Shining light on the photoactive yellow protein from halorhodospira halophila

Hendriks, J.C.

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As can be gathered from Chapter 1, in recent years a comprehensive description of the photocycle of the Photoactive Yellow Protein has become available. Such a description is of vital importance if the Photoactive Yellow Protein is to be used as a model system. Time resolved UV/Vis spectroscopy has proven to be a key technique for the description this photocycle. It is then not surprising that time resolved UV/Vis spectroscopy on a nanoseconds to seconds time-scale has been an important part of my work on the Photoactive Yellow Protein. Therefore, a description of the set-up used for these experiments is provided at the start of this chapter. The remainder of this chapter will be devoted to the description of the experiments I have been involved in that have led to a better understanding of the photocycle. The characterization of the photocycle branching reaction is described (Hendriks et al. 1999a). Though its existence was already known, it had not been characterized in detail before. Furthermore, the (kinetic) deuterium isotope effect was determined for the photocycle over a large pH range (Hendriks et al. 2002a). Here the culmination of information obtained in recent years, have made it possible in combination with these data to provide a much improved insight into the photocycle of the Photoactive Yellow Protein.
Chapter 3  Laser-flash photolysis

1 Laser-flash photolysis

1.1 The set-up

The set-up was custom build by Edinburgh Instruments Ltd. (http://www.edinst.com), and is based on their LP900 spectrometer. It incorporates a 450 Watt high pressure Xenon arc lamp, which acts as the probe light source. This lamp is powered by a power supply (Xe900) that provides a constant power. This constant power can be supplemented with a millisecond power pulse from a different unit (Xp900), resulting in a temporary significant increase in light output, more UV output, less structure of the lamp emission spectrum, and a shorter lifetime of the Xenon lamp. After the probe light has passed through the sample chamber it enters the TM300 monochromator. In this monochromator two gratings are present, one with 300 grooves per millimeter and another with 1800 grooves per millimeter. The latter is used in combination with the photomultiplier detector, the former is used in combination with the CCD camera detector.

For excitation of the sample a nanosecond tunable laser was used. A Continuum I-10 YAG laser (output intensity 140 mJ at 355 nm) was used to pump a Continuum Surelite I OPO (output range 410 to 2200 nm), which was usually tuned to 446 nm. Pulse width of the obtained laser pulse was 6 ns. The laser pulse was guided to the sample chamber via prisms.

For the measurements two types of sample holder have been used. One can be water-cooled on one side of the cuvette, and was supplied with the set-up by Edinburgh Instruments Ltd. The other, is a home build cuvette holder, with which the sample temperature can be Peltier controlled. Also, a feature was incorporated that allows the sample to be illuminated via the bottom of the cuvette, e.g. with a LED or via a light guide. This home-build cuvette holder was used for experiments described in Chapter 2 section 3, and Chapter 3 section 3.

1.2 CCD measurements

The advantage of the CCD measurements is that a large spectral range can be detected at once. The disadvantage is that only one time slice can be recorded at once. Time gated spectra are obtained as follows. Light refracted by the grating in the monochromator (light of different wavelength is refracted at different angles by this grating) is projected onto an image intensifier (DiDEC unit). Here a wavelength range encompassing approximately 260 nm is projected (when the grating with 300 grooves per millimeter is used). Only if the projected light is amplified, a phosphor image is created that is intense enough for the CCD camera to read. Consequently, by adjusting the delay and width of the gain pulse of the image intensifier, a spectrum at a specific point in time can be recorded. The image intensifier is able to generate gain pulses with a delay up to 1 ms and a width (or gate) down to 10 ns. Spectra with longer delays are obtained by supplying the image intensifier with external pulses from a delay generator (BNC model 500 pulse generator). Typically several images are accumulated per spectrum to reduce the signal to noise ratio. Time gated spectra were usually recorded pseudo-randomly with the CCD camera in order to prevent long-term trends in the data. Also, time points were usually chosen, evenly spaced on a logarithmic time-scale. Typically a gate of 5% of the delay time was chosen with 10 ns as minimum and 10 ms as maximum.

1.3 Photomultiplier measurements

The advantage of the photomultiplier measurements is that traces are recorded with a high temporal resolution. The disadvantage is that only one wavelength can be recorded at a time. Time traces are obtained as follows. Light refracted by the grating in the monochromator is projected, through a slit, onto the photomultiplier tube. The resulting signal is subsequently monitored by a Oscilloscope (Tektronix TDS 340A). Typically several traces were averaged to reduce the signal to noise ratio.

