Kinetics of and intermediates in a photocycle branching reaction of the photoactive yellow protein from *Ectothiorhodospira halophila*

Johnny Hendriks[a], Ivo H.M. van Stokkum[b], Wim Crielaard[a], Klaas J. Hellingwerf[a],*

[a]*Laboratory for Microbiology, E.C. Slater Institute, BioCentrum, University of Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands*
[b]*Department of Physics Applied Computer Science, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands*

Abstract We have studied the kinetics of the blue light-induced branching reaction in the photocycle of photoactive yellow protein (PYP) from *Ectothiorhodospira halophila*, by nanosecond time-resolved UV/Vis spectroscopy. As compared to the parallel dark recovery reaction of the presumed blue-shifted signaling state pB, the light-induced branching reaction showed a 1000-fold higher rate. In addition, a new intermediate was detected in this branching pathway, which, compared to pB, showed a larger extinction coefficient and a blue-shifted absorption maximum. This substantiates the conclusion that isomerization of the chromophore is the rate-controlling step in the thermal photocycle reactions of PYP and implies that absorption of a blue photon leads to *cis* → *trans* isomerization of the 4-hydroxy-cinnamyl chromophore of PYP in its pB state.
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Key words: Transient spectroscopy; Photocycle intermediate; Branching reaction; Blue light; Photoactive yellow protein; Global analysis

1. Introduction

The photoactive yellow protein (PYP), first identified in the purple sulfur bacterium *Ectothiorhodospira halophila* [1], is a blue light photoreceptor and a member of the family of the xanthopsins [2]. *E. halophila* shows a negative phototactic response to blue light, and it has been proposed that PYP is the photoreceptor facilitating this negative tactile response, on the basis of single cell behavioral analyses [3]. Other photoreceptors involved in bacterial phototaxis are the well-studied sensory rhodopsins from the archaeon *Halobacterium salinarum* (see e.g. [4]).

The photocycles of PYP and the sensory rhodopsins can be divided into two stages. In stage one, light excitation of the ground state of the photoreceptor protein quickly leads to the formation of a relatively stable photocycle intermediate (usually a microsecond event [5,6]), which functions as a signaling state [7]. The relative stability of this signaling state is important since this will give the cell enough time to read this state and pass on a signal through a signal transduction pathway. This first stage is initiated by photoisomerization of the chromophore of these photoactive proteins [8–10]. In stage two of the photocycle, the protein recovers slowly to its ground state structure (in most cases a millisecond event (e.g. [6,11]). During this stage the chromophore re-isomerizes, in a dark reaction, by the auto-isomerase activity of the photoreceptor protein.

Re-isomerization of the chromophore can also take place via photoisomerization. This manifests itself as a branching reaction. For three members of the bacterial rhodopsin family, bacteriorhodopsin, halorhodopsin and sensory rhodopsin I, branching reactions have already been characterized [12–14]. Besides their biological function (such as the generation of a repellent signal in sensory rhodopsin I [14] or the fine-tuning of transmembrane ion gradients for bacteriorhodopsin and halorhodopsin (e.g. [15])), these branching reactions can also be useful in materials science applications (particularly of bacteriorhodopsin), such as in optical memories and optical switches [16]. The branching reaction in sensory rhodopsin I, which branches from the attractant signaling state by blue light excitation of the photoreceptor protein, does result in a faster recovery of the ground state, although its kinetics are still in the millisecond time scale [11].

In PYP a branching reaction has also been identified [17,18], but this reaction pathway has not yet been characterized in detail. Here we report more detailed studies of this photocycle branching reaction of wild type *E. halophila* PYP, based upon laser-induced flash photolysis experiments. The results obtained not only show a 1000-fold increase in the speed of recovery when PYP progresses through the branching pathway, but also reveal a new photocycle intermediate that is formed transiently in the branching pathway.

