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On the Configurational and Conformational Changes in Photoactive Yellow Protein that Leads to Signal Generation in *Ectothiorhodospira halophila*

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Abstract. Photoactive Yellow Protein (PYP), a phototaxis photoreceptor from *Ectothiorhodospira halophila*, is a small water-soluble protein that is crystallisable and excellently photo-stable. It can be activated with light (λ^{max} = 446 nm), to enter a series of transient intermediates that jointly form the photocycle of this photosensor protein. The most stable of these transient states is the signalling state for phototaxis, pB. The spatial structure of the ground state of PYP, pG and the spectral properties of the photocycle intermediates have been very well resolved. Owing to its excellent chemical- and photochemical stability, also the spatial structure of its photocycle intermediates has been characterised with X-ray diffraction and multinuclear NMR spectroscopy. Surprisingly, the results obtained showed that their structure is dependent on the molecular context in which they are formed. Therefore, a large range of diffraction-, scattering- and spectroscopic techniques is now being employed to resolve in detail the dynamical changes of the structure of PYP while it progresses through its photocycle. This approach has led to considerable progress, although some techniques still result in mutually inconsistent conclusions regarding aspects of the structure of particular intermediates. Recently, significant progress has also been made with simulations with molecular dynamics analyses of the initial events that occur in PYP upon photo activation. The great challenge in this field is to eventually obtain agreement between predicted dynamical alterations in PYP structure, as obtained with the MD approach and the actually measured dynamical changes in its structure as evolving during photocycle progression.

Key words: 4-hydroxy-cinnamic acid, contact dependence, hysteresis, photoactive yellow protein, photo-isomerisation, polarization spectroscopy, protein dynamics, time-resolved X-ray diffraction, time-resolved FTIR spectroscopy, transient intermediates

1. Introduction

Structural biology has advanced to such an extent that several well-established methods are available for the resolution of the time-average structure of biological

macromolecules [1, 2]. These different techniques in most cases provide us with mutually consistent information so that detailed and reliable information about bio-molecular structure has become available. From these, one can often also extract information concerning dynamical fluctuations in these time-average structures. When it comes to function, however, not only the time average-, but also the functionally relevant dynamical alterations in bio-molecular structure and conformation are of crucial importance. Regarding the latter, several different methods have been developed during the past few years. This now allows us to compare the results of their application to accessible model systems from the realm of biology, to assess the nature and implications of protein conformational change in enzyme catalysis and biological signal transduction.

The possibility to trigger a (bio)chemical reaction with light allows one to perform studies with extremely high time-resolution and to synchronize the activity of all light-sensitive molecules in a certain reaction volume. Accordingly, the study of photoactive molecules provides some of the advantages present in the single-molecule approach. By selecting a photoactive molecule that has a function in signal transfer, i.e. a photosensor protein, one may in addition expect to be able to trigger significant changes in protein conformation. Accordingly, photosensor proteins provide an interesting material for studies of the dynamical changes in protein structure [3].

The extremophilic purple phototrophic bacterium *Ectothiorhodospira halophila* (*E. halophila*) produces a small yellow water-soluble protein. Because it is also photoactive, it was named Photoactive Yellow Protein (PYP) [4]. Similar proteins have also been characterized from several related phototrophic bacteria. Jointly these are referred to as the family of the xanthopsins [5]. Their yellow colour is due to a unique chromophore, an anionic cinnamin derivative, so far only detected in this family of photosensor proteins. PYP functions in bacterial behaviour [6], as the photoreceptor of a repellent response of *E. halophila* towards blue light. Nevertheless, other members of the xanthopsin family have (an) other biological function(s) [7].

PYP from *E. halophila* displays excellent stability, both chemically and photochemically. Furthermore, it can be heterologously expressed in *Escherichia coli* in large quantities in wild type- and various mutant- and hybrid forms. These opportunities have led to an avalanche of biophysical studies on the mechanism of the **intramolecular** signal transduction in the PYP protein, initiated by photoactivation and leading to a transient partial unfolding of the protein. These experiments have revealed great detail of the mechanism of signalling state formation in PYP and have made this protein a model system for studies in photochemistry and in protein folding [8].

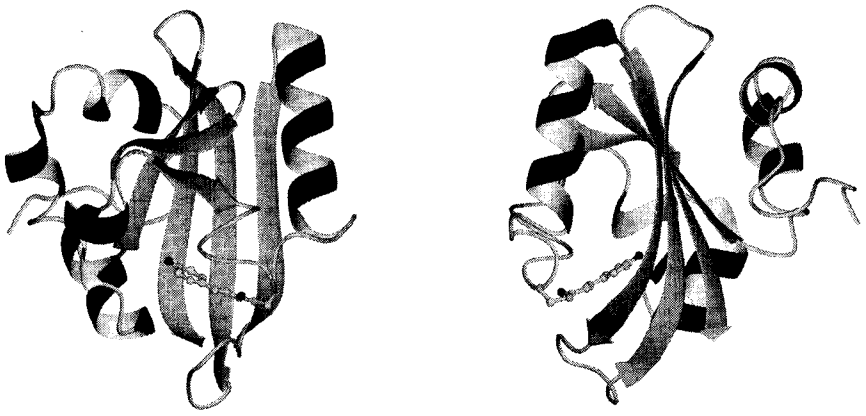

Figure 1. Structure of PYP. Spatial structure of the (backbone of) Photoactive Yellow Protein, viewed from two perpendicular orientations, to emphasise its two hydrophobic domains. The helical segments represent α-helices and the arrows β-strands.

