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Investigations of the primary events in a bacterial photoreceptor for photomotility: photoactive yellow protein (PYP)

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PYP, the Photoactive Yellow Protein, is a small water-soluble protein extracted from the cytosol of the halophilic purple bacterium *Halorhodospira halophila*. PYP is thought to mediate the phototactic response of the bacterium against blue light. Its chromophore is the deprotonated *trans-p*-hydroxycinnamic acid covalently linked, *via* a thioester bond, to the unique cysteine residue of the protein. Upon blue-light irradiation, PYP undergoes a photocycle. As for rhodopsins, the *trans* to *cis* isomerization of the chromophore was shown to be the first overall step of this photocycle. From time-resolved spectroscopy measurements on native PYP in solution, it emerged that the reaction involves a series of fast events on the subpicosecond and picosecond timescales, but the reaction path that leads to the formation of the *cis* isomer is not clear yet. A few years ago, we initiated a comparative study of native PYP and several chromophore analogues in solution in order to try to further clarify the early steps of the photocycle. Our experimental approach consists in probing, in real-time, the ultrafast photoinduced events by transient absorption and gain spectroscopy using the pump–probe technique. In the present paper, we review our experimental results and discuss them within the context of the recent literature.

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Pascale Changenet-Barret obtained her PhD in 1996 at the University of Orsay (France), studying by subpicosecond spectroscopy intramolecular charge transfer in dimethylamino-benzonitrile (DMABN) and dimethylamino-substituted triphenylphosphines. In 1997, she spent one year as a post-doctoral researcher in the group of Prof. M. Glasbeek at the University of Amsterdam (The Netherlands), working on the ultrafast events in Photoactive Yellow Protein in collaboration with Prof. K. Hellingwerf. Then, she spent one year in Prof. R. Hochstrasser's group at the Department of Chemistry at the University of Pennsylvania, in Philadelphia (USA), working on the ultrafast contributions of protein motions to the dielectric response. In 1999, she came back to France as CNRS researcher in Monique Martin's group at the Laboratoire de Photophysique Moléculaire at the University of Orsay. In 2000, the group moved to the Department of Chemistry at Ecole Normale Supérieure in Paris (France). Pascale Changenet-Barret received the CNRS Bronze medal in 2002 for her work on PYP and its chromophore analogues.

Introduction

Many microorganisms are highly sensitive to signals from their environment. They are able to detect and to respond to various external stimuli (mechanical impact, concentration gradients of various chemical species, pH, gravity, *etc.*). Particularly interesting is the ability of some free-living microorganisms like algae and bacteria to respond to light irradiation by a cellular movement (photomotility). Different kinds of photomotile responses have been reported (for a review see ref. 1). Among them, phototaxis produces an oriented movement with respect to the light source; the cell swims either toward it (positive phototaxis) or against it (negative phototaxis). At the molecular level, the photomotility is initiated by a photoreceptor that is composed of a chromophore linked to a protein. The absorption of a photon by the chromophore initiates a cyclic chain of reactions, called a photocycle, localized first on the chromophore itself, then extended to the whole protein structure. This leads to the formation of a long-lived state (the signalling state) which is stable enough to interact with another partner downstream in the signal transduction chain, ultimately leading to the observed directed change in the motility. In addition to its fundamental interest, the understanding, at the molecular level, of light-induced behavioural processes, such as phototaxis, is of crucial importance for future advances in microbial ecology and biotechnology. It opens up interesting perspectives for the development of new nanodevices mimicking the capabilities of biological systems to convert light into mechanical, chemical, or electrical energy (see for example ref. 2).

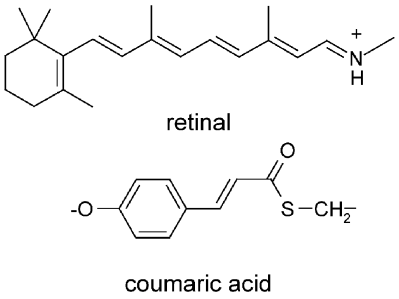

Fig. 1 Structure of Schiff-base linked retinal and methyl thioester linked 4-hydroxycinnamic acid.

A limited number of photoreceptor proteins for photomotility have been identified.[1,3] They have in common broad absorption spectra with high extinction coefficients which explains their high sensitivity to sunlight. Rhodopsins form a well-known family of photoreceptors found in many photomotile microorganisms. Rhodopsins have been extensively studied since they are also involved in another light transduction phenomenon of higher organisms, vision.[4,5] The chromophore of rhodopsin is retinal, a polyene derivative bound to the protein *via* a protonated Schiff base linkage (see Fig. 1). As to vision, it is generally admitted that the primary photochemical reaction of rhodopsin is the ultrafast *cis–trans* isomerization of retinal in a few hundred femtoseconds, followed by a protein conformational change.[6]

Photoactive Yellow Protein (PYP) is a photoreceptor protein isolated from the phototrophic purple bacterium *Halorhodospira halophila* (formerly named *Ectothiorhodospira halophila*).[7] PYP is thought to be the photoreceptor of the bacterium's negative phototactic response towards blue light.[8] It contains a new type of chromophore, the deprotonated *trans-p*-hydroxycinnamic acid (see Fig. 1) covalently linked, *via* a thioester bond, to the unique cysteine residue of the protein.[9–12] Studying PYP provides thus a renewed opportunity to better understand light transduction phenomena, with the advantage that the PYP chromophore is simpler than that of rhodopsins.