2. Materials and methods

2.1. Materials

Recombinant apoPYP was produced heterologously in *Escherichia coli*, as described previously [2]. ApoPYP was reconstituted with the anhydride derivative of 4-hydroxy-cinnamic acid, according to Imamoto et al. [19]. This PYP was used after cleavage and removal of its poly-histidine tail.

Samples for the different experiments were taken from a PYP batch solution with $OD_{446} = 1$. PYP was buffered at pH 5.6 (± 0.1) with 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES; Sigma). A mixture of PYP in the pG and pB states was obtained by continuous illumination of a PYP sample with a Schott KL1500 light source (containing a 150 W halogen lamp) through a glass fiber (i.e. $\lambda > 350$ nm). The pH selected (see also [11]) allowed for ∼70% pB

*Corresponding author. Fax: (31) (20) 5257056.
E-mail: k.hellingwerf@chem.uva.nl

Abbreviations: PYP, photoactive yellow protein; pG, dark-adapted ground state photocycle intermediate of PYP; pR, red-shifted photocycle intermediate of PYP; pB, blue-shifted photocycle intermediate of PYP; pB^t, blue-shifted intermediate formed after UVA light absorption by pB

to be accumulated. At pH = 8 only 20% pB is accumulated, which is insufficient for our purposes.

2.2. Nanosecond time-resolved absorption spectroscopy

Laser-induced transient absorption spectra were measured with a system custom made by Edinburgh Instruments Ltd. (Edinburgh, UK), composed of a Continuum Surelite I-10 Nd:YAG laser (output intensity 140 mJ at 355 nm; pulse width 6 ns), a Continuum Surelite OPO (output range 410–2200 nm) and an Edinburgh Instruments LP900 Spectrometer. The spectrometer contains a 450 W short-arc Xe lamp, providing the measuring beam, in combination with a pulser power supply and a Peltier cooled CCD camera (Wright Instruments) for read-out. The time resolution attainable with this system is 10 ns. The samples were kept at $\sim 20°C$ during the measurements, using a water-cooled cuvette holder. Time-resolved spectra were recorded pseudo-randomly, with delays between 119 ns and 8 s. The gate width was selected between 10 ns and 10 ms at 5% of the delay time. For each time point between 60 and 10 spectra were accumulated using a cycle time of 20 s. The wavelength scale of the spectrophotometer was calibrated with a holmium filter; the position of the 355 nm line of the Nd:YAG laser was consistent with this calibration.

2.3. Photocycle measurements

Measurements were performed either with a steady state mixture of the pG and the pB states of PYP (obtained with continuous actinic illumination), or with the pG state present exclusively. The steady state mixture of PYP intermediates was excited with either a 355 nm laser flash (Nd:YAG, 5 (± 1) mJ/pulse) or with a 446 nm laser flash (OPO, 7 (± 1) mJ/pulse). The sample containing pG exclusively was excited with a 446 nm laser flash (OPO, 7 (± 1) mJ/pulse). Flash intensities were varied through adjustment of the laser pump voltage. With 355 nm laser flash excitation a new sample was used regularly to minimize the effects of sample deterioration. For all samples used, the decrease in absorbance at 446 nm due to photo-deterioration of the sample was less than 2%.

2.4. Analyses of time-gated spectra

Global analysis was performed as described in [20]. The transient absorption data, accumulated in the presence of background light, were modeled as follows. All spectra before and after the excitation were analyzed simultaneously. The kinetic model used described the background light excitation by a pseudo rate constant k_I, which depended upon the effective background light intensity I. This k_I was a fitting parameter for each time delay, to allow for the fluctuations in the equilibrium concentrations of pB and pG, caused by slight differences in cuvette position with respect to the continuous actinic light

source. Typically k_I was 2–3 times as large as the rate of the recovery reaction of pB back to pG. The concentrations of the four species, pR, pB, pB^t and pG, obey the following set of coupled differential equations:

$$\frac{d}{dt}\begin{pmatrix} pR \\ pB^t \\ pB \\ pG \end{pmatrix} = \begin{pmatrix} -k_1 & 0 & 0 & k_I \\ 0 & -k_3 & 0 & 0 \\ k_1 & 0 & -k_2 & 0 \\ 0 & k_3 & k_2 & -k_I \end{pmatrix} \begin{pmatrix} pR \\ pB^t \\ pB \\ pG \end{pmatrix} + \delta(t)\begin{pmatrix} \beta \\ \alpha \\ -\alpha \\ -\beta \end{pmatrix}$$