2. Structure of PYP

Functional holo-PYP can be reconstituted from apo-PYP by the addition of an anhydride derivative of 4-hydroxy-cinnamic acid [9], or with activated cinnamyl-derivatives derived from N,N-carbonyl-di-imidazole [10].

Detailed information on the 3-D structure of PYP from *E. halophila* (i.e. E-PYP) has become available via X-ray crystallography and multi-nuclear NMR spectroscopy [11, 12]. Although initially its structure was incorrectly resolved [13], in 1995 the proper structure became available at 1.4 Å resolution. Recently this resolution was improved to 0.82 Å [14]. E-PYP is a relatively small protein containing 125 amino acids, with a relatively high abundance of negatively charged residues, as is common for proteins from halophilic organisms. PYP displays a typical α/β fold, with an open, twisted, 6-stranded, antiparallel β-sheet, flanked on one side by 3 α-helices and a long, well-defined loop that forms a π-helix and flanks the chromophore-attachment site C69. This secondary structure of E-PYP confirms a very early CD spectrum of the protein [4], which predicted this α-helical content. This part forms the major hydrophobic core of the protein. Two additional α-helical segments (D10 to L15 and D19 to L23) are folded independently at the back of the central β-sheet and cover a second, minor, hydrophobic core (Figure 1). In the solution- (i.e. NMR-) structure [12] this latter helix is disordered. Other differences between the structure obtained with X-ray diffraction and NMR spectroscopy are the two possible orientations of R52 in the NMR structure, that both are different from the orientation found with X-ray crystallography. Additionally, also the Y98 side chain has different orientations. In one orientation of R52 in the NMR (solution) structure, its two free amino groups are about 4 Å away from the aromatic ring of the chromophore. In the other orientation they are about 4 Å away from

the aromatic ring of Y98. These positions are in line with the observation that positively charged amino groups like to pack within 3.4 to 6 Å of the centroids of an aromatic ring [15]. Site-directed mutants [10, 16] have shown that the cationic R52 is not of critical importance for neither the tuning of the absorbance maximum of PYP, nor for functional photocycle activity. This shows that interaction between the anionic chromophore and cationic R52 is not of critical importance for the functional properties of PYP and is more in line with the NMR data.

In terms of the 'PAS fold' (see further below; [17]), the major hydrophobic core is composed of (i) the β-scaffold, (ii) the helical connector (i.e. the longest α-helix of PYP (α-5)) and the PAS core. The latter contains a π-helix (flanking C69). This helix may be crucial for the flexibility of the protein backbone required for signalling state formation [18]. The structure of E-PYP was recently also determined in crystals of a second space group, $P6_5$ rather than the initial $P6_3$. There are only small differences in the structure of the protein in these two different environments, which are well explained by flexibility of the protein along the eigenvectors of motion predicted from a molecular dynamics analysis [19]. The same eigenvectors also can account for most of the difference between the crystal- and solution structure of PYP.

As already mentioned above, PYP carries a light absorbing chromophore. Through the initial confusion about the structure of PYP [13], it took rather long to resolve its structure. Initially it was presumed that PYP would be a retinal protein. That this could not be true was evident from the fact that it was linked to C69 [20]. One year later this chromophore was shown to be *trans* 4-hydroxy-cinnamic acid [21, 22]. The 4-hydroxy cinnamyl chromophore is present in the anionic form in the ground state (pG_{446}) of the protein [22, 23], buried within the major hydrophobic core of the protein, where it is stabilised via a hydrogen-bonding network, involving the amino acids Y42, T50 and (protonated) E46 [11]. With site-directed mutagenesis the role of most of the amino acid side chains lining the chromophore-binding pocket has been investigated. Particularly the E46Q mutant protein has been instrumental in resolving various aspects of proton transfer within the protein initiated by photoactivation. The chromophore, with a pK_a in solution of 8.9 [24], is deprotonated at neutral pH in the protein (apparent pK equals 2.8 [24]) and E46, typically with a pK_a of around 4.4, is protonated. Therefore, the pK_a's of the chromophore and E46 are significantly tuned away from their solution values. This also holds for the absorption maximum of the chromophore in the protein, as compared to the free chromophore in aqueous solution [25].

