

FEATURE ARTICLE

Photoactive Yellow Protein, A New Type of Photoreceptor Protein: Will This “Yellow Lab” Bring Us Where We Want to Go?^{||}Klaas J. Hellingwerf,^{*,†} Johnny Hendriks,^{‡,§} and Thomas Gensch^{†,‡}

Laboratory for Microbiology, Swammerdam Institute for Life Sciences (SILS), BioCentrum,
University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands,
and Institute of Biological Information Processing 1, Research Centre Jülich, D-52425 Jülich, Germany

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Photoactive Yellow Protein (PYP), discovered almost 20 years ago in *Ectothiorhodospira* (*Halorhodospira halophila*),¹ is a 4-hydroxycinnamic acid-containing protein that functions as a blue-light photoreceptor in a behavioral avoidance response in this organism. During the past 10 years, PYP has become a model system for studies in photochemistry and protein folding, to the extent that it has become competitive with the rhodopsins. This is because PYP is small and very water-soluble, forms crystals readily (diffracting to high resolution), and shows excellent chemical- and photo-stability. These overall characteristics have allowed the application of an array of physicochemical techniques to analyze the biological function of PYP, i.e., the translation of a change of the configuration of its 4-hydroxycinnamic acid chromophore into an altered conformation of the surrounding protein. This has led to detailed insight into this process, both temporally and spatially, with respect to the structure of the transient intermediates involved, although we are still quite far from being able to track the position of all atoms in space, upon light activation of the protein in the relevant time domain. Nevertheless, the data already obtained may function as a calibration set in future work, to extend the time span of molecular dynamics simulations of conformational transitions in proteins to the time scale relevant for catalytic turnover. Occasionally, the application of multiple biophysical techniques has led to (seemingly) conflicting results. In one example, this has revealed the fact that the light-induced conformational transitions in this photoreceptor protein can become restricted by the mesoscopic context, e.g., via a crystal lattice. Other inconsistencies, such as those regarding the radius of gyration of the protein, still remain to be explained. Below, we discuss the spatial and temporal details of the series of steps initiated in PYP by a short pulse of blue light, as revealed with this array of biophysical techniques, thereby highlighting contributions from our own group.

Introduction: Discovery, Biological Function, and Impact of PYP

In 1985, while aiming at making an inventory of all colored proteins present in an anoxygenic phototrophic and extremophilic bacterium, *Ectothiorhodospira* (now: *Halorhodospira halophila*), Terry Meyer discovered a small (14 kDa; 125 amino acids) yellow-colored protein that looked different from the well-known flavoproteins.¹ Soon afterward, the protein was shown

to be photoactive,² hence it was named Photoactive Yellow Protein (PYP). The yellow color of these proteins (Figure 1) turned out to be due to their unique chromophore, an anionic cinnamoyl derivative.³ Until that time, a chemical structure of this class of chromophores had never been shown to play a role in a photobiological signal transduction process.

In the years following its discovery, evidence was presented that PYP has a role in the process of bacterial phototaxis in *H. halophila*, in an avoidance response to blue light.⁴ Similar proteins, and genes encoding such proteins, were found in a number of bacterial species belonging to the family of the proteobacteria.^{5–8} This has led to the proposal to group these proteins in the family of the Xanthopsins.⁷ This then is a group of blue-light photoreceptor proteins, which contain 4-hydroxycinnamic acid as their photoactive chromophore. This review will deal with the Xanthopsin from *H. halophila*, the Photoactive Yellow Protein.

A key structural element in many signal transduction pathways in prokaryotic and eukaryotic organisms, including man, is the PAS domain.^{6,9–11} J. C. Lagarias was the first to note that there is identifiable sequence conservation between the PAS

* Corresponding author. Tel.: +31-20-5257055. Fax: +31-20-5257056. E-mail: K.Hellingwerf@science.uva.nl.

[†] Laboratory for Microbiology, Swammerdam Institute for Life Sciences (SILS), BioCentrum, University of Amsterdam.

[‡] Institute of Biological Information Processing 1, Research Centre Jülich.

[§] Present address: Department of Biochemistry and Molecular Biology, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

^{||} ABBREVIATIONS. PYP: Photoactive Yellow Protein; pG: stable ground state of PYP; pR and pB: transient intermediates of PYP, with red- and blue-shifted absorption maxima, respectively; pB_{dark}: ground-state of PYP obtained upon acid denaturation; CW: continuous wave; RT and LT: room- and low-temperature, respectively; PAS: acronym derived from the genes *Per*, *Arnt*, and *Sim*; PhAS: photo acoustic spectroscopy; TL: thermal lensing; TG: thermal grating; 4HCA: 4-hydroxycinnamic acid.

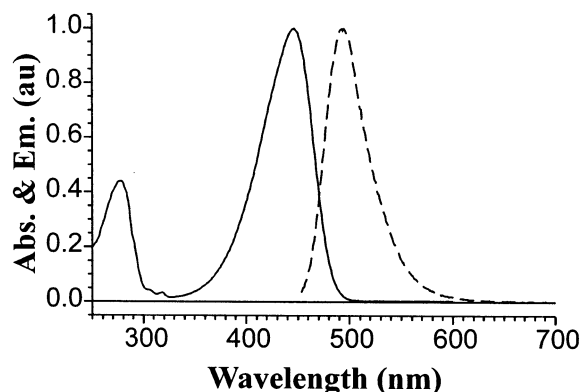


Figure 1. UV/Vis absorption (solid line) and fluorescence emission spectrum (excitation at 446 nm; dashed line) of PYP. Further spectral characteristics of PYP are $\epsilon = 45.5 \text{ mM}^{-1} \text{ cm}^{-1}$; $\lambda_{\text{max}} = 446 \text{ nm}$; $\Phi_{\text{fl}} = \sim 0.002$; $\Phi_{\text{photochem}} = 0.35^{1,32,120}$.

consensus sequence and PYP.¹² Meanwhile, of four members of the PAS-domain family (including PYP) the spatial structure has been resolved with excellent resolution.^{13–16} They indeed all show a strongly similar fold of their backbone. Furthermore, PYP has been nominated as *the* structural prototype for the complete (i.e., not only the PAS-core) three-dimensional fold of the PAS domain super-family,⁹ of which it displays all the structural and functional features (i.e., the N-terminal cap, PAS core, helical connector, and β -scaffold). This, together with its excellent physicochemical (e.g., with respect to pH and salt concentrations) and photochemical stability (in particular toward visible radiation¹⁷), has made PYP a prominent model system for understanding receptor activation in biological signal transduction in general.

PYP Basics

Before we go into detail, it is important to explain some of the basic characteristics of PYP. The initial characterization of PYP was performed with protein isolated from *Ectothiorhodospira* (now: *Halorhodospira*) *halophila*. As can be anticipated on the basis of its function,⁴ this organism produces only very small amounts of this protein physiologically. Gene cloning, heterologous overproduction in *Escherichia coli*, and in vitro reconstitution of apo-protein into holo-protein^{7,18} have boosted PYP research enormously. Various procedures can now be used to achieve this heterologous production. In one, the apo-protein is overproduced extra-cellularly;¹⁹ in most, however, intracellularly.^{7,20} In addition, PYP is in most cases produced with a poly-histidine containing N-terminal tag, to facilitate purification. This tag can be selectively removed by proteolytic digestion.⁷ Upon heterologous overproduction in *E. coli* (up to 2500-fold, compared to the expression level in *H. halophila*) apo-PYP is produced and the chromophore (4-hydroxycinnamic acid, 4HCA) can be attached, e.g., via an activated (e.g., imidazole) intermediate, to obtain holo-PYP. By consequence, it is straightforward to engineer PYP both genetically, via site-directed mutagenesis, and chemically, through the use of alternate chromophores, respectively.^{19–24}

PYP is a relatively small (14 kDa; 125 amino acids) and highly water-soluble protein. The structure of the stable ground state of PYP has been determined via X-ray crystallography,¹³ which shows that it has an α/β -fold, containing a six-stranded antiparallel β -sheet as a scaffold, flanked by several helices (see Figure 2). It contains two hydrophobic cores, one on each side of the β -scaffold. The smaller of these two comprises the N-terminus, while the larger contains the chromophore-binding