The 355 nm laser flash $\delta(t)$ excites a proportion α of pB to pB^t, and a (much smaller) proportion β of pG to pR, whereas the background light only excites pG to pR (with pseudo rate k_I). A contribution of the background light to the branching reaction can be ignored, because of the lack of overlap between the color of the actinic beam and the absorbance spectrum of pB. Furthermore, an independently measured ground state spectrum was added to the data, and the amplitudes of the spectra of pB and pB^t were assumed to be zero at wavelengths larger than 425 nm [6,20]. This latter constraint is necessary in order to be able to estimate all kinetic and spectral parameters. The 446 nm excitation data are analyzed according to the same procedure, except that pB^t and α are omitted from the above equations.

In the present study, in which time-gated spectra were recorded, all transitions in the photocycle of PYP were fitted with mono-exponential kinetics, largely because of the lack of a sufficiently large signal to noise ratio to discriminate between mono- and bi-exponential kinetics of these transitions, or even more complex kinetics (compare [11,20]).

3. Results

To study the PYP photocycle branching reaction, originating from the pB intermediate, a steady state mixture of pG and pB was generated with actinic illumination. Typically, in such mixtures about 70% of the PYP molecules are in the pB state. Subsequently, a 355 nm laser flash (FWHM = 6 ns) was used to selectively excite the pB intermediate. Absorbance transients with nanosecond time resolution were measured up to 10 s after the flash and global analysis was performed on the data obtained (Fig. 1). This analysis showed the presence of a previously undetected intermediate that we propose to name pB^t. This intermediate is slightly blue-shifted with respect to pB (Fig. 1D). Within the time resolution of our set-up pB^t is formed instantaneously after laser flash excita-
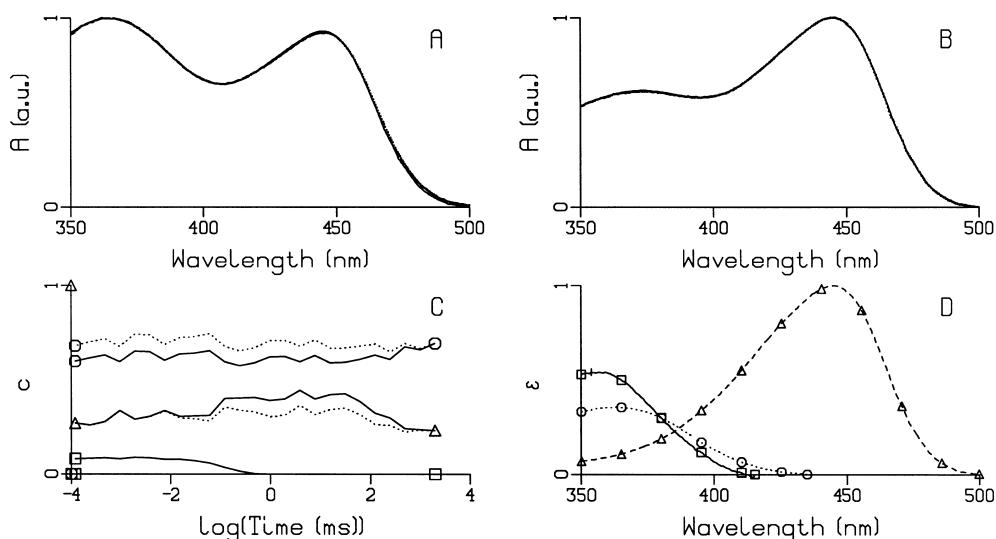