Based on the (electrostatics of) the structure of the surface of PYP detailed speculations about a possible specific surface region, involved in signal transfer to a downstream partner, already were published [11]. The analyses of the structure of photocycle intermediates and the molecular dynamics analysis of E-PYP [26] reveal, however, that such conclusions still must be considered as preliminary, the more so, because its downstream partner(s) has not been identified yet.

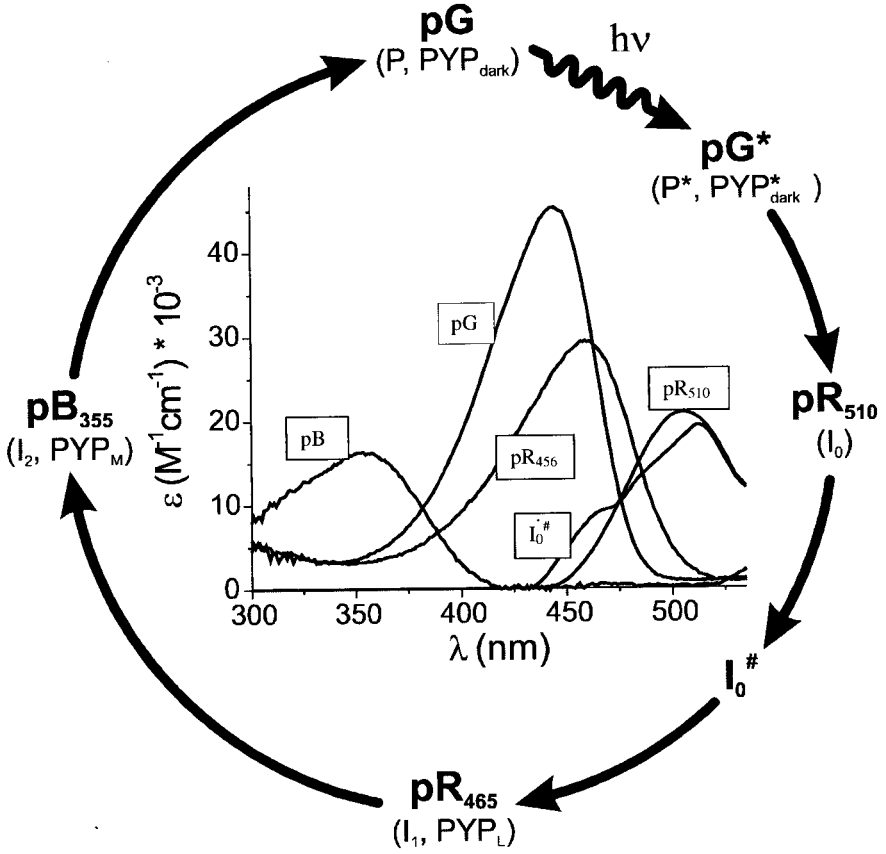

Figure 2. Overview of the photocycle of PYP. The key intermediates in the photocycle of PYP as detected with transient absorption spectroscopy at ambient temperature are indicated in the (redundant) nomenclature that is currently in use in the scientific literature. The inset shows the UV/vis spectra of these intermediates.

3. Photo Activation of PYP

Functional activity of almost any protein, particularly of all enzymes, requires rapid dynamical fluctuations in its structure. Signal-transduction proteins are ideal model systems to study such dynamic transitions, because of their intrinsic capacity to transiently form a signalling state, which has to have a long-enough lifetime to be recognised by the downstream signal transduction partner. In a photoreceptor protein these dynamical changes are initiated by photon absorption and therefore can be resolved – through the use of pulsed lasers – with high time resolution. In most cases these dynamical transitions in structure are reflected in the colour of the photosensor protein and can therefore be studied with transient UV/Vis spectroscopy. Nevertheless, many additional techniques are available. Below we will discuss these studies as performed on the xanthopsin E-PYP.

In photoactive proteins light absorption by the chromophore leads to excited state formation, followed by a series of transient and reversible colour changes, usually referred to as its photocycle (see Figure 2). This reversibility facilitates kinetic studies through the application of signal-averaging techniques in e.g. laser-induced transient absorption spectroscopy (TAS). The measured light-induced absorption (and fluorescence) changes as a function of time can be subjected to advanced mathematical analyses like singular-value decomposition and global analysis (e.g. [27] and references therein), to derive (kinetic) models of relevant intermediates and their associated spectra.