Upon irradiation with blue light, PYP undergoes a photocycle characterized by several intermediates formed on a timescale spanning from several hundred femtoseconds to seconds. From the earliest experimental observations, it emerged that, as for rhodopsins, the primary events in the PYP photocycle are related to the *trans* to *cis* isomerization of the chromophore,[13,14] but the reaction path that leads to the formation of the *cis* isomer is nevertheless not clear yet.[15–26]

Our experimental approach

In order to shed light on the isomerization mechanism involved in the PYP photocycle, it is crucial to know the intrinsic photophysics of the chromophore. Until very recently, little was known about the photophysics of hydroxycinnamic acids in solution,[27,28] whereas for rhodopsins, the intrinsic relaxation properties of retinal had been the subject of detailed time-resolved studies (see for example ref. 29, and references therein). For the last few years, we have been carrying out a comparative study of the photophysics of native PYP, guanidinium-denatured PYP and several chromophore analogues in solution.[24,25,30–32] In denatured PYP, the chromophore is still linked to the protein by the thioester bond but exposed to the solvent. This comparative study addresses the role of both the intrinsic properties of the chromophore and the local environment on the short-time dynamics of the PYP photocycle. The ultimate goal is to obtain some direct information on the nature of the reacting potential energy surfaces.

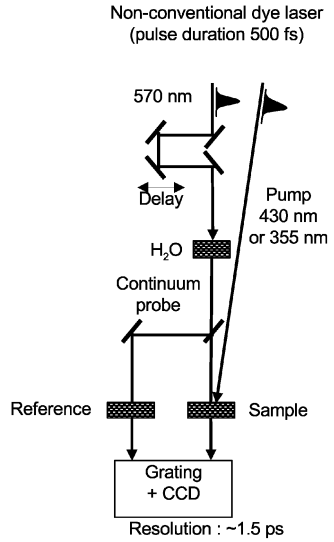

Fig. 2 Scheme of the pump–probe experiments. For the present experiments, 500 fs pulses were generated simultaneously at 355 nm (or 430 nm) and 570 nm. The pulses at 355 nm (or 430 nm) were used as the pump, and a white-light continuum probe was produced by focusing the 570 nm pulses in a 1 cm water cell.

Our experimental approach consists in probing, in real-time, the ultrafast photoinduced events by transient absorption and gain spectroscopy using the pump–probe technique. Compared to fluorescence up-conversion techniques, the transient absorption technique has the advantage of detecting both fluorescent and non-fluorescent species. A subpicosecond laser[33] provides the pump pulse for exciting the sample and the probe pulse is a continuum of white light produced by focusing part of the laser output in a water cell. For illustration, our pump–probe set-up is sketched in Fig. 2. Production of the appropriate wavelength for excitation has been described in detail elsewhere.[30,31] Our set-up allows the measurement of differential absorbance spectra on a broad spectral range, from 340 nm to 750 nm. The delay between the pump and probe pulses is optically varied to follow the temporal evolution of the differential absorbance spectra with a time resolution of about 1.5 ps. The differential absorbance is the difference of the excited sample absorbance, A_p and the steady-state absorbance, A_0:

$$\Delta A(\lambda,t) = A_p(\lambda,t) - A_0(\lambda)$$
$$A_0(\lambda) = 0.43 l \sigma_a(\lambda) N$$

where σ_a is the absorption cross section of the ground state S_0, N is the total population and l is the sample thickness. For example, in a simple three-state model (see Fig. 3), the expression for the excited sample absorbance is the following:

$$A_p(\lambda,t) = 0.43 l[\sigma_a(\lambda,t)N_0(t) + \sigma_u(\lambda,t)N_1(t) - \sigma_e(\lambda,t)N_1(t)]$$

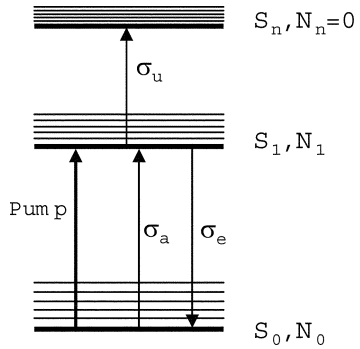

Fig. 3 Three energetic state model with σ_a the absorption cross section of the ground state S_0, σ_u and σ_e the absorption and stimulated-emission cross sections of the first excited state S_1. N is the population associated with each state.

where $N = N_0(t) + N_1(t)$, with N_1 being the excited-state population at time t and N_0 the ground-state population at time t. σ_u is the absorption cross section of the excited state S_1 and σ_e is the stimulated-emission cross section. The differential absorbance is then:

$$\Delta A(\lambda,t) = 0.43l[\sigma_u(\lambda,t) - \sigma_a(\lambda,t) - \sigma_e(\lambda,t)]N_1(t)$$

At a given wavelength, the sign of ΔA depends on the value of σ_u, σ_e and σ_u. ΔA is positive when the excited state absorption σ_u dominates. ΔA is negative when the stimulated emission σ_e dominates or when the ground-state absorption dominates (bleaching signal). When the cross sections are constant, the spectro-temporal evolution of the ΔA spectra is directly related to the dynamics of the transient populations. On the contrary, in polar solvents, the stimulated-emission cross section of the solute can vary with time. When the charge distribution of the solute is significantly altered by the excitation, the solvent dipoles suddenly become out of equilibrium. They subsequently relax toward a new equilibrium distribution with a lower free energy of solvation. This induces a red shift of the stimulated emission cross spectrum. The rate of this shift is directly correlated to the solvation dynamics.