Fig. 1. Global analysis of the data obtained with transient absorbance measurements with 355 nm laser flash excitation. A,B: Fit of the absorption spectrum obtained after 0.5 µs and 31 ms respectively. C: Concentration profiles of pG (triangles), pB (circles), and pB^t (squares), before (dashed lines) and after (solid lines) the laser flash. Data points in this panel have been omitted for clarity. D: Estimated spectra of pG (triangles), pB (circles), and pB^t (squares). The absorbance has been plotted relative to pG ($\varepsilon_{pG, 446} = 45.5$ mM^{-1} cm^{-1} [30]).
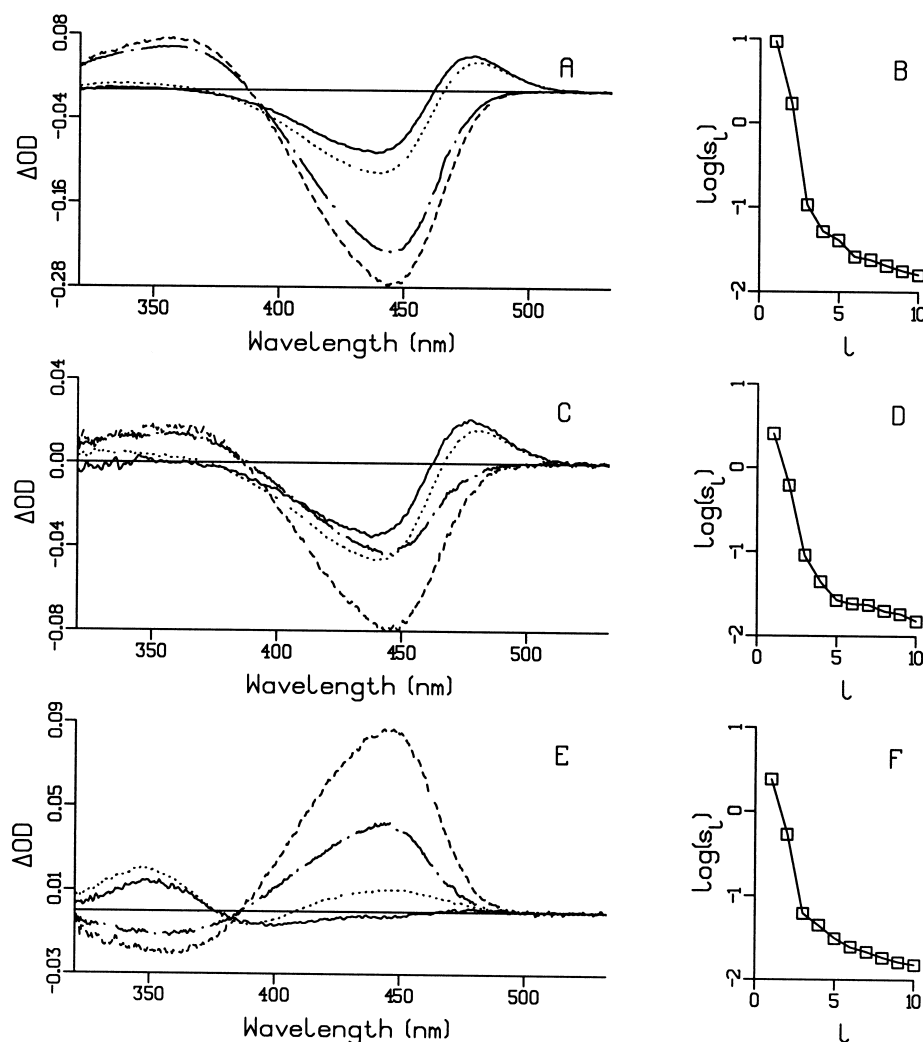
Fig. 2. Difference spectra at 119 ns (solid line), 15 μs (dotted line), 1.95 ms (dashed line) and 250 ms (dot-dashed line) and singular values of the matrix of time-gated spectra. A,B: pG sample excited with 446 nm laser flashes. C,D: Steady state mixture of pG and pB, excited with 446 nm laser flashes. E,F: Steady state mixture of pG and pB, excited with 355 nm laser flashes.