It was shown with femtosecond TAS (λ_{exc} = 400, 460 nm), observing the first 10 ps of the photocycle, that an intermediate with a red-shifted absorption spectrum compared to pG, is formed with a time constant of 2.8×10^{11} s^{-1} ([28], see also Figure 2). This process is preceded by an ultra-fast relaxation process on the excited state surface with a rate constant of 1.4×10^{12} s^{-1}, which produces an excited state intermediate in which the *trans/cis* isomerisation may already be underway. The subsequent intermediate, formed within picoseconds, was tentatively assigned to pR_{465}, the first intermediate reported in TAS studies with nanosecond time-resolution [27]. A subsequent experiment with picosecond time resolution and a nanosecond observation time window (λ_{exc} = 452 nm), however, established the appearance of an intermediate (I_0) preceding the formation of pR_{465}, which was formed within 3 ps [29], whereas pR_{465} is formed within 3 ns. In the same report another intermediate ($I_0^{\#}$) – either a real state with a broader absorption spectrum, very similar to I_0, or a thermal equilibrium of pR_{465} and I_0 – was detected in between I_0 and pR_{465}. More recently, Devanathan et al. reported a wavelength dependence of PYP excitation in a TAS study with femtosecond-excitation and -time resolution [30]. They observed that excitation at 395 nm leads to a much more red-shifted stimulated emission signal than excitation at 460 nm. From this they concluded that a higher-lying excited state is involved, which – when excited – forms I_0 via an excited state I_0^*. In their interpretation excitation at 400 nm leads to the formation of a higher laying pG^{**} which can relax to pG^* or cross to I_0^*. The I_0^*- I_0 energy difference is assumed to be smaller than that of pG^*-pG, leading to red-shifted stimulated emission. In other words, they propose that isomerisation initiates on the excited state surface. The time-dependence of the difference absorption signal within the first seven picoseconds was described in a global fit with a mono-exponential function. The estimated rate constant of I_0 formation is 4.4×10^{11} s^{-1}.

In similar investigations of the E46Q mutant, the same sequence of appearance of intermediates (pG -> I_0 -> $I_0^{\#}$ -> pR_{465}) was observed although spectra were not determined [30, 31]. E46 forms a strong hydrogen bond with the negatively charged phenolate of the chromophore in the ground state of PYP. Replacement of E46 by a glutamine is supposed to weaken this hydrogen bond substantially [32, 33]. The rate constant of I_0 formation in the mutant protein was very similar to the corresponding one in wild type PYP. The two slower transitions were significantly

faster in the two studies (although they mutually differ by almost a factor of two). These differences are assigned to a significant difference in the signal-to-noise ratio in the two studies (being superior in [31]). In both studies it is proposed, on the basis of the larger rate constants for $I_0 \rightarrow I_0^{\#}$ and $I_0^{\#} \rightarrow pR_{465}$ of the E46Q mutant, that these two processes involve movements of the phenolate ring of the chromophore. The larger rate constants, however, could also be due to the fact that for the mutant protein movements of this phenolate ring are possible, that would be largely blocked in wild-type E-PYP, due to stronger hydrogen bonding. That the rate of the primary reaction [30] ($pG \rightarrow I_0$) is not altered, is interpreted by Devanathan et al. as support for the rotation of the carbonyl group of the chromophore as the primary photoreaction [30] (first proposed in [34]). The alternative, photo-isomerisation of exclusively the 7,8-vinyl bond of the chromophore, would require release of the entire phenolate ring from its binding pocket. On the contrary, Zhou et al. derive from the differences between wt-PYP and the E46Q mutant a hypothetical model were the C = C isomerisation takes place after formation of $I_0^{\#}$ [31]. Both interpretations are rather speculative. Since there is evidence for chromophore isomerisation in low-temperature intermediates preceding pR_{465} [35], we consider it most plausible that the isomerisation takes place before the formation of pR_{465}.

Low-temperature spectroscopy allows one to trap intermediates that are formed upon photoactivation. These can subsequently be investigated with spectroscopy and/or X-ray diffraction. Accordingly, the early steps after photoactivation become accessible for structural studies. This has led to a rather complex photophysical scheme of the first part of the PYP photocycle, in particular through an analysis of the excitation-wavelength dependence of its primary photochemistry. It is postulated that two different intermediates, PYP_H and PYP_B, are formed in parallel, which both subsequently relax via yet another intermediate in each pathway (PYP_{HL} and PYP_{BL}, respectively) to the same pR_{465} [35, 36]. Recent support for this model was obtained in a TAS experiment [37], in which PYP_B corresponds to I_0, while PYP_H was not observed before. This is the first report in which continuous illumination has been used to characterize the initial branching in the photocycle.

The conversion of pR into pB and the subsequent recovery reaction, has extensively been characterized with UV/Vis transient absorption spectroscopy in the nanosecond to millisecond time range (e.g. [38, 8, 27, 10]). Various models can be used to fit the observed transitions, from single exponential fits, via multi-exponential deconvolution, up to distributed kinetics (i.e. a Gaussian distribution of the rate constants), depending on the signal to noise ratio present in the available data. For instance, in a study of the pH-dependence of the recovery kinetics the best fit was obtained with a mono-exponential fit at acidic and alkaline pH values, but with a bi-exponential fit for the neutral pH range (J. Hendriks et al., unpublished). Additional complexity is added to this photocycle by the observed light-sensitivity of the pB state [39, 40]. Intriguingly, the illumination history of PYP has an influence on pB→pG recovery. This recovery in a fresh PYP sample shows only small deviations from a mono-exponential behaviour (97.4% relative