Photoactive yellow protein from *H. halophila*

PYP isolated from the cytosol of *H. halophila* is a small (14 kDa) water-soluble protein that contains 125 amino-acid residues.[34] Fig. 4 sketches the structure of PYP in the vicinity of the chromophore. The latter, the deprotonated *trans-p*-hydroxycinnamic acid, is embedded in the main PYP hydrophobic core and protected from the solvent by the Arg52 residue. The negatively charged oxygen of the chromophore is involved in a hydrogen-bond network with the hydroxy groups of Glu46 and Tyr42 and is further stabilized by the nearby positively charged Arg52.[12] In protein monocrystals, Arg52 is linked by two hydrogen bonds to the carbonyl oxygen atoms of Thr50 and Tyr98.[11] In solution, the protein structure differs slightly since these two hydrogen-bonds between Arg52 and Thr50 and Tyr98 do not exist any more.[35] However, the main hydrophobic core containing the chromophore remains rather rigid and solvent inaccessible.[35]

Although the complete signal-transduction chain in which PYP functions has not been elucidated yet, the photophysics of PYP has drawn much interest these last ten years. Detailed reviews have been already published on PYP photoactivity (for example see refs. 36 and 37). In this section, we will thus only give a brief description of the protein photocycle. We will focus on the early events and discuss our experimental results on native PYP within the context of this recent literature.

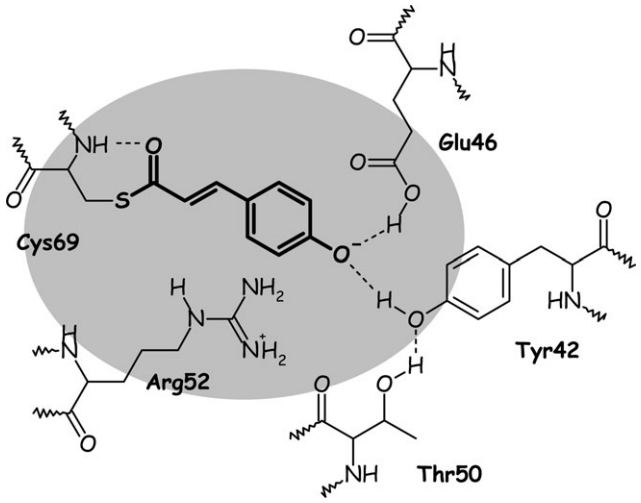

Fig. 4 Schematic representation of PYP structure in the ground state in the vicinity of the chromophore.

PYP photocycle: a brief overview

After the absorption of blue light, PYP in solution exhibits a photocycle involving at least two well characterized intermediates I_1 and I_2 (see Fig. 5; please note that I_1 and I_2 are also referred to as pR and pB).[9,38] I_1 is an intermediate whose absorption spectrum is red-shifted (λ_{max} = 465 nm) with respect to that of the ground state PYP, pG (λ_{max} = 446 nm). I_1 is formed in 3 ns and is converted in a few hundred microseconds into I_2, a blue-shifted intermediate (λ_{max} = 355 nm) that returns to the dark state pG in the millisecond regime. Over the years, the PYP photocycle has become significantly more complex and new intermediates have been identified (for details see ref. 37).

It was shown that the chromophore in the intermediate I_1 has a *cis* conformation.[39,40] The structure of I_1, as determined with time-resolved FTIR, is characterized by the absence of the hydrogen bond between the chromophore and the Cys69 residue while those with Glu46 and Tyr42 are preserved.[39–42] This observation lends weight to a *trans* to *cis* isomerization mechanism involving the rotation of the carbonyl group rather than the large-scale rotation of the phenolate group.[39–42] DFT computations estimated that the energy of I_1 is about 25 kcal mol^{-1} higher than that of pG. This strong destabilization is due to the protein environment which induces a large deviation from the initial planar configuration of the chromophore.[43] This, in addition to the disruption of the hydrogen bond between the chromophore and Cys69, accounts for the red shift of the I_1 absorption spectrum with respect to that of the dark state, pG.

The formation of I_2 involves an important conformational change of the protein, leading to the exposure of a hydrophobic surface to the solvent and to the reprotonation of the chromophore (see ref. 37, and references therein).

Time-resolved X-ray diffraction showed that the PYP photocycle occurs also in protein monocrystals but that the kinetics and the nature of the protein conformational changes are altered by the constraints of the crystal lattice.[44] The intermediates I_1 and I_2 have been clearly identified.[45–47] In addition, an early intermediate with a highly distorted geometry, formed by flipping the thio-ester group and rotating the vinyl bond about half way between the *trans* and *cis* conformations, has been cryotrapped in PYP crystals and characterized by X-ray diffraction.[48] This species has been proposed to be a precursor of the well characterized *cis* isomer I_1.[48,49] However, the existence of this highly distorted intermediate is now questioned since recent X-ray diffraction measurements gave evidence of an early cryotrapped intermediate with a full *cis* conformation.[50]

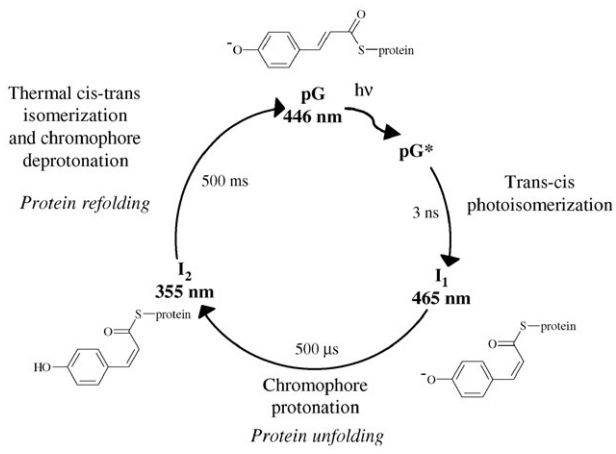

Fig. 5 Simplified scheme of the photocycle of native PYP. Only the two main intermediate states, initially identified in the PYP photocycle at room temperature, are represented.

