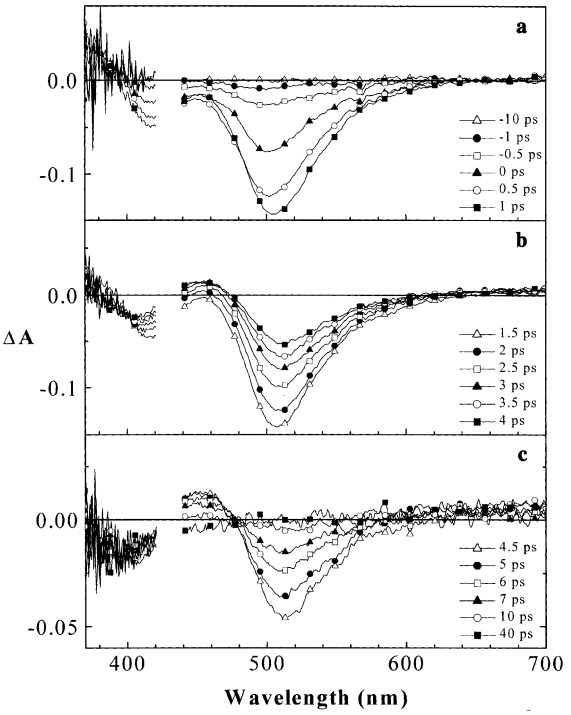

Fig. 3. Time-resolved differential absorption spectra $\Delta A(\lambda, t)$ of *trans*-pCT in a borate buffer at pH 10.6, at room temperature, after excitation at 428 nm, for different pump–probe delays between (a) −10 and +1 ps, (b) 1.5 and 4 ps, (c) 4.5 and 40 ps. The scattered pump light signal around 428 nm was masked.

ps). The spectral changes are characterized by three different phases:

- During the excitation pulse (Fig. 3a), three bands are present. The positive band below 400 nm is assigned to excited-state absorption (ESA). This UV ESA band is quite noisy because in that region the probe light is strongly absorbed by the sample but its presence is consistent in various experiments. Between 400 and 450 nm, a small negative band due to the ground-state bleaching peaks at 420 nm. This band is slightly red-shifted compared to the steady-state absorption due to the overlapping UV ESA band that modifies its shape. Between 450 and 650 nm, in the steady-state fluorescence region, the ΔA spectra are dominated by stimulated emission (SE). The continuous red-shift up to about 5 nm of the SE band is attributed to solvation dynamics in the excited-state. The charge-redistributed character of the excited state is indeed expected from the steady-state fluorescence solvatochromism (see Section 3.1). In water, the average solvation time lies in the subpicosecond regime [29,30]. With the present experimental resolution, only the slower component (close to 1 ps and accounting for 35% of the total solvation dynamics) can be detected. This could explain the small amplitude of the red-shift.
- For probe delays between 1.5 and 4 ps (Fig. 3b), the former three bands decay simultaneously while a transient absorption band rises at 450 nm, between the bleaching and the SE bands. This rising band leads to an apparent red-shift of the SE band and a decrease of its bandwidth. On the other hand, the decrease of the UV ESA band leads to an apparent blue-shift of the bleaching band and to the observation of an isosbestic point at 400 nm ($\Delta A < 0$).
- Finally, at longer probe delays (>4 ps, Fig. 3c), the SE band and the 450-nm ESA band decrease simultaneously in a few picoseconds, with an isosbestic point ($\Delta A = 0$) around 480 nm. In the ground-state absorption region, a small persistent bleaching signal remains after 10 ps but the isosbestic point at 400 nm is no longer observed.

The kinetics recorded between −10 and +40 ps in the three regions of the ΔA spectra are represented in Fig. 4. All kinetics have been fitted simultaneously to a biexponential function followed by a small plateau, convoluted with the apparatus response function. The plateau accounts for the observed persistent bleaching signal. The UV ESA, the bleaching and the SE bands decay with both 1.7 and 2.8 ps time constants. The 450-nm band appears to develop in 1.7 ps and to decay in 2.8 ps.

3.4. Nature of the 450 nm transient absorption band

Since the bleaching, SE and 450-nm ESA bands overlap in the 400–500 nm region, we examined the possibility that the delayed rise at 450 nm could be due to the solvation-induced red-shift of the SE band. In that picture, the SE and the 450-nm ESA bands would come from the same excited state and would decay with the same time constant, roughly 2.8 ps. Simulating a red-shift of the SE band of 5 nm at a rate constant of 1 ps^{-1}, we calculated that the amplitude of the 450-nm band should be dominant (i.e., $\Delta A > 0$) at times below 1 ps. Since we did not observe such a behavior, we consider another explanation which involves the

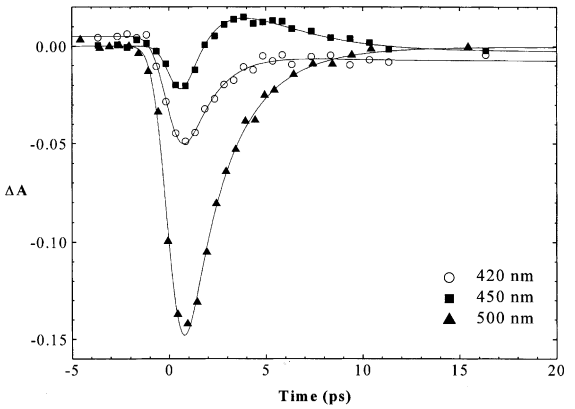

Fig. 4. $\Delta A(t)$ kinetics measured at 420, 450, 510 nm for *trans*-pCT in a borate buffer at pH 10.6, after excitation at 428 nm. The data were fitted simultaneously with a multiexponential function convoluted by a 1.5-ps (FWHM) Gaussian function representing the experimental response function. The fits led to two short components, respectively, 1.7 and 2.8 ps followed by a small plateau attributed to the persistent bleaching signal observed after 10 ps. The fits are represented by the solid lines.