tion (Fig. 1C). pB^t subsequently relaxes to pG in the microsecond time scale (Fig. 1C and Table 1).

With 355 nm laser flash excitation it is also possible to excite pG (see e.g. [11]). This will result in the subsequent formation of the intermediates pR and pB, before the system returns to the steady state. However, when the intensity of the laser flash is chosen sufficiently low, and the steady state mixture of intermediates predominantly contains pB, such a contribution by pG excitation can be ignored. This latter approximation holds for the measurements presented here. When, in

Table 1
Results from global analysis on the data of the three separate data sets

Sample		pB/pG	pB/pG	pG
λ_{ex} (nm)		355	446	446
excited (%)		12 (of pB present)	27 (of pG present)	32
$k_{pR \to pB}$ (ms^{-1})		–	4.2	3.3 (3)
$k_{pB \to pG}$ (s^{-1})		0.74	0.60	0.72 (7)
$k^t_{pB} \to pG$ (ms^{-1})		6.8	–	–
pG	λ_{max} (nm)	445	445	445
	ε_{rel}	1	1	1
pR	λ_{max} (nm)	–	463 (2)	460 (2)
	ε_{rel}	–	0.67 (3)	0.62 (3)
pB	λ_{max} (nm)	363	363	363
	ε_{rel}	0.36	0.34	0.35
pB^t	λ_{max} (nm)	354	–	–
	ε_{rel}	0.54 (2)	–	–

The estimated standard error has been indicated in parentheses only when it is larger than 1 in the last decimal.
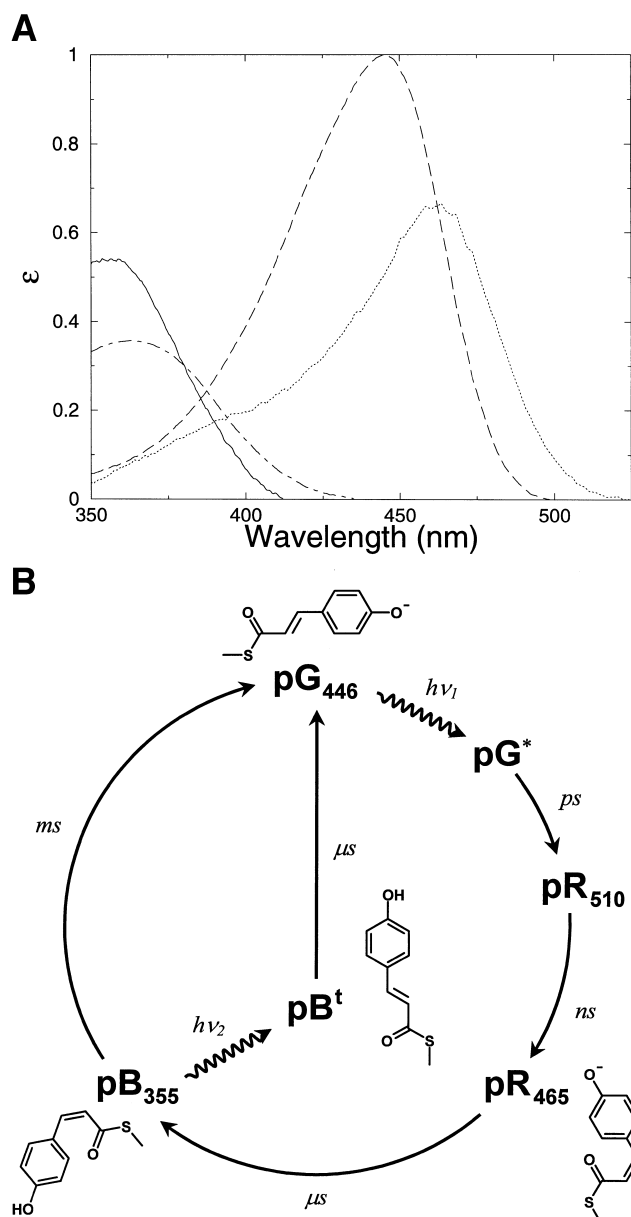
A