Early events in PYP photocycle: precursors of I_1

Although much experimental work in solution has been devoted to the characterization of the early photocycle intermediates, *i.e.* the precursors of I_1, the number and the nature of these precursors are not clear yet.[18,20,21,26] Time-resolved spectroscopy showed that the excited-state deactivation of PYP involves a series of fast events on the subpicosecond and picosecond timescales, prior to the formation of I_1.[14–26,51,52] As an illustration, Fig. 6 displays the differential absorbance spectra (ΔA) we measured for native PYP in buffer, together with the corresponding steady-state absorption and emission spectra. A detailed analysis of these spectra has already been published in refs. 24 and 25. Upon excitation, one observes the rise of the excited-state absorption band peaking at 370 nm and of the ground-state bleaching and the excited-state stimulated emission bands, both located in the region of the steady-state spectra. For pump–probe delays up to 50 ps, the three bands exhibit a biexponential decays with 1.9 ps and 16 ps lifetimes. After 50 ps, the 370 nm transient absorption band is no longer observed and the stimulated emission band has been replaced by a broad transient absorption band at 500 nm. At longer delays, while the bleaching band is still decaying, the 500 nm band turns into a blue-shifted transient absorption band corresponding quite well to the absorption spectrum of I_1.[17,53]

The ΔA spectra of native PYP are quite complex, exhibiting multiexponential decays likely due to the contribution of several overlapping absorption bands especially in the 480–540 nm spectral region. Consequently, modeling the ΔA spectra leads to several possible kinetic schemes with competing and cascading steps.[18,20,21,26] Generally, the multiexponential decays in the 480–540 nm spectral region are attributed to the formation of one or two distinct precursors of I_1.[17,18,20–22,24–26] The earliest one, named I_0, has been associated with the cryotrapped intermediate reported in refs. 18, 19, 48 and 54 but recent time-resolved pump–probe anisotropy measurements as well as visible pump–mid-IR probe spectroscopy gave support to the hypothesis that the precursors of I_1 could have already the *cis* conformation.[21,22] The geometrical change of the PYP chromophore would occur in 2 ps or less.[22]

In addition to the hypothesis of a sequential formation of the precursors of I_1, the multiexponential decays of the ΔA signals have also been attributed to competing pathways due to the existence of close-lying excited states, simultaneously populated upon excitation.[17,21,25,32] The nature of these excited states has not been identified yet, but they could be related to the steady-state spectra of native PYP.[25] We will discuss this point further in the next section.

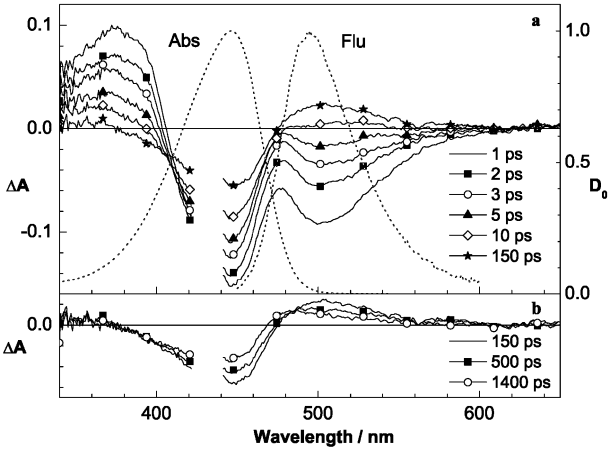

Fig. 6 Differential absorbance $\Delta A(\lambda,t)$ of native PYP in water (pH 7.5) between (a) 1 and 150 ps and (b) 150 ps and 1.4 ns after excitation at 430 nm with 0.5 ps laser pulses. The scattered pump light around 430 nm has been masked. The steady-state absorption and fluorescence spectra are presented as dotted lines.

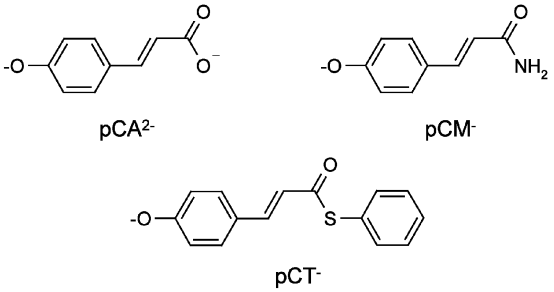

Fig. 7 Structure of deprotonated *trans*-pCAH$_2$, *trans*-pCMH and *trans*-pCTH.