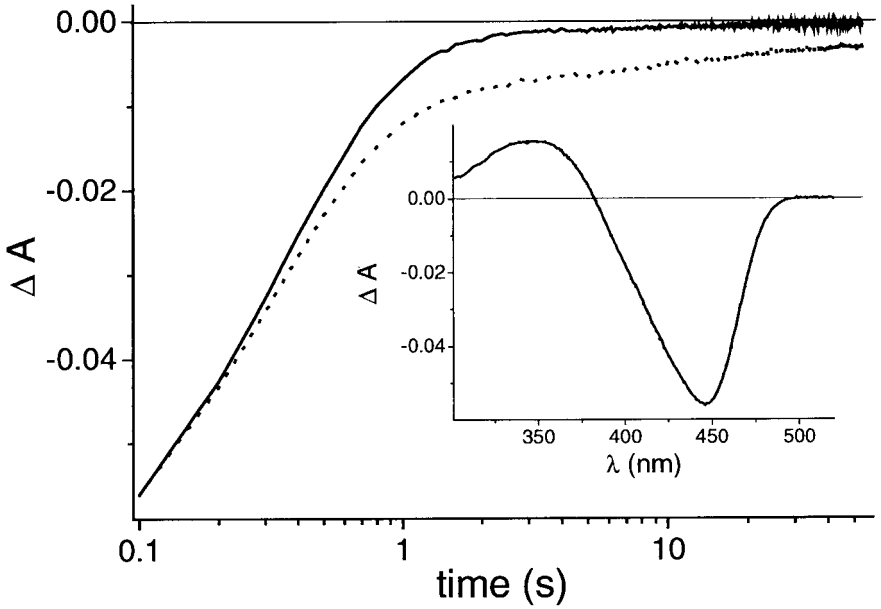

Figure 3. Dependence of the pB→pG recovery on illumination history. Before the illumination period the pB→pG recovery of the PYP sample (2.4 μM in 50 mM Tris buffer (pH 8)) was recorded as the absorption change at 446 nm (solid line). Excitation was achieved with the light of a photoflash (0.5 ms duration) that was send through a bandpass filter (465 $\pm$ 5 nm). The cw illumination source (462 $\pm$ 11 nm) exclusively excites pG and established initially a photo-equilibrium with 20% pB and 80% pG. The relative pB/pG concentration varied anti-parallel during the illumination time (13.5 h) to final values of 30%/ 70%. After the sample was kept in the dark for 5 h, its absorption spectrum was identical to that at the start of the experiment (see inset). The pB→pG recovery was again recorded as described above (dotted line). Visual inspection of the two curves already proves, that the sample after the long illumination had a higher extent of slow pB→pG recovery. Global analysis of the two traces with a three-exponential function $\Delta A = \alpha_1{}^*\exp(-t/k_1) + \alpha_2{}^*\exp(-t/k_2) + \alpha_3{}^*\exp(-t/k_3))$ leads to 2.7 s^{-1}, 0.14 s^{-1} and 0.0076 s^{-1} as recovery rates. The relative amplitudes of the two unusually slow recovery rates have increased by 10% (1.8%, and 0.8% before illumination vs. 6.2% and 6.4% after illumination) due to the illumination. The inset depicts the difference aborption spectra prior to and after cw illumination. They are are identical, which demonstrates that the spectral properties of PYP, i.e. the pG and pB spectra, were not affected by the illumination.

amplitude for the dominating fast recovery rate, Figure 3). When the sample at pH 8 is irradiated for an extended period with blue light (462 nm), the amplitude of additional slow recovery components increase (only 87.4% relative amplitude for the fast recovery rate after 13.5 hours, Figure 3). The pG absorption spectrum of the sample after the illumination is indistinguishable from that before, as well as the light-induced difference spectra (inset in Figure 3). This demonstrates that a fraction of the PYP molecules is transformed into a form with identical spectra

of ground and signalling state, but a significantly decelerated recovery rate. This process causes hysteresis in the PYP photocycle.

The distributed kinetics [41] in PYP may have its basis in the 'energy-landscape' description of protein conformation [42]. This may bestow PYP with characteristics of complexity, because individual protein molecules may reside in different conformational sub-states, from which they cannot escape with the thermal energy available at low temperature.

Both photocycle kinetics [10] and spectra of photocycle intermediates [24, 43] are strongly dependent on pH. For instance the maximum of pB shifts from 365 nm (at low pH), via 355 nm (at neutral pH) to ∼420 nm at high pH [24, 43], through deprotonation of the chromophore. Low recovery rates at low pH can be exploited to accumulate the signalling state of PYP (i.e. pB; see e.g. [44, 45]). As an alternative, one may use proteins altered through site-directed mutagenesis (e.g. M100A [46]) and/or chemical engineering [25, 47].