B

Fig. 3. Absorption spectra of intermediates and update of the schematic representation of the photocycle of PYP. A: Scaled absorption spectra of pG (dashed line), pR (dotted line), pB (dot-dashed line), and pB^t (solid line). Molar extinction has been plotted relative to pG ($\varepsilon_{pG, 446} = 45.5$ mM^{-1} cm^{-1} [30]). The spectra of pB and pR have been taken from the data set in which a mixture of pG and pB was excited with a 446 nm flash; for pB^t the corresponding data set with 355 nm excitation was used. B: Simplified photocycle scheme with the blue light-induced branching reaction, originating from pB, included. Approximate lifetimes have been indicated. The symbols hv$_1$ and hv$_2$ refer to blue (i.e. 446 nm) and UVA (i.e. 355 nm) photons, respectively. The subscripts of the intermediates refer to the approximate position of the λ_{max} of these intermediates. They have been selected according to previously published values [6,20,31].

contrast, the 355 nm laser energy was high, contributions from both pB and pG excitation were indeed observed (data not shown).

To determine whether or not the use of a steady state mixture of pG and pB influences the regular photocycle of PYP, measurements with 446 nm laser excitation were compared

between a sample containing a light-induced steady state mixture of pB and pG and a sample containing only pG. Difference spectra of these experiments are shown, together with those of the experiment with 355 nm excitation, in parts A, C, and E of Fig. 2. The difference between the difference spectra obtained with 446 nm excitation (Fig. 2A,C) and those obtained with 355 nm excitation (Fig. 2E) is striking. The two sets of difference spectra obtained with 446 nm excitation (Fig. 2A,C) look very similar and both show the formation of the pB intermediate (dashed and dot-dashed) from the pR intermediate (solid and dotted). For all three experiments the singular value decomposition showed two significant singular values, implying that the data contain two relevant components (panels B, D and F of Fig. 2). For excitation with a 446 nm laser flash, these components are the formation of pB from pR and the return from pB to pG and to the steady state mixture of pG and pB, respectively (panels A, B and C, D of Fig. 2, respectively). For excitation with a 355 nm laser flash these are the formation of pG from pB^t, and the return to the steady state mixture from pG (Fig. 2E,F).

In Table 1 the results of the global analysis of the three different experiments are shown. Comparison of the rates of corresponding photocycle transitions shows minor variations, which we consider insignificant. The absolute values of these rate constants are largely in agreement with previously published values [6,11,20]. We therefore conclude that the photocycle kinetics of PYP are not significantly affected by the actinic illumination.

In each of the three experiments the extinction coefficient and shape of the UV/Vis spectra of the different intermediates were also estimated in the global analysis (e.g. Fig. 1D and Table 1). Of all four intermediates a spectrum is shown in Fig. 3A. The spectra of pB and pR have been taken from the data set in which the mixture of pG and pB was excited with a 446 nm flash; for pB^t the corresponding data set with 355 nm excitation was used. The pG spectrum is shown only for comparison and was obtained with static UV/Vis absorption spectroscopy.