PYP chromophore analogues

A complementary way to further investigate the complex short-time dynamics of the PYP photocycle is to characterize the intrinsic photophysics of the free chromophore in solution, in order to separate the internal and external coordinates that may be involved in the early processes. In this section, we will review the results we obtained in comparative experiments on three different chromophore analogues and on denatured PYP. We will compare their photophysics with that of native PYP and discuss the isomerization mechanism. Most of these results have been previously published.[24,25,30–32] Fig. 7 shows the chemical structure of the studied analogues, the fully deprotonated *trans-p*-hydroxycinnamic acid (pCA^{2-}), the *trans-p*-hydroxycinnamide (pCM$^-$) and the *trans-S*-phenyl-*p*-hydroxythiocinnamate (pCT$^-$). At this point, it is worth mentioning that other PYP chromophore analogues, one of them being the deprotonated *trans-S*-methyl-*p*-hydroxythiocinnamate (pCTMe$^-$), have recently been studied by transient absorption and time-resolved fluorescence up-conversion by another group.[55–57]

Electronic transitions and solvent effects

Fig. 8 gives an overview of the normalized steady-state absorption and emission spectra recorded in water for native and denatured PYP and for the three deprotonated chromophore analogues sketched in Fig. 7. We estimated the fluorescence quantum yield of all these molecules to be lower than 1%.

The absorption spectrum of denatured PYP, even at pH values considerably higher than the pK of its coumaryl chromophore (*i.e.* ~8.8), is considerably blue-shifted with respect to that of native PYP. This difference is likely due to the environment of the chromophore, since denaturation disrupts the specific interactions of the chromophore with the protein and exposes it to the solvent.[28,54,58] The absorption spectra of denatured PYP and pCT$^-$ are quite similar, which suggests that pCT$^-$ is a good model compound for the PYP chromophore. The massive blue shift of the absorption spectra of pCA^{2-} and pCM$^-$ with respect to that of denatured PYP can be explained, both by the absence of the thioester group[43] and the presence of a strong hydrogen-bond network formed by the solvent with the carboxylic acid and amid groups, respectively. As a matter of fact, in dimethylformamide, an aprotic solvent, the absorption spectrum of pCM$^-$ is red-shifted by about 3700 cm^{-1} as compared to that in water.[59] On the other hand, DFT computations performed on pCA^{2-} in a vacuum gave a similar red-shift (about 3890 cm^{-1}) for the lowest transition.[43]

In addition, we have reported that the steady-state emission spectrum of pCM$^-$ and pCT$^-$ undergoes a red shift with increasing the solvent polarity.[25,30,32] This positive solvatochromism is due to the electronic transition promoting an electron transfer from the phenolate oxygen to the carbonyl group.[60,61] In pCA^{2-}, the presence of the negatively charged carboxylic acid group probably affects this charge displacement.

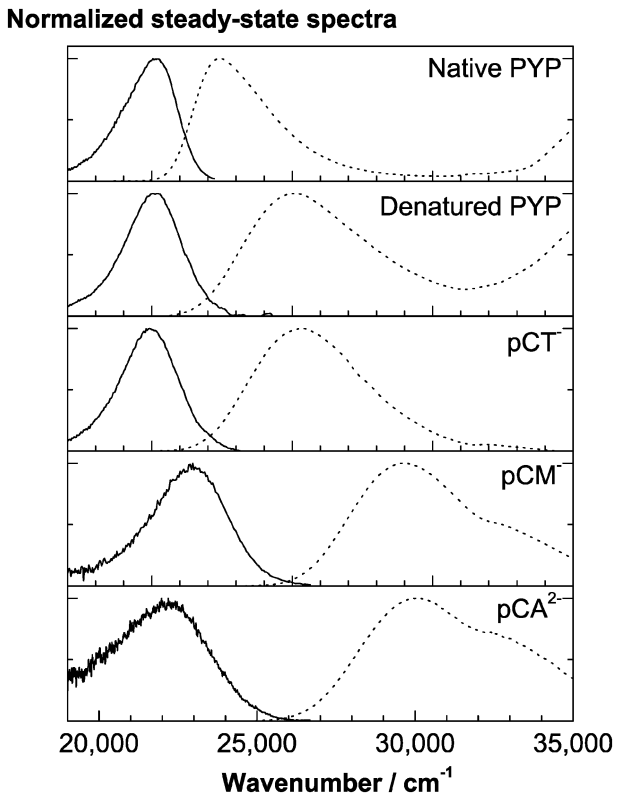

Fig. 8 Steady-state absorption (dotted line) and fluorescence spectra (solid line) of the native and guanidinium chloride denatured PYP and of the three analogues (pCT^-, pCM^- and pCA^{2-}) in water.

Finally, coming back to the point discussed in the PYP section, we attributed the distinct shoulder in the blue side of the absorption spectra of pCA^{2-} and pCM^- to the presence of a second electronic transition rather than to a vibrational structure.[25] This also explains why the absorption spectra of pCT^-, denatured PYP and native PYP are broader than the corresponding emission spectra, which arises from only one of the two states.[25] *Ab initio* and DFT computations performed on $pCTMe^-$ have indeed shown the existence of an $n\pi^*$ transition localized on the phenolate oxygen, close to the $\pi\pi^*$ lowest transition.[60,62]

pCA^{2-} and pCM^-: a "stilbene-like" isomerization mechanism

Transient spectroscopy. Fig. 9 illustrates the differential spectra measured for pCA^{2-} in water and the corresponding normalized steady-state spectra; pCM^- in water exhibits rather similar spectra.[25,32] As was previously reported, the transient spectroscopy of these two analogues is quite different from that of native PYP (for comparison see Fig. 6).[25,30,32] The rapid and concomitant decay of the stimulated emission band at 450 nm and of the excited-state absorption band below 420 nm, in 10 ps for $pCA^{2-}$and 4 ps for pCM^-, indicates that an efficient non-radiative process occurs from the excited state of those two molecules. On the other hand, the broad positive band observed above 550 nm is attributed to the absorption of the solvated electron formed by a biphotonic ionization of the phenolate group.[25,30,32] For pCA^{2-}, the associated radical absorption band, which is expected around 330 nm,[63] could not be observed since it is outside the probed region (Fig. 9). Taking into account our experimental resolution, the formation of the radical-solvated electron pair can be considered as an "instantaneous" process initiated upon excitation. This non-linear process has no relationship with the one-photon process contributing to the differential spectra in the 340–550 nm spectral region.