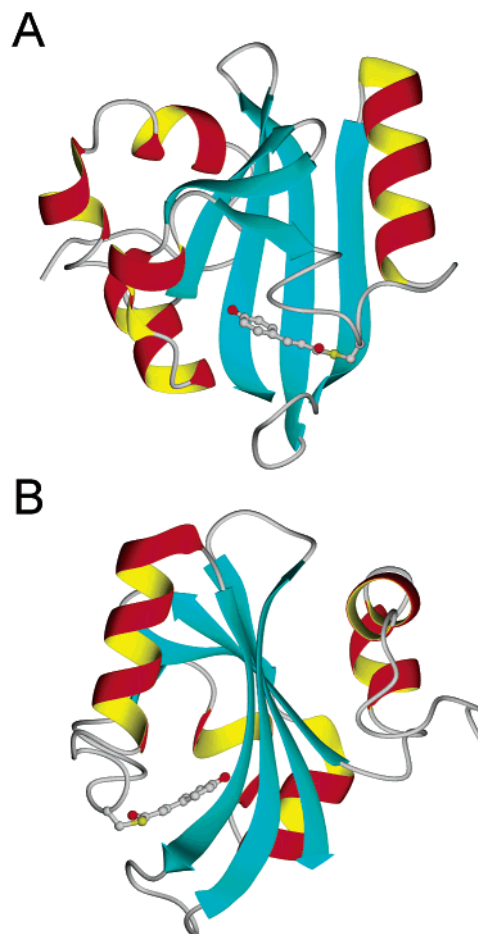


Figure 2. Two orientations of a ribbon representation of the structure of Photoactive Yellow Protein from *Halorhodospira halophila* are presented. The figure was prepared using the program MOLMOL¹²¹ with the structure coordinate file deposited at the Protein Data Bank¹²² (<http://www.rcsb.org/pdb>) with PDB ID: 2PHY.¹³ The program POV-Ray (<http://www.povray.org>) was used to generate the images.

pocket. The chromophore (4HCA) is linked through a thiol ester linkage to the sole cysteine in the protein, Cys69.^{25–27} In the ground state this chromophore resides in the *trans* configuration and is deprotonated.^{27,28} The resulting negative charge on 4HCA is stabilized via a hydrogen-bonding network, involving the residues Tyr42, Glu46, and Thr50.¹³ Additional stabilization may be provided by the positive charge on Arg52.^{13,29} Besides a crystal structure, also a solution structure was determined, using multinuclear NMR analyses.³⁰ Though minor differences exist, in particular in the region of the N-terminal helices of the protein, molecular dynamics simulations have shown that both structures are essentially the same.³¹

The key functional characteristic of PYP *in vitro* is that it goes—after absorption of a blue photon—through a reversible photocycle. This photocycle can be divided into three basic steps (see Figure 3A): (A) Initial photocycle events, in which the chromophore is isomerized. These initial events are considered completed once the intermediate pR is formed. (B) Protonation of 4HCA through intramolecular proton transfer and subsequent significant structural change of the protein, which under many circumstances is equivalent to partial unfolding of the protein. In this step the signaling state, pB, is formed. As we shall see later, the extent of structural change in this step depends on the mesoscopic (= molecular) environment. (C) De-protonation and re-isomerization of the chromophore, and refolding of the protein. In this/these latter step(s) the ground state, pG, is recovered.

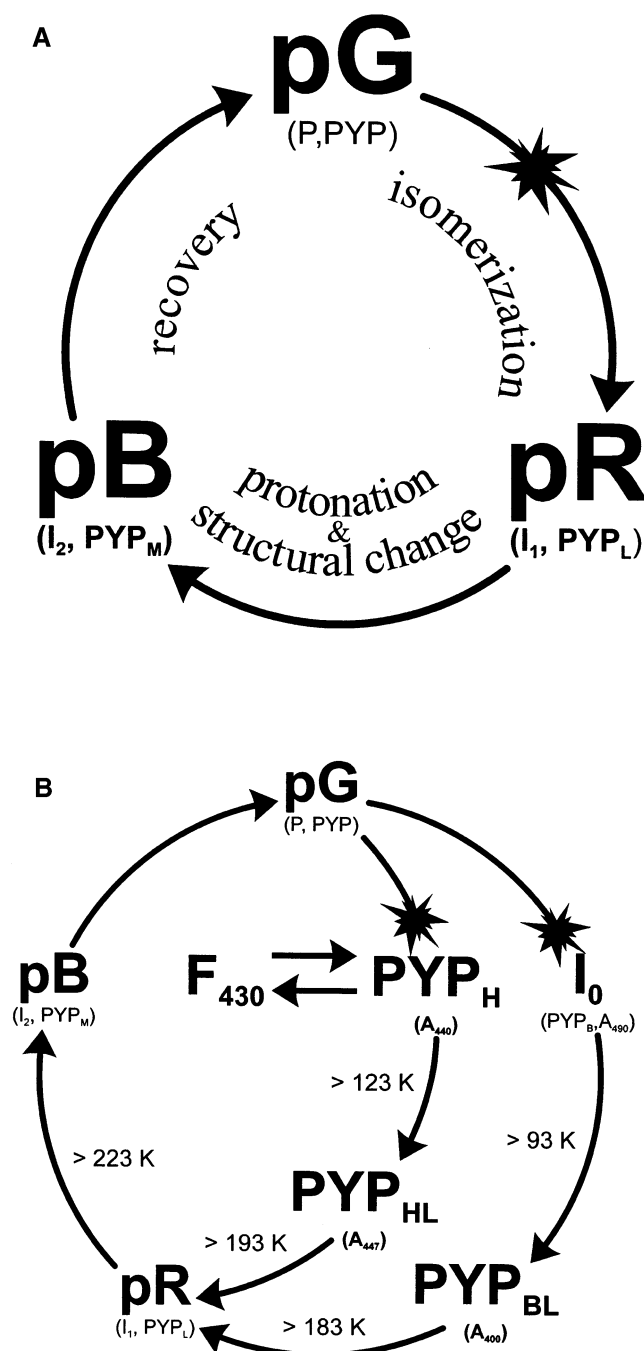


Figure 3. The photocycle of Photoactive Yellow Protein. (A) The key intermediates and events in the photocycle of the Photoactive Yellow Protein at ambient conditions. (B) Intermediates involved in the photocycle of PYP at low temperature (for further details see refs 33, 52).

Over the years, three different nomenclatures for the photocycle have evolved. Though this may be confusing at times, and a single nomenclature would be helpful, it is beyond the scope of this review to suggest one. It is therefore relevant to note the alternative nomenclatures for the three basic intermediates pG, pR, and pB introduced above: Besides the basic scheme $pG \rightarrow pR \rightarrow pB \rightarrow pG$, the schemes $P \rightarrow I_1 \rightarrow I_2 \rightarrow P$ ³² and $PYP \rightarrow PYP_L \rightarrow PYP_M \rightarrow PYP$ ³³ are also regularly used.

Effect of Experimental Conditions on the Structure of the Signaling State

Solvent conditions have a pronounced effect on the (dynamic) properties of Photoactive Yellow Protein. This may lead to

confusion when experiments performed under different conditions must be compared, but it has also led to a better understanding of the molecular functioning of PYP. The effect of ionic strength, viscosity, hydrophobicity of the solvent, as well as the effects of denaturing agents on the photocycle kinetics were studied from the very beginning.² As a function of increasing ionic strength, all reactions seem to be slow, be it that recovery of the ground state seems to be affected most.² Viscosity and hydrophobicity have an even larger effect on the photocycle kinetics. The rate of formation of pB decreases with increasing viscosity, and increases with increasing hydrophobicity. Recovery of the ground state shows complex viscosity dependence—initially the rate increases, but then it decreases upon further increase of the viscosity. The dependence on hydrophobicity is relatively straightforward and shows a decrease in rate upon increase of hydrophobicity.³² In the presence of a denaturing agent the rate of signaling state formation seems hardly affected.² However, the rate of recovery of the ground state dramatically decreases upon increased denaturant concentration, long before denaturation of the protein takes place. Combined, these results suggest that formation of the signaling state involves a significant structural change of the protein, exposing a hydrophobic surface in the process. Several additional experiments, among others based on kinetics analyses of the photocycle recovery reaction, H/D exchange experiments, and ¹H NMR characterization of the signaling state of PYP (refs 34–36, and see further below) have confirmed this hypothesis.

In contrast to this, diffraction experiments on the Photoactive Yellow Protein (i.e., with the protein in the crystalline state) have revealed only very small light-induced structural changes, by far not large enough to characterize them as partial unfolding of the protein.^{37–40} Since in these experiments the mesoscopic context of the protein (i.e., its immediate molecular environment) is completely different from that in solution, this needs not to come as a big surprise. Nevertheless, this issue was hotly debated and only settled after it was confirmed independently with (time-resolved) FTIR experiments that in crystalline PYP only relatively small light-induced structural changes take place, while in solution these structural changes are much larger.⁴¹ Accordingly, one must conclude that the extent of structural change can be modulated by the mesoscopic context of the PYP protein.