4. Discussion

Here we have used a steady state mixture of pB and pG to characterize a branching reaction in wild type PYP from *E. halophila* that is induced by excitation of the pB intermediate. We detected a new intermediate in this branching pathway, which is slightly blue-shifted with respect to pB and which recovers to pG in the microsecond time scale. This latter rate is approximately 1000-fold faster than the thermal recovery from pB back to pG in the dark. The branching reaction was not observed when 446 nm light was used to excite the steady state mixture of pB and pG. This is as predicted when the observed branching reaction, elicited with 355 nm light excitation, indeed originates from pB and is in agreement with our assumption that the pB intermediate does not show absorption at wavelengths above 425 nm (see Section 2). Fig. 3B shows the photocycle of PYP with this new branching reaction incorporated. The protonation and isomerization state of the chromophore of the different intermediates is also indicated. The protonation state of the new pB^t intermediate (with a λ_{max} at 354 nm) is evident from its strongly blue-shifted absorbance maximum with respect to pG, similar to that of pB. It should be noted that the λ_{max}

determined for pB in the current data set is at a slightly longer wavelength than previously determined values (i.e. 363 nm vs. $\sim$ 340–357 nm [6,11,20]). Nevertheless, the difference in λ_{max} between pB and pB^t, measured within one data set, is significant (the standard error in the determination of λ_{max} is ~ 2 nm for pB-like intermediates; see Table 1).

The isomerization state of the chromophore in pG, pR and pB has been resolved with capillary electrophoresis, X-ray diffraction and NMR [10,21–24]. As long as similar techniques have not yet been used for the intermediate discovered in this study (i.e. pB^t), we presume that its chromophore is in the *trans* configuration. By comparing the spectral characteristics of pG and pR, as well as of those of pB and pB^t (Fig. 3A), it is then evident that, when bound to the protein, the *trans* form of the chromophore (e.g. pG) is blue-shifted with respect to its *cis* form (e.g. pR). This shift corresponds to an energy difference of approximately 0.1 eV. In addition, the extinction coefficient in the *trans* form is higher than that of the *cis* form. This extrapolates well to the comparison between the pB and the pB^t intermediate. In aqueous solution, chromophore model compounds like 4-hydroxy-cinnamic acid, have an extinction coefficient that is highest in the *trans* form of these compounds [25]. These compounds show the opposite behavior, however, with respect to the wavelength of maximal absorbance, which is red-shifted approximately 0.4 eV in the *trans* derivatives.

The kinetics of the recovery from pB^t to pG are 1000-fold faster than those of the dark recovery from pB. This implies that chromophore isomerization is the major rate-controlling factor in the dark recovery of pG from pB. This was also concluded from experiments performed on the Met100Ala mutant of PYP [18] and also implies that the change in exposure of a hydrophobic patch in the protein, as reported by van Brederode et al. [26], must be intimately linked to isomerization of the chromophore. The change in heat capacity observed in the latter study may therefore have been caused to a significant extent by the exposure of the chromophore and the surrounding amino acids to the aqueous solvent.

The kinetics of pG formation from pB^t are very similar to those of pB formation from pR, although these transitions proceed in opposite direction with respect to the partial unfolding of PYP [26,27]. The combination of conformational change and (de)protonation in both reactions is a microsecond event. The absolute value of these two time constants (i.e. several hundreds of microseconds) is compatible with the assumption that this transition is accompanied by a rearrangement of a considerable part of the protein (i.e. equivalent to a loop of 40–50 amino acids, see [28]).

The intermediate(s) in the main branching pathway in sensory rhodopsin I has/have a clear biological significance [14]. For PYP this is not known yet, since phototactic responses to UV light have not been investigated. However, given the fact that this branching reaction displays microsecond kinetics (note that the ground state recovery in sensory rhodopsin I displays millisecond kinetics) it will be interesting to see whether or not these microsecond kinetics are too fast for a signal to be passed on from PYP to its downstream signal transduction component. Nevertheless, this branching reaction may have important implications for technological applications of PYP in information processing technology [16,29].

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