4. Dynamical Alterations in the Structure of PYP

The intrinsic dynamics of the ground state of a protein is reflected in the correlated motions of groups of atoms, around their equilibrium position, as determined by e.g. X-ray diffraction, neutron scattering and/or NMR spectroscopy. These dynamics can be analysed *in silicio* by molecular dynamics modelling. In this modelling approach the trajectories of each atom of the molecule are simulated on the basis of classical rate laws. Given current limitations in computer capacity these calculations can realistically be extended only up to the nanosecond time scale for a single protein molecule in a box of water molecules.

From the correlated motion of different atoms, the eigenvectors of the intrinsic dynamics in a protein can be calculated. For PYP this analysis revealed several striking features. The protein shows correlated motion of its chromophore and the surrounding amino acid side chains; this dynamic flexibility is largely described by the three main eigenvectors from the essential dynamics analysis; in each eigenvector the majority of the atoms of the protein is involved [26]. For some proteins the eigenvectors derived from essential dynamics analyses describe the initial part of the trajectory of the conformational change that is important for their functional activity [48, 49]. It is a major challenge to relate the eigenvectors describing the dynamics of PYP to the conformational transitions, relevant for signalling.

Changes in the structure of PYP that occur upon initiation of photocycling have been studied with various techniques. Most extensive has been the application of X-ray crystallography, which has been applied in various forms, e.g. with continuous laser illumination (for pB; [50]), cryo-trapping (for an early intermediate prior to pR_{465}; [14]]) and with time resolved X-ray diffraction at ambient temperatures (for pR, [51, 52]). The cryo-trapped intermediate was identified as PYP_{BL} but considering the temperature and illumination conditions used, could also be composed of a mixture of intermediates. The initial alterations of the structure of PYP, elicited by

light, can be described as a flip of the carbonyl group around the long axis of the chromophore. This is accompanied by a 3.4 Å displacement of its carbonyl-oxygen atom and rearrangement of the dihedral angles around the sulphur atom (leading to a 1.4 Å displacement). The chromophore itself has a stretched *cis* configuration, showing that isomerisation already has taken place. Initial movement of the carbonyl-oxygen, as proposed from FTIR data [34], is evident from the structure of this cryo-trapped intermediate. The aromatic ring of the chromophore moves slightly (0.4 Å). To accommodate these changes, the most notable alteration in the residues lining the chromophore is a 0.5 Å shift in the position of the aromatic ring of Phe-96. Together with the small packing gaps that flank this residue, this allows the carbonyl 'flip', without significant collisions.

The thiolester carbonyl oxygen is rotating **clockwise** towards the backbone nitrogen of Y98, when viewed from C69, but no hydrogen bond is obtained yet with its backbone nitrogen. In the cryo-trapped intermediate the degree of rotation is 166 of the 282° needed to reach its position in pR [51]. The time-resolved 1 ns structure also shows changes predominantly around the chromophore, but with the carbonyl oxygen in hydrogen-bonding contact with Y98. In addition, the *cis* configuration of the chromophore is less stretched. In the cryo-trapped intermediate the hydrogen bonding between the chromophore and E46 and Y42 is still intact. For the pR structure obtained at room temperature [51] initially it was concluded that the hydrogen bond between the chromophore and E46 had been disrupted (because their mutual distance is > 5 Å). More recent refinements, however, show that E46 and the chromophore move in concert, so that this hydrogen bond presumably can remain intact during pR formation, which is in full agreement with FTIR results [34, 52–54].

The transition to the pB state is subsequently initiated by transfer of a proton from E46 to the chromophore [34, 55]. The buried charge that is generated in this process destabilizes PYP to such an extent that PYP partially unfolds [54]. The resulting structure of the pB intermediate, however, is dependent on the mesoscopic context in which PYP functions (see further below).

Re-isomerisation of the chromophore to the *trans* configuration (which also readily proceeds in crystalline PYP) is initiated from the pB state. In this conformation the carbonyl-oxygen of the chromophore group is again hydrogen bonded to the backbone nitrogen of C69 [50]. However, no detailed rationalization on how the PYP protein may facilitate this re-isomerisation can be given yet.