Presumably the most important experimental condition that can be modulated with respect to the functioning of PYP is the pH. The dependence of the ground-state recovery kinetics on pH has a bell-shaped form, with a maximum recovery rate at pH 8. Both at lower and at higher pH values, a decreased rate is observed, with implied pK_a values of 6.4 and 9.4, respectively.¹⁹ When comparing data from different experiments the pH is a factor that definitely has to be taken into account. In fact, the pH is frequently adjusted to obtain favorable photocycle kinetic properties for a specific measurement; e.g., at low pH more of the signaling state can be accumulated under continuous illumination.^{42,43} However, at extremely low pH values acid denaturation of PYP occurs with an apparent pK_a of 2.7.⁴⁴

Configurational and conformational transitions in PYP have been studied with a large array of biophysical techniques, using radiation with wavelengths ranging from X-rays to the infrared and with measurements based on transient-absorption, -scattering, and -diffraction. In these experiments, frequently deuterium oxide has been used as a solvent rather than water. When comparing data obtained in these two solvents, it is important to take into account that pH and pD values cannot always be directly compared. The difference of the dissociation constant

between water and deuterium oxide⁴⁵ has to be taken into account. In fact, photocycle kinetics in PYP seem to be mostly dependent on the concentration of hydroxide ions and are therefore pOH dependent, rather than pH dependent—an important distinction when comparing data obtained in water and deuterium oxide (Hendriks et al. *Biophys. J.*, in press). Also, signaling state formation is slightly slower and ground-state recovery slightly faster, when deuterium oxide is used as the solvent (Hendriks et al. *Biophys. J.*, in press).

Particularly with FTIR spectroscopy it is very important to consider the exact mesoscopic context of the sample during measurements. The following conditions have been most frequently used: solutions at very high protein concentration,^{35,41} hydrated films,^{35,46–48} and even crunched crystals.⁴¹ Special care should be taken with hydrated films, since the degree of hydration has pronounced effects on the characteristics of the Photoactive Yellow Protein;³⁵ (M. van der Horst et al., unpublished experiments).

Primary Events

Understanding the initial phase of the response of a photoreceptor to light absorption—which occurs on the femtosecond to nanosecond time scale—is a prerequisite for understanding the formation of its signaling state at the molecular level. The absorbed light-energy is initially, when the photoreceptor is brought to its Franck–Condon state, exclusively changing chromophore properties. Although multiple excited states may be involved, for this discussion we will assume that PYP will arrive within ultra-short time at an excited-state position from which it returns to a (transient) ground state (but note that barrier crossing does play a role even in the excited state⁴⁹ (M. van der Horst and L. L. Premvardhan, unpublished experiments). In an excited-state, various reaction pathways, such as isomerization (if a double bond is present) or electron and/or proton transfer can be initiated, but always the trivial nonradiative and radiative deactivation processes will take place in parallel.

For many photoreceptors, including PYP, photoisomerization has been identified as the primary photoreaction. However, the electronegativity of the groups linked to the two carbon atoms of the C₇=C₈ double bond of 4HCA is rather different.⁵⁰ Therefore, considerable charge transfer may occur during photoexcitation. In agreement with this, a very large excited-state dipole moment (i.e., 26 D) was measured in PYP, using Stark spectroscopy (Dr. L. Premvardhan, unpublished observation; note, however, that this value is significantly larger than the calculated value, e.g., of 9 D⁵¹).

The configurational change accompanying isomerization of 4HCA in PYP, of course, will lead to dramatic changes of the interaction of the chromophore with the shell of amino acids surrounding it. The initial isomerization event, typically occurring in the subpicosecond to few picoseconds time-range, in most photoreceptor proteins is accompanied by a red shift of the absorption maximum of the protein, and followed by rearrangements of the position and orientation of the surrounding amino acid side chains. This often leads to changes in the hydrogen-bonding interactions in the chromophore-binding pocket. In PYP such relaxation processes continue up to 10 ns after photoexcitation, while the actual photoisomerization is completed already in a few picoseconds (note, however, that further rearrangements can be detected in the protein, while the pR spectrum is retained, far into the microsecond time domain; see further below).

The study of subnanosecond events requires dedicated techniques. In the case of PYP, time-resolved and low-

temperature absorption and fluorescence spectroscopy and time-resolved and low-temperature X-ray diffraction analyses have been applied. The first two exclusively probe the chromophore. In the low-temperature experiments the conformational relaxation upon photoisomerization is interrupted at a specific stage (characterized by (an) intermediate(s) with specific spectroscopic and structural properties). For the time-resolved experiments, the so-called pump–probe measurement principle is used, where two very short pulses of electromagnetic radiation (e.g., visible or X-ray) impinge on the sample, such that the first one initiates the photoreaction and the second one is delayed and probes a specific property (absorption, fluorescence, or structure) at a known time after the excitation. The duration of the pulses limits and determines the time resolution in such experiments.

A complicated picture of the first part of the photocycle—the formation of pR—emerges from absorption and fluorescence spectroscopy performed at LT. In those experiments the photoreaction is typically triggered at 77 K with continuous irradiation, which leads to formation of (a) primary photoproduct(s). Subsequently, the sample is warmed gently, until a spectral change is observed. In such experiments, pR is formed via two parallel pathways with two intermediates each (Figure 3B). In one, the first intermediate shows a red-shifted absorption spectrum around 490 nm (A₄₉₀ or PYP_B^{33,52}). Such a red-shift is also observed in all other known photoreceptors with photoisomerization as the initial step (i.e., phytochromes and the retinal proteins from the rhodopsin family^{53–56}). It is followed by an intermediate with blue-shifted absorption, peaking around 400 nm (PYP_{BL}) similar to the BSI intermediate in rhodopsin⁵⁷ and transforms into pR at around 183 K.³³ By using various wavelengths of illumination, a second pathway was identified that forms pR via two intermediates with similar absorption maximum and slightly lower extinction coefficient (A₄₄₀ or PYP_H, and PYP_{HL}^{33,52}) as the ground-state pG. PYP_{HL} transforms into pR at about 193 K. This second and unusual pathway predominates at excitation wavelengths above 460 nm.

Recently, some aspects of the chromophore in the different LT intermediates were elucidated with the help of infrared difference absorption spectroscopy.⁴⁸ It was shown that for both branches trans-to-cis photoisomerization is the primary event, i.e., 4HCA is in cis-configuration in both PYP_B and PYP_H. In addition, the two intermediates share with pR a very similar hydrogen-bonding interaction of Glu46 with the negatively charged phenolic oxygen of the chromophore. From this one can conclude that the distance between the phenolic part of 4HCA and Glu46 does not change dramatically in all three intermediates, since the hydrogen bond remains intact during these transitions. This is only compatible with a photoisomerization mechanism with a concerted isomerization of the C₇=C₈-ethylene double bond and (a) neighboring single bond(s).⁵⁸ H/D substitution experiments revealed⁴⁸ that the major (structural) differences between the three intermediates are localized near the ethylene bond of 4HCA. This may be explained by noncomplete rotation of the ethylene bond and/or the adjacent single bond(s) in the different intermediates (see also below) or left- and right-hand rotations of the carbonyl moiety for PYP_H and PYP_B formation, respectively. Alternatively, it may be explained by two different orientations of Arg52, which might not be interconvertible at low temperature. NMR analyses have shown Arg52 to be present in two different orientations,³⁰ and a recent spectral-tuning study has shown the influence of the position of Arg52 on the absorption spectrum, in which it can induce either a blue or a red shift, depending on its position.^{29,59}

The continuous wave (CW) excitation light sources used in LT spectroscopy can cause a problem for the interpretation of these results, however. Since the ground state and the early intermediates have overlapping spectra, both are excited in parallel and may therefore all undergo photoreactions themselves. In fact, in many photoreceptors with an isomerizable chromophore the photo back-reaction, i.e. reformation of the ground state, has a higher quantum yield than the forward reaction.^{60–62} This may explain the excitation wavelength dependence described above, but surely it is the reason a third species, F_{430} , observed at 77 K and with a strong fluorescence-excitation maximum around 430 nm,⁵² is formed. Only the comparison with time-resolved measurements at room temperature (RT) will show whether the observed excitation wavelength dependence at low temperature is an intrinsic property of PYP or a consequence of photo back- and/or side-reactions.