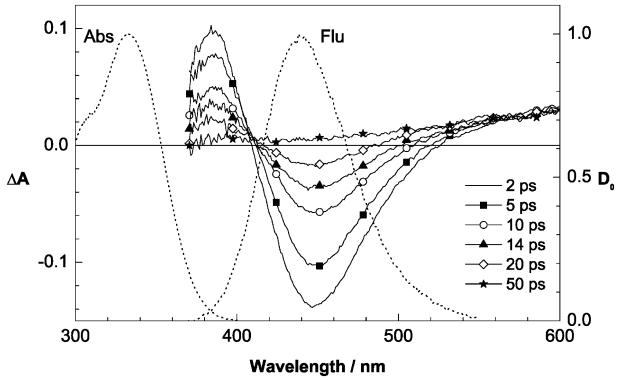

Fig. 9 Differential absorbance $\Delta A(\lambda,t)$ of pCA^{2-} in buffer (pH > 12) between 2 and 50 ps, after excitation at 355 nm with 0.5 ps laser pulses. The steady-state absorption and fluorescence spectra are presented as dotted lines.

As expected from steady-state fluorescence, a positive dynamic Stokes shift of the stimulated emission band has been observed for pCM^-.[25,32] It is due to the solvation dynamics induced by the charge displacement in the chromophore upon excitation.

Isomerization mechanism. We demonstrated previously that the efficient non-radiative process that causes the rapid deactivation of the excited state of pCA^{2-} and pCM^- is the *trans* to *cis* isomerization reaction.[25,30,32] The *trans* excited state leads to the formation of the ground-state *cis* isomer without any detectable intermediate, following a mechanism comparable to that of *trans*-stilbene in solution.[64]

The ground-state absorption spectra of *cis*-pCA^{2-} and *cis*-pCM^- in water have been determined by steady-state photolysis measurements.[30,59] They are centered around 35 090 cm^{-1} and 32 790 cm^{-1}, respectively. They are blue-shifted with respect to those of the *trans* isomers. For stilbene, it is well known that the steric hindrance in the *cis*-isomer causes a decrease in the oscillator strength and a blue shift of the absorption spectrum.[64] A similar effect is expected for pCA^{2-} and pCM^-.[43] From our kinetics we can estimate that, for pCA^{2-} and pCM^- in water, the isomerization reaction barrier is a few kcal mol^{-1} in the excited state. The quantum yield of photoisomerization was found to be 0.46 for pCA^{2-}.[65] In the ground state, the isomerization barrier is estimated to be 30 kcal mol^{-1} for pCA^{2-} in vacuum.[43]

pCT^- and denatured PYP: puzzling behavior

Transient spectroscopy. Fig. 10 illustrates the differential spectra measured for pCT^- in water with the corresponding steady-state spectra. The transient spectra of pCT^- have been described in detail in two previous publications.[25,32] They are quite different from those of pCA^{2-} and pCM^-. One of the main differences is the observation, on the picosecond timescale, of a rising short-lived intermediate absorbing at 450 nm, a spectral region that falls between the bleaching and the stimulated emission bands. The lifetime of this intermediate is about 3 ps. At 15 ps, after the decay of the excited-state absorption band at 350 nm, a weak bleaching signal and a small absorption band peaking at 355 nm remain. This signal, which persists over a nanosecond timescale, is due to the formation of a long-lived photoproduct absorbing at 355 nm and causing incomplete ground-state repopulation. The formation of a similar photoproduct has been reported for $pCTMe^-$ in water by Larsen *et al.*[55,56] It has been attributed to the chromophore radical cation produced by biphotonic ionization. In addition, the absorption band of the associated solvated electron was also observed by these authors.[56] In

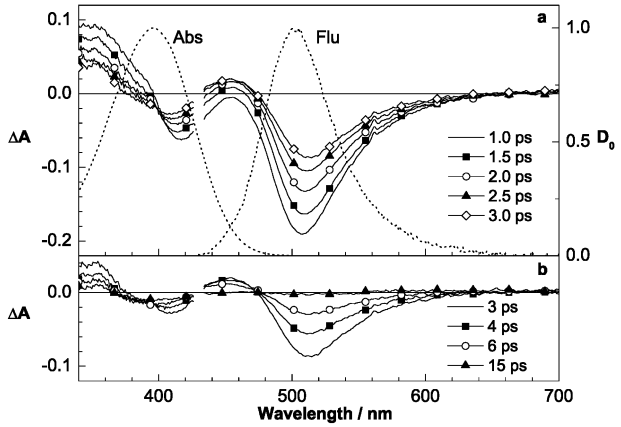

Fig. 10 Differential absorbance $\Delta A(\lambda,t)$ of pCT^- in buffer (pH 10.2) between (a) 1 and 3 ps and (b) 3 and 15 ps, after excitation at 430 nm with 0.5 ps laser pulses. The scattered pump light around 430 nm has been masked. Steady-state absorption and fluorescence spectra are presented as dotted lines.