The crucial activity of a photosensor protein is to transduce a photon absorption event into a change in conformation that can be detected by a downstream partner. To achieve this, it has to enter a so-called signalling state. Information about their nature is very scarce. Clearly identified signalling states have only been described functionally for the sensory rhodopsins, initially through the use of retinal analogues (e.g. [56]) and predominantly with respect to their spectral properties. These studies have revealed that the long-living blue-shifted intermediate functions as the signalling state for the archaeal sensory rhodopsins. Based on

their mutual similarities it has been presumed that the blue-shifted intermediate pB is the signalling state of PYP. Formation of this state shows characteristics typical for a (partial) protein unfolding reaction (see further below); its rate (i.e. 10^4 s^{-1} [27]) is compatible with this interpretation. It is therefore very exciting that the spatial structure of transient intermediates of E-PYP, with lifetimes ranging from nanoseconds to milliseconds are now available through the application of time-resolved X-ray diffraction (or: Laue diffraction) analysis, applied to crystalline PYP [50, 51]. This approach allows Å resolution at the ns time scale and in that way may reveal the structural basis of transition states in slow conformational transitions in proteins. Summarizing, upon excitation the carbonyl group starts rotating around the long axis of the chromophore; subsequently the phenolate moiety is partially (at the sub ms time scale) exposed to solvent. This structure relaxes to the ground state on the s time scale. Surprisingly, the backbone structure of PYP is hardly affected by these transitions, except for some very small displacements near the chromophore-binding site. A limited number of side chains, in particular R52 and R124 do significantly alter their orientation during pB formation, however, which leads to a structure with a chromophore partially exposed to the surrounding solvent.

NMR studies of the structure of the pB intermediate of E-PYP, in contrast, have resulted in a very different picture of the characteristics of this intermediate. Changes in the HSQC spectrum of PYP [45] suggest that a large part of the secondary structure of the protein has been altered in pB, which actually is better described as a collection of rapidly (i.e. millisecond) exchanging states. Because of this rapid exchange it has not yet been possible to determine the spatial structure of the pB intermediate in solution. Subsequently it was shown with amide H/D exchange data that although the protein has a solid exchange-protected core, in two other regions, the chromophore loop and the N-terminal domain, the secondary structure of E-PYP in the pB state changes significantly [57].

This difference between the results of Laue diffraction- and HSQC-NMR experiments is evidence that the precise nature of the pB intermediate, formed upon illumination, depends on the mesoscopic context of PYP. So whether the protein is dissolved as a monomer in aqueous solution, or present in a crystalline lattice, has profound effect on the structure of the pB state. This conclusion has recently fully been confirmed with FTIR measurements [54], in which it was shown that the extent of the conformational change upon pB formation is far larger in solution than in crystalline PYP. This was deduced from the changes in the Amide-I region of FTIR difference spectra (Figure 4; see also [54]) of E-PYP recorded in these two mesoscopically different environments. In agreement with this we have observed that, although entrapment of E-PYP in a crystalline lattice does not affect the spectral characteristics of its intermediates, the kinetics of its photocycle transitions are significantly accelerated in a crystalline lattice (J. Hendriks et al., unpublished).

In the intact cell, PYP presumably functions in a (large) signalling complex. Therefore, *in vivo,* PYP may have characteristics in-between those of the crystalline-

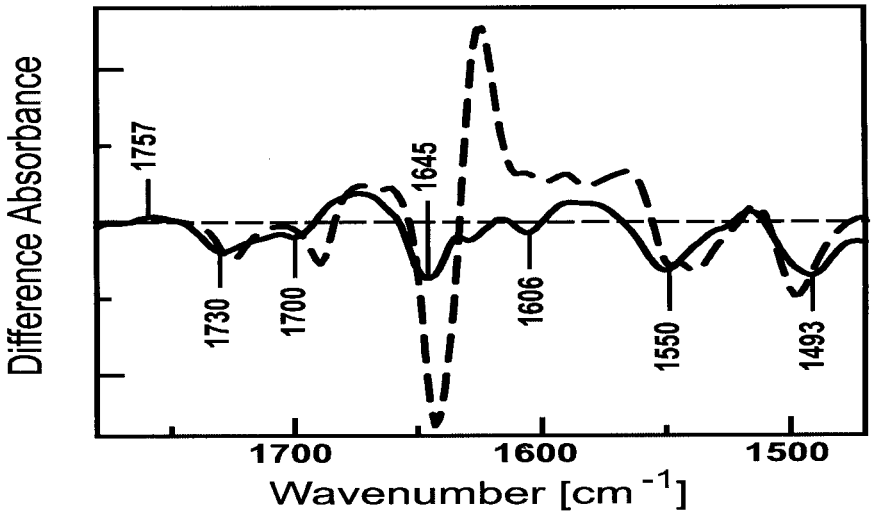

Figure 4. Mesoscopic dependence of conformational transitions in E-PYP. pB-pG infrared spectra of PYP in solution (dashed spectrum) and in $P6_3$ crystals (uniterrupted spectrum). This figure is modified from Figure 3 in: Xie et al. (2001) Biochemistry 40: 1510–1517, in which also further details can be found.

and the dissolved state. We consider it a major challenge to resolve these *in vivo* characteristics of PYP and its intermediates and to elucidate their role(s) in xanthopsin-mediated signal transduction. Such studies have additional relevance because of the prototype function of PYP for all PAS proteins and therefore may elucidate key features of regulation of (eukaryotic) signal transfer in general. The crucial parameter may be the number of intermolecular contacts in the various environments. This number also differs between the two space groups in which PYP crystals can be obtained (i.e. $P6_3$ and $P6_5$). It is therefore of interest also to compare the characteristics of PYP in these two types of crystal.