Intermediates found with LT spectroscopy will not necessarily be observable in RT time-resolved experiments. In fact, while pR can be detected at LT and RT, only for PYP_B (i.e., only for one of the four LT precursors of pR), intermediates with similar absorption properties at RT have been identified with certainty. They were named I_0 and I_0^\ddagger , first found in a pioneering study covering the first 4 ns after excitation of PYP, while using discrete time points at 25 and 50 ns in a pump–probe absorption experiment.⁶³ I_0 has an absorption maximum near 495 nm and is formed within a few picoseconds.^{63–67} A relaxation process on the excited-state surface with a time constant of about 5 ps is also observed.^{64,66,67} I_0 transforms into an intermediate, I_0^\ddagger , which has a similar absorption maximum but a different absorption coefficient and a time constant of 200 ps.^{63,67} The absorption coefficient of I_0^\ddagger is smaller⁶³ or larger⁶⁷ compared to I_0 , depending on the photophysical model used to describe the experimental data. Finally, pR is formed from I_0^\ddagger within a few nanoseconds.^{63,66} These two transitions—most probably—reflect 4HCA relaxations and/or initial responses of the neighboring amino acids caused by the isomerized chromophore. This is supported by results obtained with several PYP mutants, in which formation of both I_0 and I_0^\ddagger is observed, but with a significantly altered rate of formation and decay in some of them.^{68–70}

In contrast to all previous investigations, the production of a RT intermediate similar to PYP_H was reported recently.⁶⁶ However, the experimental data presented in this study are clearly different from those of most other studies, in which no indications for the involvement of a PYP_H intermediate were obtained. In a recent study we have confirmed the one-path model of Ujj et al.⁶³ with—at least—two intermediates between the excited state and pR.⁶⁷ These intermediates (I_0 and I_0^\ddagger) are spectroscopically similar to PYP_B. By comparing femtosecond transient absorption measurements with excitation at 400 and 485 nm, it was tested whether the excitation wavelength dependence found at LT had its origin in a fundamentally different photophysical behavior at different excitation wavelengths, or in secondary photochemistry induced by the CW excitation used in the LT experiments. In fact, very similar absorption changes (with respect to spectra and quantum yield) in the region above 490 nm were observed in the ultrafast experiments at RT. The excitation-wavelength dependence at LT is therefore most probably caused by secondary photochemistry.

Polarization-sensitive ultrafast transient absorption spectroscopy has been used to investigate the time at which isomerization of the chromophore takes place after light absorption. The idea behind this experiment is that if the transition dipole

moment of a photointermediate and its parent state would be different, a decrease in the anisotropy (a function of the absorption measured with parallel and perpendicular observation light with respect to the linearly polarized excitation light) is expected. Indeed, it was found that the anisotropy decreases already for the first intermediate (formed within 3 ps), reflecting a change in transition dipole moment of about 25 degrees.⁶⁷ It then stays nearly constant for the next 500 ps. It is straightforward to explain these findings with the photoisomerization of 4HCA being the primary event of the PYP-photocycle and the subsequent events being mainly rearrangements of neighboring amino acids and their interactions with the chromophore.

It has to be noted that the different ultrafast transient absorption studies on PYP yielded—in part—very different experimental results.^{63–67} The differences in the time of formation of I_0 (below 1 ps^{64,66,67} and ~ 2 ps^{63,65}) and the presence/absence of an additional relaxation process around 5 ps^{63–67} can be explained reasonably well by the differences in time resolution of the various experiments. For the quantum yield of the I_0 formation, however, values between 0.2 and 0.55 have been reported. A closer look at the published experimental data reveals that not only different data analysis, but also obvious differences in the data itself are responsible for this wide range of reported quantum yields. The reason for these different findings is at present unknown.

Fluorescence analysis can be a very valuable tool for the investigation of the primary step(s) of a photoreaction, i.e., as long as excited states are involved. It reports not only about the formation of primary photoproducts—although in an indirect manner via the disappearance of the excited state(s)—but also makes it possible to identify reaction mechanisms involved, via the properties of the excited states themselves. The fluorescence spectrum of PYP (see Figure 1) has a maximum at 495 nm with a normal Stokes shift of 46 nm (2100 cm⁻¹). The fluorescence quantum yield—as for most photoreceptor proteins—is very low ($\sim 10^{-3}$ ^{52,71}). Nevertheless, it was possible to characterize the fluorescence of PYP with excellent spectral and temporal resolution. The fluorescence decays multiexponentially with the two fastest processes (time constants of 700 fs and 3–4 ps) being responsible for 80% of the total fluorescence.^{72–74} A third component with a decay time of 30 to 80 ps carries another 15% of the signal.^{73,74} The fluorescence decay characteristic was studied in detail as a function of temperature.⁷⁴ The subpicosecond process was found to have no activation energy, while the second and third component showed normal Arrhenius behavior with activation energies of 8 and 30 kJ mol⁻¹, respectively. The fast component was assigned to the formation of a twisted state of 4HCA, i.e., a noncomplete isomerization.^{72–74} This issue has been addressed recently by replacing the hydrogen atoms of the C₇=C₈ double bond by heavier and more bulky atoms.⁷⁵ No significant effect was found, ruling out a rotation around the C₇=C₈ double bond in this hypothetical twisted state.

In addition, a number of mutations in the 4HCA-binding pocket, as well as various chromophore analogues, were investigated. For all the mutants the subpicosecond component disappears (or slows down significantly).^{49,76} A chromophore analogue with a covalent bridge across the isomerizable double bond also completely lost the first two components.⁷³ The latter results suggest that the picosecond component in fluorescence reflects the formation of the isomerized photoproduct I_0 . The fluorescence experiments support the findings from the transient absorption studies, i.e., formation of the primary isomerized photoproduct I_0 within a picosecond and off-pathway relaxations

on a longer time scale. They also suggest that the protein environment accelerates the photoisomerization.

As described above, isomerization of the chromophore is not accompanied by dramatic movements of the phenolic part of 4HCA via rotation around the $C_7=C_8$ double bond. Isomerization is basically achieved via rotation of the carbonyl group of the chromophore, as first suggested on the basis of a LT FTIR study on PYP.⁵⁸ This can be achieved via isomerization around the $C_7=C_8$ double bond and the C_9-S_γ single bond simultaneously, i.e., from $C_7=C_8$ -trans C_9-S_γ -cis in pG to $C_7=C_8$ -cis C_9-S_γ -trans in pR. This is further corroborated by LT and time-resolved X-ray diffraction experiments.^{38–40} Although the interpretation of these X-ray data is difficult and complicated by the presence of mixtures of intermediates (e.g., through the existence of a photoequilibrium), the rotation of the carbonyl moiety is clearly observable. PYP was the first photoreceptor for which the structure of photocycle intermediates has been determined successfully. As new and improved data sets are expected to be available soon, we will not go into further detail here.

In summary, the primary event in the photoactivation of PYP is the photoisomerization of its coumaryl chromophore, which is completed within a few picoseconds. The state formed shows a red-shifted absorption spectrum and is named I_0 . From there, several relaxation processes of both the strained chromophore and the hydrogen-bonding network between the chromophore and the surrounding amino acids occurs, which is reflected in the $I_0 \rightarrow I_0^\dagger$ and $I_0^\dagger \rightarrow$ pR transitions. pR represents the relaxed PYP structure with the deprotonated chromophore in cis configuration (see also Figure 4). This structure, however, is only semi-stable and leads, e.g., to the displacement of Arg52, away from the chromophore and Tyr98, which is observed at a very early phase in the time-resolved X-ray diffraction experiments.⁴⁰ Protonation of 4HCA (supposedly by Glu46) and large conformational changes occur on a time scale, ranging between 3 and 7 orders of magnitude slower than pR formation. Details of these reactions will be discussed in the next section.