the case of pCT^-, the presence of the solvated electron band is not obvious in Fig. 10 but it has been confirmed very recently in additional measurements carried out by exciting the sample at 355 nm.[59]

As for native PYP, the transient spectra of pCT^- exhibit complex kinetics. For instance, the formation of the 450 nm absorption band in 1.1 ps is concomitant with the decay of the red side of the 350 nm excited-state absorption band. Meanwhile, the decay of the blue side of the excited-state absorption band in 2 ps corresponds quite well to the decay of the stimulated emission band. The complexity of the transient spectroscopy of pCT^- is not clarified yet. It might be due to the presence of several overlapping absorption bands related to the two electronic transitions underlying the ground-state absorption spectrum.[25,32]

The early differential spectra of denatured PYP are displayed in Fig. 11. They look similar to those of pCT^-. However, some differences can be seen. The relative amplitude of the absorption band of the 450 nm intermediate is smaller and the stimulated emission exhibits a slower decay with an averaged lifetime of about 10 ps. These differences have been attributed to some solvent effects due to the high concentration of denaturing agent required for PYP denaturation.[25] On the other hand, a clear dynamic Stokes shift of the denatured PYP emission band has been observed by time-resolved fluorescence measurements.[54] We have also reported a similar behavior for pCT^- in alcohols, as expected from its steady-state spectroscopy.[25,32] Moreover, the excited-state lifetime is found to be related in part to the solvent polarity.[59]

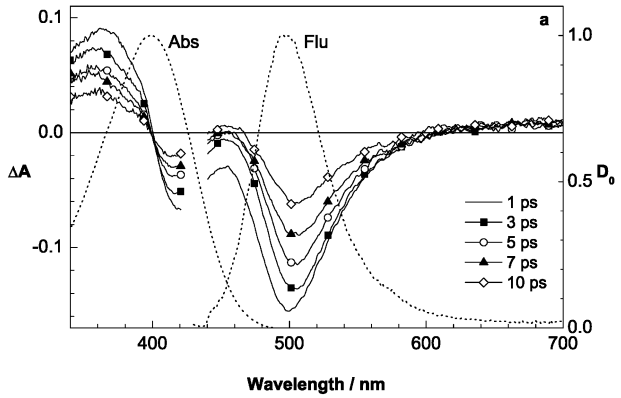

Fig. 11 Differential absorbance $\Delta A(\lambda,t)$ of denatured PYP in buffer (pH 9.6, guanidinium chloride 7 M) between 1 and 10 ps, after excitation at 430 nm with 0.5 ps laser pulses. The scattered pump light around 430 nm has been masked. The steady-state absorption and fluorescence spectra are presented as dotted lines.

Nature of the 450 nm intermediate. Making the hypothesis that an isomerization reaction occurs from the excited state of pCT^- or denatured PYP, it is tempting to attribute the 450 nm intermediate to the ground-state *cis* isomer. However, unlike pCA^{2-} and pCM^-, the formation of a stable *cis* isomer has not been observed by steady-state photolysis of pCT^- in water.[31] The presence of the thioester group has been proposed to be responsible in part for a possible destabilization of the ground-state *cis* isomer making its observation difficult by steady-state photolysis. In the light of recent calculations, this first hypothesis is now questioned because the energy of the isolated pCT^- and $pCTMe^-$ in their ground state *cis* conformation is estimated to be only 5 kcal mol^{-1} higher than in their *trans* conformation, like pCA^{2-}.[43,49] Moreover, the isomerization barrier of the isolated pCT^- and $pCTMe^-$ is estimated to be about 30 kcal mol^{-1},[43,49] *a priori* excluding the existence of a ground-state *cis* isomer with a short lifetime.

Another possibility is that the excited-state deactivation of pCT^- involves another non-radiative process faster than the photoisomerization reaction. This would explain why the *cis* isomer was not observed by steady-state photolysis. For instance, it is known that asymmetrically substituted stilbenes with electron donor and acceptor groups undergo an efficient charge transfer in the excited state. In such compounds, it is proposed that a photoinduced mechanism involving the formation of a twisted intramolecular charge transfer state can occur in competition with the photoisomerization process.[66] Ultrafast photoinduced charge transfer has also been reported for the merocyanine dye DCM in a few polar solvents where the photoisomerization yield of this molecule is low.[67] Competition between photoisomerization and photoinduced charge transfer with a possible change in geometry was also reported recently for some push–pull polyenes, depending on the solvent.[68,69] In the latter case isomerization can also occur from the charge transfer state. The solvent polarity and the electron donor–acceptor character of the substituents are then determining factors able to affect both the decay kinetics and the deactivation pathway of the PYP chromophore analogues studied here. The excited-state deactivation of pCM^- is unambiguously correlated with the photoisomerization process, but one might infer that the formation of such a twisted charge transfer intermediate might become the dominant relaxation pathway for pCT^-. In the hypothesis that the photoisomerization reaction is prevented in the free PYP chromophore, it would be clear, then, that the protein environment forces the chromophore to deactivate along a particular reaction path that cannot be reached in polar solution. At present, our experimental data in water do not allow us to reach a conclusion on the exact nature of the non-radiative process involved in pCT^- and denatured PYP. A careful data analysis of the experiments carried out on pCT^- in several alcohols is in progress in order to clarify this point.