The initial observation that suggested the occurrence of a large structural change in E-PYP during pB formation was obtained from quantitative studies of the temperature dependence of the kinetics of photocycle transitions [41]. In that study it is reported that the recovery reaction of the PYP photocycle shows strongly non-linear Arrhenius behaviour (i.e. a plot of the natural logarithm of the rate constant versus reciprocal temperature is concave). Simple (gas-phase) chemical reactions usually show linear Arrhenius behaviour, whereas complex protein-folding reactions typically show (strongly) curved plots. This is usually explained by invoking a change in heat capacity (ΔC_p) in the transition under study [58], which arises from the exposure of hydrophobic surface area to water, in the denatured state. The hydrophobic side chains are surrounded by 'icebergs' of water that melt with increasing temperature, thus making a large contribution to the heat capacity of the denatured state and a smaller one to the compact transition state for folding [59]. In this interpretation the thermodynamic characteristics of the transition are assumed

to be independent of temperature; as an alternative one may also assume that ΔS and/or ΔH are strongly temperature dependent [60].

In PYP the recovery of pG shows a strongly curved Arrhenius plot. If this transition in the photocycle is considered as a pseudo equilibrium transition with the transition state, thermodynamic activation parameters can be calculated, from which the area of the exposed hydrophobic contact surface can be derived. For the recovery reaction this results in a ΔC_p value that indicates that about one-third of the protein unfolds in the signalling state pB ([41, 61], see also [62]).

Recently, two experiments were performed that allow one to further characterise the unfolding event of signalling state formation in PYP. First, genetic truncations of PYP were constructed, that lack their N-terminal domain [63]. This does not impair photocycling, nor signalling state formation, although the kinetics of the recovery reaction in the photocycle of these truncated variants is strongly decelerated. Surprisingly, the extent of curvature of the Arrhenius plot of the recovery reaction of these truncated proteins has significantly decreased; to the extent the Arrhenius curve for $\Delta 25$ is essentially linear [63]. This suggests that the transient functional unfolding of PYP in the pB state is predominantly taking place in the intrinsically unstable N-terminal domain of the protein. The conformational changes during pB formation, however, are not restricted to the N-terminal domain. Using Nile Red as a probe we have detected the formation of a hydrophobic patch, paralleling formation of the pB state, which appears to be identical in the intact protein and the truncated variants (J. Hendriks et al., Biophys J., in press). In combination with the NMR experiments this leads to the conclusion that the Nile Red-binding pocket is near to the chromophore.

5. Concluding Remarks

Because of its extraordinary photo/chemical stability and the ease with which it forms crystals, the PYP from *Ectothiorhodospira* has become a photochemistry lab in itself: PYP is a very attractive model system for studies of the photochemistry of photosensing and for studies of (functional) protein (un)folding reactions. Consequently, a wide range of biophysical techniques is currently being used in its further characterization. Among these are: Molecular modelling, time-resolved X-ray diffraction, NMR- and fluorescence spectroscopy, small-angle X-ray- and neutron scattering experiments, time-resolved Optical Rotary Dispersion measurements, etc. Some of these techniques even owe part of their development on the availability of such suitable model systems as PYP.

Application of these techniques leads to exciting developments. The most important of these are: **(i)** Quantum chemical calculations: These are used to predict the trajectory of the photo-isomerisation process (e.g. [64]); even entire protein molecules come within reach of this approach [65, 66]. **(ii)** Various forms of (polarized) ultra-fast absorption and fluorescence spectroscopy will have to answer questions like the involvement of a bi-radical pair and/or the hula-twist mechan-

ism [67] in the photo-isomerisation of the chromophore. **(iii)** Time-resolved Laue diffraction: By applying the mathematics techniques that are extensively used in spectroscopy, like global analysis and singular value decomposition, this technique is at the point of revealing all relevant structures of PYP photocycling in a crystal lattice. **(iv)** FTIR measurements: The many assigned features in difference spectra of PYP intermediates make this a very powerful technique for more detailed characterization of the structure of (very short-living) transient intermediates. **(v)** Small-angle scattering experiments may provide valuable information regarding the structure of the signalling state of PYP in solution, perhaps even up to the extent that spatial structures may be reconstructed from X-ray scattering curves.

The experiments reported on PYP have made it clear that a major condition that must hold before a conclusion can be accepted is that separate, independent techniques must give the same answer to a particular question. The importance of this approach can be illustrated with the radius of gyration of the pB state. Describing this state as partially unfolded, leads to the expectation that its radius of gyration has increased. Whereas this indeed is observed with some techniques (e.g. [68]), it is not with others (like ^{1}H NMR spectroscopy, D.R.A. Marks et al., unpublished observation). Such discrepancies have to be resolved before we can really be confident to understand key properties of the signalling state of PYP.

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