Signaling State Formation

The transition from pR to the signaling state pB consists of two distinct events, i.e., protonation of the chromophore and structural change of the protein. It has been demonstrated with FTIR spectroscopy that protonation of 4HCA precedes the majority of the structural changes in the protein.⁴¹ Transfer of a proton from Glu46 to the chromophore leads to formation of the intermediate pB', that has very similar spectroscopic properties in the visible part of the spectrum as pB (Hendriks et al. *Biophys. J.*, in press). Presumably, this is the reason pB' was not clearly identified with UV/Vis spectroscopy earlier, although the existence of an intermediate with spectroscopic properties similar to those of pB was proposed on the basis of the biexponential character of pB formation.² The pB' intermediate differs from pR only in one respect, i.e., whereas in pR a buried negative charge resides on 4HCA, where it can be effectively stabilized via delocalization of the charge over the conjugated system of the chromophore, via the hydrogen-bonding network with Glu46 and Tyr42, and via the vicinity of the positive charge from Arg52, in pB' this buried negative charge resides on Glu46 where it no longer can be effectively stabilized. This is a stressful situation that can be resolved in two different ways: (i) Glu46 can be protonated again by the 4HCA, i.e., return to the pR state, or (ii) formation of pB. In this latter process the chromophore becomes exposed to solvent, which can be accompanied by a large structural change of the

protein. The extent of this structural change may then be dependent on whether Glu46 becomes protonated in this step. This latter concept is supported by the observations on the Glu46Gln mutant protein.⁴¹

In a recent study exploring the kinetic deuterium isotope effect in PYP (Hendriks et al. *Biophys. J.*, in press), a photocycle model incorporating these possibilities (Figure 4) was tested and found to fit the data very well. In this model the reversible character of the pR-to-pB' transition explains the biexponential character of pB formation observed before. However, the existence of an alternative route, in which pB is formed directly from pR, and where the chromophore is not protonated by Glu46, cannot be disregarded. It then most likely depends on the measurement conditions (such as ambient pH, etc.) which route is preferred for the formation of pB. Nonetheless, for most measurement conditions, protonation of the chromophore by Glu46 is the most likely route.

Many experiments have been carried out to characterize the structural change that occurs upon formation of pB. It must be noted, however, that a major structural change is observed only for PYP in aqueous solution. For PYP in a crystalline matrix,³⁷ at low temperature,⁴⁶ and in PYP films with reduced hydration,³⁵ much less structural change is observed. In a temperature-dependent study of the photocycle kinetics it was shown that recovery of the ground state exhibits clear non-Arrhenius behavior.³² This was later explained as being caused by changes in the heat capacity of the system of protein plus solvent, that accompany the partial unfolding of the protein, through application of a model derived to explain non-Arrhenius kinetics in protein folding.³⁴ In this approach the (simplifying) assumption is made that all thermodynamic parameters are independent of temperature.

From this difference in heat capacity, the surface area of exposure of previously buried hydrophobic residues to aqueous solvent, upon formation of pB, can be calculated. In contrast to this, the same analysis applied to a mutant protein with the first 25 N-terminal residues genetically deleted ($\Delta 25$ -PYP) shows close to normal Arrhenius behavior.²⁴ This indicates that the N-terminus of PYP is largely responsible for the observed partial unfolding (i.e., the large structural changes) of the protein.

An alternative way to study exposure of hydrophobic surface is via the use of fluorescent probes sensitive to the hydrophobicity of their environment. Two different probes have been used to study PYP, 8-anilino-1-naphthalene-sulfonate,⁷⁷ and Nile Red.⁷⁸ For both, it was shown that they transiently bind to pB. However, only for Nile Red was a possible binding site determined. Interestingly, Nile Red does not bind to the pG-nor to the pB'-state of PYP; it binds only to pB. Furthermore, analysis of $\Delta 25$ -PYP shows that Nile Red binding is not sensitive to the structural change in the N-terminus of the protein. Therefore, also considering available NMR results,⁷⁹ it was concluded that Nile Red must bind close to the 4HCA-binding site.

In an NMR study of the pB intermediate (i.e., in aqueous solution) it was shown that pB exhibits structural and dynamic disorder with respect to the ground state.³⁶ A subsequent NMR study on pB_{dark} (i.e., the unfolded state that is formed at very low pH) formation⁷⁹ showed that the photocycle intermediates pB and pB_{dark} have several features in common. It was also revealed that upon formation of pB_{dark} the backbone of the protein could be divided into three parts: a relatively stable core (residues 32–41, 80–94, and 113–122) and two areas that display large structural perturbation. These latter are the N-terminal helices (residues 6–18 and 26–29) and the area

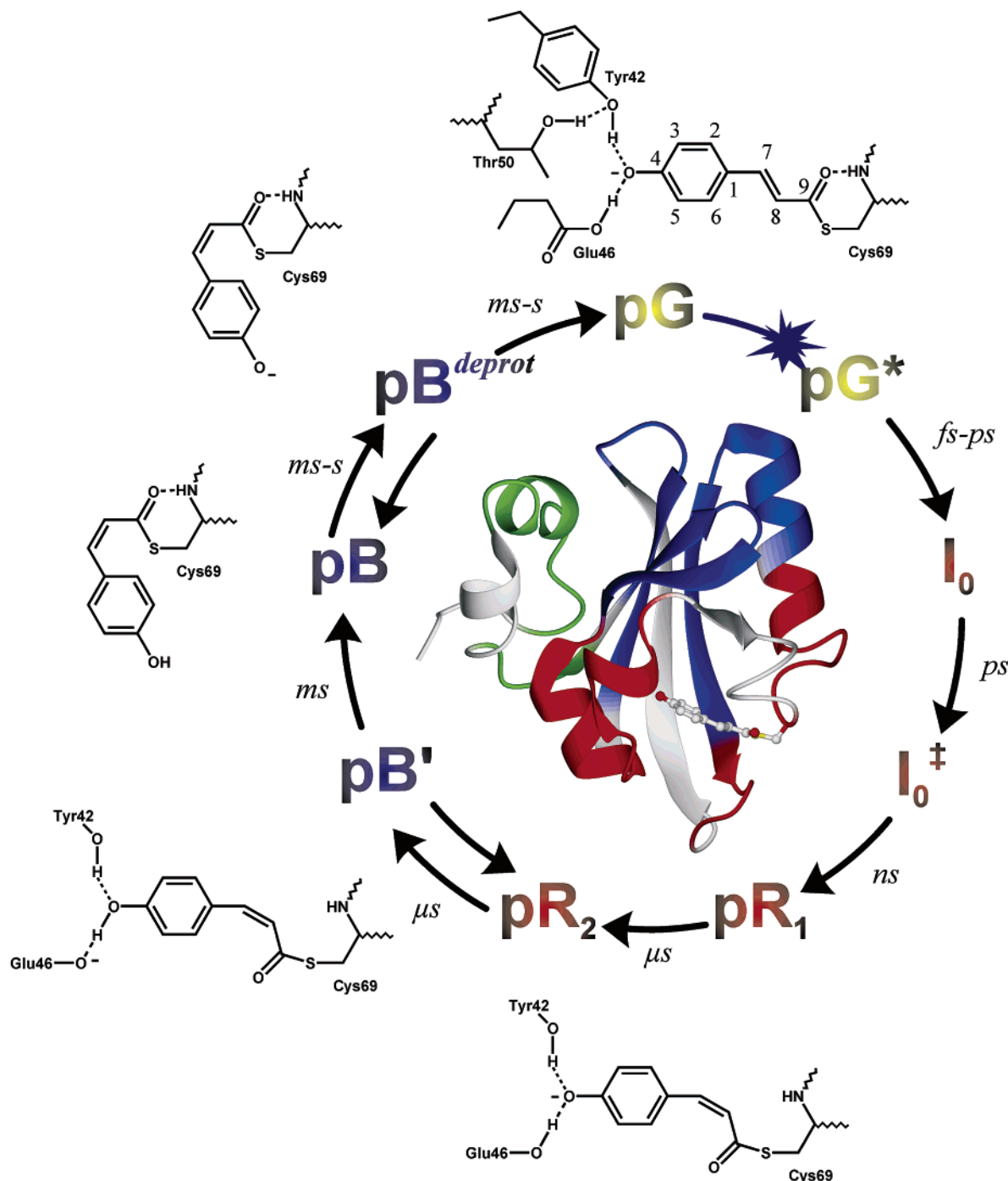


Figure 4. Detailed model of all distinguishable steps (with transient UV/Vis spectroscopy) in the photocycle of PYP. The relevant changes in the configuration of the chromophore and in the surrounding functional groups, at the various steps, are indicated in the structural diagrams. The inner part of the figure shows the structure of PYP, color-coded according to the extent of structural change in pB, as measured with NMR.⁷⁹ Atom numbering of the carbon atoms of the chromophore is given in the inset corresponding to pG. Color code: green: large structural change in the N-terminal domain; red: identifiable residues with large structural change surrounding the chromophore; blue: no structural change. For further details: see text.

around the 4HCA-binding site (residues 42–58, 69–78, and 95–100). As discussed above, the structural perturbations of the N-terminus are largely responsible for the observed non-Arrhenius behavior of the photocycle kinetics of PYP. The structural perturbation around the chromophore-binding site is most likely monitored by the hydrophobicity probe Nile Red (see above). Furthermore, the NMR data suggest that the pB intermediate is a mixture of (a) structurally perturbed form(s) and a form structurally similar to pB formed in crystalline PYP.³⁷ This model is supported by the results obtained with

Nile Red⁷⁸ and by a molecular dynamics study on the crystal structure of pB in a box of water molecules.⁸⁰

Significant structural changes have also been detected by means of circular dichroism (CD) and optical rotary dispersion (ORD) spectroscopy in the far UV and visible spectral region. A decrease in secondary structure was detected for both, wt-PYP at pH 4⁷⁷ and a mutant (M100L),⁸¹ in CD experiments exploiting the enrichment of pB under blue light illumination. We have recently performed a study combining CD and time-resolved ORD spectroscopy on wt-PYP at pH 8 in which we

confirmed this loss in secondary structure (Chen et al. *Biochemistry*, in press). In addition, we could identify this loss as a decrease in α -helix content. More importantly, we have investigated the time course of the change in secondary structure, which revealed—as in the case of the FTIR and Nile Red experiments—that only pB, but not the precursor pB', shows this structural change. Time-resolved ORD measurement in the visible spectral region, which monitors the conformation of 4HCA, shows that a large rearrangement of 4HCA takes place in a few microseconds. This transition is hardly reflected in the UV/Vis spectrum of pR, but may reflect a very late rearrangement of the latter.