Comparison with native PYP. It is worth mentioning the similarity of the 450 nm intermediate of pCT^- and denatured PYP with the earliest intermediate of the PYP photocycle found to appear in the same spectral region, on the same timescale. This similarity led us to propose that the early events in PYP could be, in part, controlled by the intrinsic properties of the chromophore.[25] These intrinsic properties are closely related to the presence of the thioester group since pCA^{2-} and pCM^{2-} exhibit a quite different transient spectroscopy. From Fig. 8, it is clear that the two electronic transitions underlying the steady-state absorption spectra are quasi-degenerate when the chromophore structure contains the thioester group. In addition to the fact that the two excited states may both be directly excited in most of the reported experiments, the

proximity of these two states may strongly affect the shape of the lowest excited adiabatic surface, providing channels to escape the Franck–Condon region that are common to pCT^- and PYP and differ from those of pCA^{2-} and pCM^{2-}.

On the other hand, we showed that solvent properties such as hydrogen-bonding ability and polarity strongly affect the steady state and the transient spectroscopy of the PYP chromophore analogues, independently of their chemical structure. In the protein, the effect of the environment on the excited-state deactivation of the chromophore may be rather different. This has been demonstrated in studies of the photodynamics of several mutants.[19,23,52,54,70–73] These studies showed that a partial disruption of the H-bonding network in the chromophore binding pocket slows down the early kinetics of PYP.[19,54,73] Moreover, an oscillatory behavior of the time-resolved spontaneous emission has been observed for native PYP and several mutants but not in denatured PYP. These oscillations, which are found to depend strongly on the local environment, are supposed to be coupled to the chromophore reconfiguration and to enhance the isomerization process.[23,73] On the other hand, recent calculations show that the isomerization reaction is facilitated by the electronic stabilization of the chromophore excited state by the nearby positively charged Arg52. Interestingly, the isomerization reaction of the chromophore is not found to occur in a vacuum.[74] The presence of a thioester group in the chromophore structure and the nature of the environment both influence the relaxation path and kinetics.

Conclusions and perspectives

Throughout the years, PYP has become a model system for studying the primary photochemistry involved in bacterial photomotility since it is highly photostable and because its high resolution 3D structure is known.[11] In our work, the excited-state deactivation of native PYP is found to involve the formation, in the picosecond regime, of at least one distinct precursor of the well-characterized *cis* isomer, I_1, absorbing in the 480–500 nm spectral region, in agreement with the recent literature. The complicated kinetics associated with the differential spectra likely result from the existence of competing pathways in the PYP photocycle possibly due to two close-lying excited states populated upon excitation.

In a first attempt to approach the intrinsic properties of the PYP chromophore, we carried out subpicosecond transient absorption on the fully deprotonated *trans-p*-hydroxycinnamic acid (pCA^{2-}) and *trans-p*-hydroxycinnamide (pCM^-) in solution. These two analogues exhibit a quite different transient spectroscopy from native PYP. The ground-state *cis* isomer was found to be formed directly from the excited *trans* conformation without any detectable intermediate with a mechanism comparable to that of *trans*-stilbene.

In contrast, the photophysical behaviors of *trans-S*-phenyl-*p*-hydroxythiocinnamate (pCT^-) and denatured PYP in water are found to be close to that of the protein, highlighting the determining role of the thioester bond in the primary molecular events of PYP. The main excited-state deactivation channel is found to involve the formation of an early intermediate absorbing around 450 nm with a picosecond lifetime. As for native PYP, the complex kinetic behavior of pCT^- and of denatured PYP is proposed to result from competing relaxation pathways likely due to the presence of two close-lying excited states simultaneously populated by the excitation pulse. The main excited-state deactivation channel of pCT^- and denatured PYP has not been elucidated yet since the formation of the ground-state *cis* isomer has not been proved. This puzzling behavior is tentatively explained by the existence in polar solutions of another very efficient non-radiative process in competition with the photoisomerization process. According to this hypothesis, it would be clear that the protein environment would force the chromophore to deactivate along a particular reaction path, leading to the formation of the *cis* isomer, that cannot be reached in polar solutions. The solvent effects (hydrogen-bond and polarity) are indeed determining factors in the spectroscopy and the photophysics of pCT^- and denatured PYP.

If the ultrafast events on the femto–picosecond time scale in native PYP seem related to the chromophore restructuring, the reaction path that leads to the formation of the *cis* isomer is still unclear. The determination of the structure of the I_1 precursors is then crucial. Time-resolved vibrational spectroscopy can be a useful tool to probe the structure of the transients of PYP photocycle and a first study by UV-pump–IR-probe spectroscopy has already been reported.[22] In addition, theoretical approaches should address the possible existence of two degenerate excited states and their role in the deactivation path.

The elucidation of the early kinetics of pCT^- is a potentially efficient way to understand the isomerization mechanism of PYP. Further studies are nevertheless still needed to understand what coordinate (internal or external, or both) provides the driving force for the excited-state evolution of this analog of the PYP chromophore. The study of the solvent effects on the excited-state deactivation of pCT^- is thus crucial. This study will bring important elements to understand the effect of the chromophore constrained environment in native PYP. It is known that the hydrogen-bond network involving the chromophore plays a key role in the excited-state deactivation of PYP.[19,54,73] On the other hand, the determination of the structure of the early intermediate observed by the transient absorption spectroscopy will bring important information on the nature of the non-radiative processes possibly in competition with the isomerization reaction. A recent study of pCT^- by UV-pump–IR-probe spectroscopy gave promising results since it led to the observation of a modification in the stretching motions of the phenolate group upon electronic excitation.[75]

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