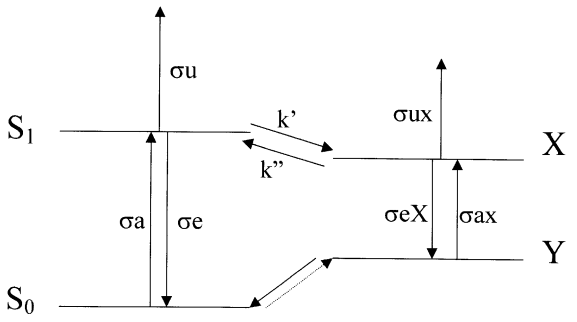

Fig. 5. Four electronic state model proposed to explain the evolution of ΔA spectra of pCT^-. The transient state X is formed in 1.7 ps from S_1 and decays non-radiatively toward Y in 2.8 ps after equilibration with S_1.

formation of a transient state from the first emitting state.

In a first attempt to interpret the time evolution of the ΔA spectra, we propose a four-state kinetic scheme (Fig. 5). The quenching of the stimulated emission is attributed to the formation of a transient non-emissive state ($\sigma_{eX} \sim 0$) which absorbs at 450 nm. Since, after 4 ps, both transient absorption and SE bands decay identically, a fast equilibrium between the fluorescent state and the non-emissive state must be reached. Although, one does not know the nature of this transient dark state, the present results bring a definitive evidence that it is a non-emissive excited state. In addition, the small persistent bleaching observed after 10 ps gives evidence that there is no complete ground-state repopulation after the decay of the excited states. This is an indication of the formation of a long-lived photoproduct. The most likely photoproduct should be the *cis* isomer of pCT^-.

3.5. Comparison with pCA^{2-} and PYP

The intrinsic relaxation of pCT^- in water is different from that of pCA^{2-}. The transient absorption experiments carried out on pCA^{2-} gave evidence for the formation of a *cis* isomer in 10 ps, with the same rate as the excited-state decay [22]. No intermediate state was detected as in the case of the well-documented *trans*-stilbene isomerization process [31]. The excited-state relaxation of pCT^- in water is much faster than that of pCA^{2-} and proceeds through an intermediate state prior to the formation of a long-lived photoproduct. This observation stresses the important role of the thioester group on the excited-state deactivation of the PYP chromophore.

Interestingly, the excited-state relaxation kinetics of pCT^- differs markedly from that of the denatured PYP. The decay rate of the latter in solution is in fact comparable to that of pCA^{2-} [22]. This was interpreted as a strong indication that an isomerization around the vinyl bond also takes place in the denatured PYP [22]. The difference between the decay rates of pCT^- and the denatured PYP remain then obscure since both compounds contain the thioester group. However, pCT^- provides a better representation of the charge distribution of the native PYP chromophore than pCA^{2-}. As matter of fact, a dynamical Stokes shift of the fluorescence spectrum is observed for pCT^-, indicating that a photoinduced charge redistribution occurs like in the denatured PYP [21]. For pCA^{2-} in water and alcohols, no solvation dynamics have been detected indicating little electronic redistribution in the excited state, likely due to the presence of the charged carboxylic group [23].

It is worth noting that the transient absorption spectroscopy of pCT^- is apparently similar to that of the native PYP. In the protein, a transient absorption band was observed to appear in 3 ps between the bleaching and the stimulated emission bands and associated to the formation of an intermediate state, possibly I_0, prior to the *cis* isomer I_1. This comparison might nevertheless be fortuitous because the I_0 intermediate of PYP is a ground state and that of pCT^- is, from the present work, an excited state in equilibrium with the fluorescent state.

4. Conclusion

The deprotonated *trans* *S*-phenyl thio-*p*-hydroxycinnamate (pCT) has been studied in a basic aqueous solution by subpicosecond transient absorption and gain spectroscopy. The excited-state relaxation of pCT^- is found to involve the formation in 1.7 ps of a dark excited-state that decays

in 2.8 ps, partly forming a long-lived (>40 ps) photoproduct. Our experiment provides evidence for a rapid equilibrium between the initial fluorescent state and the dark excited state. The photoproduct has not been characterized yet but is thought to be the *cis* isomer. Surprisingly, the excited-state dynamics of pCT^- differs markedly from that of the denatured PYP but is close to that of the native PYP. The present study suggests that both the intrinsic properties of the chromophore, especially the thioester group, and the effect of the protein environment account for the primary processes in the native PYP.

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