Another way to monitor structural change within the protein is to study hydrogen/deuterium (H/D) exchange (rates). Exchangeable protons are abundant and can be divided into three groups: (1) protein backbone amine protons, (2) protons that are part of a (de)protonatable group and thus may not always be present, and (3) amino acid side chains with exchangeable protons not part of group 2. Exchangeable protons can be exchanged by other protons (i.e., hydrogen atoms) and by deuterium and tritium atoms, thus making it possible to monitor the exchange with various techniques. A buried exchangeable proton may take hours to days to exchange, whereas an exposed exchangeable proton may equilibrate with solvent within seconds or even faster. Exchangeability is influenced, e.g., by formation of hydrogen bonds, solvent accessibility, local secondary structure, and protein dynamics.⁸² PYP contains 235 exchangeable protons, 42 of which are from (de)protonatable groups. In a H/D exchange study where Electrospray Ionization Mass Spectrometry was used to monitor progress of the H/D exchange,³⁵ it was shown that in the PYP ground state 177, in pB 193 and in apo-PYP 217 hydrogen atoms were exchanged for deuterium after 30 min. It is clear that in pB more exchangeable protons are exposed to the solvent than in the pG state of PYP, confirming that a major structural change occurs within the protein upon formation of pB. In apo-PYP 29, exchangeable protons resist exchange. This implies that 29 or less of the 42 (de)protonatable groups are deprotonated. Also, a certain degree of stable structure may still be present in apo-PYP. The light-induced increase in the extent of H/D exchange was confirmed with FTIR difference spectroscopy.³⁵ These experiments, however, provide only a global picture of the rearrangements in the structure of PYP upon formation of pB and are unable to pinpoint specific parts of the protein responsible for the light-induced difference in H/D exchange. By monitoring the H/D exchange rate with ¹H NMR, a more precise picture was obtained.⁷⁹ Though only information for the exchange rate of the backbone-amide hydrogen of 51 (out of the 125) residues was obtained (in part because only a limited time-window of exchange rates was accessible), 14 of these showed a significant change (i.e., increase) in the exchange rate upon formation of pB. The latter number is only 2 smaller than the number predicted on the basis of the mass spectrometry experiment described above, which was not limited to just the backbone-amide hydrogen atoms. The backbone-amide hydrogen atoms with the most significant increase in exchange rate are from amino acids Phe28, Glu46, and Thr70. The latter two are close to the chromophore, whereas Phe28 is close to Glu46.

By monitoring pH changes as a function of signaling state formation it is possible to obtain information on uptake/release of hydrogen atoms from (de)protonatable groups. When the apparent pK_a of such a group changes upon formation of the signaling state, it is possible that a net proton release into the solvent or proton uptake by the protein takes place, resulting in a small change in the pH of the solution. In such experiments,⁴³

only (de)protonatable groups will be detected that undergo a significant change in their immediate surroundings, e.g., go from a buried state to a solvent-exposed state. For the Photoactive Yellow Protein, net proton uptake is observed at low pH and net proton release at high pH, with no or little change around the transition point at pH 7.8. Furthermore, in early experiments it has already been shown that the proton uptake events follow the formation and decay of the signaling state pB, and not that of the pB' intermediate.⁸³ This is consistent with the assumption that these proton uptake/release events are linked to altered exposure of (de)protonatable groups, rather than due to proton uptake by the chromophore upon formation of pB. In agreement with this, it was observed, using the His108Phe mutant protein, that residue His108 is involved in net proton uptake events around pH 6.6.⁴³ Above, it was already described that Glu46 donates a proton to the chromophore upon formation of pB'. Therefore, this reaction does not lead to net proton uptake nor to release. This route of intramolecular proton transfer, however, is not possible in the mutant protein Glu46Gln. In this mutant protein an increased net proton uptake is observed around pH 7,⁴³ which may indicate that 4HCA under these conditions is protonated via solvent.

In a recent study, the kinetics of proton uptake was again determined for wild-type PYP and also for the mutants Glu46Gln and Glu46Ala, using pH indicator dyes.⁸⁴ Proton uptake was monitored at pH 6.2 for wild-type PYP and the Glu46Gln mutant. The measured proton uptake was ascribed to (direct) protonation of the chromophore by the solvent. Elsewhere, however, it has been argued that, at this pH, net proton uptake is largely caused by protonation of His108.⁴³ No further experiments are reported in Borucki et al.⁸⁴ on the pH dependence of the net proton uptake signal. Measurements at higher pH (i.e., 8.3) were exclusively performed for the Glu46Ala mutant protein. Under these conditions, net proton uptake was also observed, a phenomenon that, however, was not observed with wild-type PYP at pH 8.⁴³ Borucki et al. furthermore conclude that proton uptake and the UV/Vis transition reflecting pB formation occur exactly in parallel. This, however, is in contrast to the earlier report of Meyer et al.⁸³ Binding of the pH indicator dye bromocresol purple to PYP was also observed, but could be distinguished from the effects of pH changes in the solvent, which are considerably faster. These observations are in agreement with the notion that chromophore protonation precedes structural change.

As mentioned before, the extent of structural change upon formation of the signaling state pB depends significantly on the measurement conditions (or the mesoscopic context of the protein) selected for measurements. Water may play an important role in this. Only at maximal water activity are major structural changes observed. Furthermore, residues Glu46, Arg52, and His108 have been shown to have an influence on the extent of structural change. Both the Glu46Gln⁴¹ and His108Phe⁴⁶ protein show a reduced extent of structural change upon formation of pB (as compared to the wild-type protein and based on the Amide-I signals in FTIR difference spectra). Both residues have been shown to change their state of protonation upon formation of pB. Furthermore, Arg52 also affects the extent of structural change, as a function of the anion composition of the solution (e.g., through the addition of citrate⁸⁵). It therefore seems that protonation changes of specific residues can influence the extent of structural change. As water is likely involved in such protonation changes, it is not surprising that the state of this water also has an influence on the extent of the structural change. In fact, a structural water molecule

detected near His108 in the crystal structure of the ground-state pG has been found to be involved in the structural change upon formation of pB.⁴⁶ Since the protonation state of specific residues is involved, pH will also have an influence on the extent of structural change. This is confirmed, e.g., by the pH dependence observed in the Nile Red probe binding experiments that were recently reported.⁷⁸

Ground-State Recovery

During recovery of the ground state of the Photoactive Yellow Protein, several processes have to occur. The chromophore has to re-isomerize to the trans configuration, the state of protonation of several residues and of 4HCA has to change, and the protein has to return to its ground-state fold. Though these are seemingly distinct steps, until recently they have appeared to occur simultaneously, with re-isomerization of 4HCA presumably being the rate-determining step. Via light-induced isomerization of the chromophore from cis to trans, in a photocycle branching reaction, a 1000-fold increase in the rate of recovery to pG can be achieved.⁸⁶ It is important to keep in mind that the existence of this photocycle branching reaction can influence data acquisition significantly. Specifically, the rate of ground-state recovery can be influenced by the presence of actinic- or probe light, that can be absorbed by any of the pB intermediates.⁸⁷ A dramatic example of this is shown by the Met100Ala derivative of PYP, for which the light-activated recovery rate is 6 orders of magnitude faster than dark thermal recovery.²³ In this protein, ambient light—even at moderate latitudes—already drives a considerable fraction of the protein in the pB form. In the branching pathway in wild-type PYP an additional intermediate, pB^t, was observed to form instantaneously on a nanosecond time scale, which then relaxes to the pG state on the microsecond time scale. This intermediate differs from pB only in the configuration of 4HCA. This suggests that a large change of the protein fold, and of the state of protonation of several residues, can be achieved quickly, once the chromophore has been isomerized. Interestingly, refolding kinetics of denatured PYP molecules show the same order of magnitude difference between refolding of denatured PYP molecules with the chromophore in the trans and cis states, respectively,^{88,89} as the rate observed for the difference in recovery via the photoinduced branching and the dark reaction of the photocycle, respectively.

For re-isomerization to take place at the observed rates the energy barrier for cis-to-trans isomerization of 4HCA will have to be lowered significantly. It has already been shown that when the chromophore is deprotonated, isomerization can proceed much faster.⁵⁰ It is therefore likely that deprotonation of 4HCA will precede re-isomerization (Figure 4). Chromophore deprotonation has been shown to significantly shift the absorption spectrum of pB to the red.⁴³ Until recently, no red-shifted intermediate has been observed in the recovery reaction of pG, the ground state of PYP. Therefore, the proposal that re-isomerization of the chromophore would be preceded by deprotonation has been under debate. Recently, in measurements of the kinetic deuterium isotope effect of the recovery reaction of PYP (Hendriks et al. *Biophys. J.*, in press), we obtained preliminary evidence that indeed deprotonation of 4HCA occurs before re-isomerization. From these measurements it has also become evident that the relevant intermediate, pB^{deprot}, which is formed upon deprotonation of the chromophore in pB, may have an absorption spectrum very similar to that of pG. This makes it understandable why this intermediate has not been observed before during studies of ground-state recovery.

Besides the protonation state of 4HCA, it is also important for the protein to have the correct fold in order to facilitate re-

isomerization. This is evident from the fact that at very high pH, where the chromophore can easily become deprotonated and a pB^{deprot}-like intermediate actually does accumulate,⁴³ recovery of the ground state slows down. Under these latter conditions, recovery of the ground state of PYP will be under kinetic control of the PYP protein. This conclusion is in agreement with the observed heterogeneity of the pB structure (as evidenced by the NMR results^{36,79} and corroborated by Nile Red probe binding experiments⁷⁸) at high pH. Because little structural change occurs upon formation of pB in crystalline PYP³⁷ and the photocycle recovery kinetics in crystalline PYP are faster than in aqueous solution,⁴¹ it is likely that a relatively folded state of pB is required to facilitate re-isomerization of 4HCA. Exchange between such a folded state and (the) relatively unfolded state(s) of pB in solution, is then a possible mechanism to exert kinetic control over ground-state recovery. At present, it is unclear how the protein-fold of pB, in combination with a deprotonated chromophore, facilitates re-isomerization of the chromophore, but Met100 may have an important role in this process.^{23,90}

Photoacoustic and Photothermal Analyses

Nonradiative processes are not always easily analyzed via absorption or emission techniques. Though they may coincide with events observed via absorption and emission techniques, this is not necessarily the case. These nonradiative processes can be followed in a time-resolved manner by photoacoustic and photothermal methods,^{91–95} that record among other phenomena the amount of heat released, i.e., the change in enthalpy. The time resolution of this technique covers the range from picoseconds to seconds, depending on the particular variant selected. The method is very sensitive since the energy released in nonradiative deactivation processes can easily amount to 50–95% of the energy absorbed. Although this principle has been known for a long time, only a few experimental setups have been established in the past thirty years. Three of these have been used to characterize the enthalpy changes during the PYP photocycle, in the pG-to-pR transition, i.e. photoacoustic spectroscopy (PhAS, also named light-induced optoacoustic spectroscopy (LIOAS)), thermal grating (TG), and thermal lensing (TL). All three methods suffer from the fact that processes other than heat release contribute to the signal generated, such as structural volume changes (PhAS, TG, TL) and absorption changes (TG, TL). One can, however, separate the different contributions to the signal to obtain the enthalpy change and additional information from the structural volume changes. The latter reflect alterations of bond length, solvation, protonation state, and interactions of the chromophore with surrounding amino acid side chains. Under certain circumstances, the structural volume change can be related to the difference in entropy between two photocycle intermediates.^{96,97} The PhAS and TG studies performed on the Photoactive Yellow Protein used nanosecond time-resolution. Therefore, only information about the pR intermediate was obtained, with regard to the first step of the photocycle. Information on intermediates between the ground state and pR will require the use of picosecond time resolution.

In the first photoacoustic investigation reported the energy content of the pR intermediate was determined as 120 kJ mol⁻¹. The formation of pR is accompanied by a large negative volume change (−23 Å³).⁹⁸ For the estimation of the two values it was assumed that the photocycle quantum yield, enthalpy, and structural volume changes are temperature independent. This is the standard procedure in PhAS and TL studies^{91,95} and was

found to apply to many different molecules. In a second study, PhAS was applied in combination with TG.⁹⁴ With the TG technique, it is possible to separate the heat dissipation in the first step of a reaction (i.e., from pG to pR for PYP) from the other contributions to the TG signal. This is because of the orders of magnitude faster heat diffusion compared to the lateral diffusion of the molecule under study.⁹⁹ In this way the structural volume change could be determined without the necessity to assume its independence of temperature. A value of -12 \AA^3 was obtained at 293 K; i.e., also a contraction, but only of half the size as calculated from the results obtained with PhAS by van Brederode et al.⁹⁸ In addition, an energy content of 160 kJ mol^{-1} was obtained for pR at this temperature. The structural volume change of pR formation was observed to be temperature dependent. With decreasing temperature, the magnitude of the contraction increases. At 273 K it amounts to -25 \AA^3 , a value very similar to the one measured with PhAS.⁹⁸ The TG signal was not large enough at temperatures lower than 293 K to decide whether the energy content of pR is temperature dependent as well. Because of the temperature dependence of the structural volume change, the initially estimated energy content of pR (120 kJ mol^{-1} ⁹⁸) is valid only at temperatures near $T_\beta = 0$ (i.e., 273 K). Furthermore, the difference between 160 kJ mol^{-1} and 120 kJ mol^{-1} may indicate a modest temperature dependence of the enthalpy of pR. The unusual and strong temperature dependence of the structural volume change is attributed to changes in the void volume of PYP or to a change in interactions of certain amino acid residues with 4HCA and/or the solvent in the pG-to-pR transition.

TG and PhAS measurements have also been performed on the free chromophore, 4-hydroxycinnamic acid.¹⁰⁰ It was revealed that the cis configuration has about a 50 kJ mol^{-1} higher energy content than the trans configuration. Also a volume change of -1.2 \AA^3 was observed upon trans-to-cis isomerization. This is much smaller than the volume change observed in PYP. However, a direct comparison between the free chromophore and the protein is difficult, since in measurements on the free chromophore the 4HCA was protonated, whereas it is deprotonated in the protein during the isomerization reaction. The large structural volume change in the protein is most probably directly monitoring the changes in the hydrogen-bonding network of 4HCA with the neighboring amino acids, which is absent for the free chromophore. The reason for the large temperature dependence of the structural volume change of pR formation is unknown, but it is unlikely that this should be attributed to the chromophore itself. Terazima and co-workers conclude from their studies that pR and pB are partially unfolded and that large structural rearrangements take place during the formation of these intermediates.¹⁰¹ Considering all the evidence available from spectroscopic and structural characterization of transient intermediates of PYP, this conclusion may not be warranted for pR. It must be kept in mind that a "large" structural volume change may still be smaller than a water molecule. The change of a few hydrogen bonds already leads to similarly "large" structural volume changes.^{96,102} The nature of the structural volume change of PYP during its photocycle, in particular of the early photocycle intermediates, has to be investigated in more detail in the future.

The energy content of pB was also determined with the TL technique. A value of as 60 kJ mol^{-1} was obtained (under the assumption of a temperature-independent structural volume change in the pR-to-pB transition).¹⁰¹ This result is, however, not very informative, since the temperature independence of the structural volume change in the pR-to-pB transition is

unlikely, because of the observed strong temperature dependence of the structural volume change in the pG-to-pR transition. Nevertheless, the obtained value is strikingly similar to the energy difference between the cis and trans configurations of the free (protonated) chromophore.¹⁰⁰ That would indicate that the large conformational change in pB does not lead to storage of (free) energy in the PYP protein. Interestingly, the TL signal showed kinetics for pB formation similar to that of absorption spectroscopy, i.e., the heat release is coupled to the absorption changes.

Recently, new results about the time scale of pR formation have been obtained. While pR formation is completed within 10 ns according to time-resolved absorption spectroscopy,⁶³ it extends to several microseconds as measured with PhAS (Gensch et al., unpublished results) or TG.¹⁰¹ Transitions on this time scale have also been detected by visible absorption³ (Chen et al. *Biochemistry*, in press; Hendriks et al. *Biophys. J.*, in press) and optical rotary dispersion spectroscopy (Chen et al. *Biochemistry*, in press). To account for these "late" changes in the pR spectrum, the states pR₁ and pR₂ have tentatively been introduced into the summarizing photocycle scheme shown in Figure 4.

Future studies on wild-type PYP and mutant derivatives thereof will have to reveal further detail about the optical, energetic, and structural changes that underlie the photocycle of PYP.

Comparison of PYP with Other Photoreceptors

PYP shares several homologies in spectroscopic properties, as well as in mechanistic aspects of its function, with other photoreceptors. The photoisomerization of PYP is—as for the classical photoreceptors for vision in animals and eukaryotic microorganisms (the rhodopsins^{102,103}), and for light-quality analysis in plants and algae (the phytochromes^{104–106}) and their recently found counterparts in bacteria (i.e., sensory rhodopsins,^{107–109} ion pumps (bacterio- and halorhodopsins),^{110,111} and bacterial phytochromes¹¹²)—the primary step in the photoreaction that initiates signal transduction and leads to the physiological response. Another common property is the ultrafast formation, upon excitation, of transient ground-state intermediates with red-shifted absorption spectra (compared to the parent state) and the appearance of intermediates with blue-shifted absorption spectra (compared to the parent state) at later stages of the photoreaction, which often represent the signaling state of the photoreceptor. Intramolecular proton transfer is another feature of PYP shared with the rhodopsins and sensory rhodopsins; whereas light-induced pK changes are also intrinsic to the photocycle of (bacterio)phytochromes.^{113,114}

But also significant differences exist: PYP is not an integral membrane protein as the rhodopsins, sensory rhodopsins, and rhodopsin-like ion pumps. Furthermore, the structure of the *p*-coumaryl chromophore of PYP is very different from both phytychromobilin and Schiff-base linked retinaldehyde. The conformational changes during the photocycle of PYP presumably are much larger than those that occur during the photoreaction of phytochrome¹¹² and the very subtle rearrangements in the (sensory) rhodopsins.^{107,109,115}

The PYP crystal structure is the prototype of the PAS-domain family, which can be found in proteins involved in very different intracellular signal transduction processes in all kingdoms of life. The supposed function of PAS domains ranges from cofactor binding to the facilitation of dimmer formation. It is interesting to note that the PAS domain is also present in other photoreceptors. Phytochromes contain PAS-domains (with no cofactor bound and therefore colorless) while the phototropins^{116,117}—a

recently established new class of blue-light photoreceptors from plants and algae—have two PAS domains, both binding a flavin chromophore as (blue) light-absorbing cofactor. One can speculate that in all these three photoreceptor families (i.e., including the xantopsins) the PAS domain (besides binding the chromophore) is involved in a similar (and more general) way into the signal transduction process to (a) downstream partner protein(s).

Challenges & Prospects

Because of its exceptional physicochemical- and photostability¹⁷ and the ease with which it forms well-diffracting crystals, e.g., ref 31, PYP has become a very attractive model system for studies of the primary photochemistry of light sensing and for studies of (functional) protein folding processes. This suitability as a model system subsequently was the incentive to try out several theoretical and experimental approaches on PYP, e.g., ref 39. The combination of these two facts has provided the wealth of information available regarding the physicochemical basis of PYP functioning. Nevertheless, regarding several aspects, significant further progress can be anticipated in the years to come:

(1) *Primary Events.* Quantum chemical calculations on PYP become more and more refined, to the extent that they may become useful for the prediction of absorption spectra and the initial trajectory of the photoisomerization process. In most cases, a combined quantum chemical/molecular mechanics approach is used. The α -symmetry of the visible absorption band of PYP, however, still remains to be firmly established. Although it is often explained as being caused by a vibronic sideband, further experiments may be useful to ascertain whether it is due to more than one optical transition.

(2) *Smaller, Faster.* Further application of femtosecond spectroscopy will be required to unravel the details of the primary photochemical events that are initiated by blue photons in PYP. Not only studies using visible, but particularly also vibrational spectroscopy in the mid-infrared region of the spectrum will prove rewarding. In this latter region, many functional groups of the chromophore show typical absorbance bands (e.g., the phenolate ring). Such studies can be performed in a visible pump-IR probe mode, but several alternative modes can be thought of as well. In addition, ultrafast time-resolved resonance Raman studies of photocycle intermediates will provide crucial insight into the correlation between PYP color (i.e., spectra) and the chemical structure of the chromophore during the primary photochemical transitions. Vibrational spectroscopy eventually may turn out to be better suited to improve our insight into the structural basis of primary events in PYP than structural techniques. Nevertheless, because of the power of polarization spectroscopy, it is a challenge to carry out part of these experiments in (crunched) PYP crystals.⁴¹

(3) *Catalytic Activity: Proton Transfer and Chromophore Re-isomerization.* There are two specific aspects in the PYP photocycle that are of interest to enzyme catalysis in general, and of which the atomic mechanism still remains to be resolved. The first is the intramolecular proton transfer from Glu46 to the phenolate moiety of the chromophore. This clearly is not an excited-state process but occurs in parallel to the pR-to-pB transition. Recent molecular dynamics calculations indicate that the nonplanarity of the chromophore plus the dynamical fluctuations in the pR state of PYP actually allow a downhill proton transfer to the chromophore.⁵⁹ The second is the dark-catalyzed re-isomerization of the chromophore from its *cis* to the *trans* configuration. As is argued above, the detailed

arrangement of functional groups of apo-PYP around the C₇=C₈ bond will be crucial in this process. Recent evidence has revealed⁹⁰ that the electronegativity of the residue at position #100 in the amino acid sequence of PYP is crucial for this. It is proposed that this is mediated through its interaction with a titratable group that is in hydrogen-bonding contact with the chromophore, presumably Arg52.

(4) *Tracking All Atoms of PYP during the Entire Photocycle.* The ultimate challenge regarding PYP is to give an *atomic* description of its functional dynamics. The application of time-resolved X-ray diffraction is the most direct way to achieve this.⁴⁰ However, before the results of this technique can be used to generate a “film” of the events occurring upon light activation of PYP, more detailed (spectroscopic) experiments will have to be performed to identify all relevant intermediate states, and their kinetics, in this process. Because of the dependence of the transitions in PYP on the mesoscopic context of the protein, it will be relevant to study the structure of the photocycle intermediates of PYP also with multinuclear NMR and time-resolved FTIR. The first of these suffers from a limited time window, the second from the assignment problem. For all these approaches it is important to back-up and refine the results with molecular dynamics calculations. However, particularly the results of time-resolved diffraction experiments, on the other hand, may be useful as a calibration set to lengthen the time domain that can be covered by molecular dynamics calculations.

(5) *Small-Angle Scattering Experiments on PYP and Its Radius of Gyration.* Sparked by the observed dependence of the degree of unfolding of the signaling state of PYP on the mesoscopic context of the protein, an intense interest has emerged to measure the conformation of transient intermediates of PYP under various conditions. Because of this, a number of groups have initiated the application of small-angle scattering analyses, using X-rays or neutrons. Both techniques can be applied in aqueous solution and hold promise to resolve molecular shape^{118,119} of the relevant intermediates at significant resolution.

Application of these techniques so far, however, has produced some confusing results. Although under some conditions an increase in the hydrodynamic radius of PYP, in particular in some of its mutant derivatives, was detected upon formation of the pB state,⁸⁵ in our own studies, neither with X-ray nor with neutron scattering, a significant increase in the apparent radius of gyration was detected (Hendriks et al., unpublished experiments). Surprisingly, a similar discrepancy can be noted when the difference in apparent radius of gyration of the pG and pB states of PYP are calculated from results obtained from NMR and photo acoustic spectroscopy, respectively. Using photo-thermal beam deflection, Terazima's group observed¹⁰¹ an increase of 20% in the pG-to-pB transition. In ¹H NMR experiments, however, no significant increase in the coefficient for lateral diffusion (and neither therefore in the apparent radius of gyration) was detected upon activation of PYP to the pB state (D. R. A. Marks and R. Kaptein, unpublished experiments). Whether these observations have to be explained by failing assumptions in the theoretical derivations, by subtle differences in experimental conditions, or any other factor, remains to be determined in future experiments. Nevertheless, such experiments will teach us more about both the functioning of PYP and the biophysical techniques available to characterize this small but interesting photoreceptor protein.

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