As only a limited time-range can be monitored by the oscilloscope, it is necessary to record multiple traces when information over a larger time range is necessary. These different time traces can then later be combined into a single time trace encompassing the larger time range. As the different traces usually do not overlap perfectly, it is necessary to apply a minor correction factor to these separate traces before they are combined. Unfortunately, the Tektronix TDS 340A oscilloscope does not allow the generation of data into a circular buffer as described in Chapter 2 section 4.1.4. This would allow the possibility of a logarithmic data acquisition to be programmed, and would render the combining of separate time traces unnecessary.
2 Branching reaction

The photoactivity of the Photoactive Yellow Protein (PYP) is not limited to its ground state. Also photocycle intermediates can be photoactive. It has already been shown that in the presence of 366 nm light rate of recovery becomes faster, indicating that a photon induced branching reaction exists between pB and pG (Miller et al. 1993). As it is likely that in this branching reaction the chromophore is isomerized photoactively, the study of this branching reaction may provide further insight into the photocycle recovery reaction.

2.1 Materials & Methods

2.1.1 Sample preparation

In this study only wild type PYP with its His-tag removed was used. The production and purification of PYP is described in Chapter 2 section 1. A 22 µM PYP (OD₄₄₆ is ~1) batch solution was prepared buffered at pH 5.6 with 50 mM 2-(N-Morpholino)ethanesulfonic acid (MES). Samples for the different experiments were taken from this batch solution. A steady state mixture of PYP in the pG and pB state was obtained by continuous actinic illumination of a PYP sample with a Schott KL1500 light source (containing a 150 Watt halogen lamp) through a glass fiber (i.e. λ > 350 nm). The selected pH allowed for ~70% pB to be accumulated (see also Chapter 2 section 2). At pH 8 only ~20% pB is accumulated, which is insufficient for our purposes.

2.1.2 Nanosecond time resolved absorption spectroscopy

We used an Edinburgh Instruments Ltd. LP900 spectrometer, equipped with a CCD camera, in combination with a Continuum Surelite OPO laser (for further details see section 1). The samples were kept at ~20ºC 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.

2.1.3 Photocycle measurements

Measurements were performed either with a steady state mixture of the pG and the pB state 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%.

2.1.4 Analyses of time gated spectra:

Global analysis was performed as described in (Hoff et al. 1994a). 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 used to create the background light. Typically $k_I$ was 2 to 3 times as large as the rate of the recovery reaction of pB back to pG. The concentrations of the four species, pR, pB, pB¹ and pG, obey a set of coupled differential equations as described in Equation 5.
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 the spectra obtained with 446 nm excitation (Figure 32) are omitted from Equation 5. 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 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 section 3 and (Hoff et al. 1994a; Hoff et al. 1997a)).

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

Equation 5

The 355 nm laser flash \( \delta(t) \) excites a proportion \( \alpha \) of \( pB \) to \( pB^t \), and a (much smaller) proportion \( \beta \) of \( pG \) to \( pR \), whereas the background light only excites \( pG \) to \( pR \) (with pseudo rate \( k_f \)). A contribution of the background light to the branching reaction can be neglected, because of the lack of overlap between the color of this background light and the absorbance spectrum of \( pB \). Furthermore, an independently measured ground state spectrum was added to the data, and the amplitude of the spectra of \( pB \) and \( pB^t \) were assumed to be zero at wavelengths larger than 425 nm (Meyer et al. 1987; Hoff et al. 1994a). 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 \( \alpha \) are omitted from Equation 5. 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 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 section 3 and (Hoff et al. 1994a; Hoff et al. 1997a)).

2.2 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 ns time resolution were measured up to 10 s after the flash and global analysis was performed on the data obtained (Figure 31). 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 \( pB \) (Figure 31 d). Within the time-resolution of our set-up \( pB^t \) is formed instantaneously after laser flash excitation (Figure 31 c). \( pB^t \) subsequently relaxes to \( pG \) on the \( \mu s \) time scale (Figure 31 c and Table 11).

With 355 nm laser flash excitation it is also possible to excite \( pG \) (see e.g. (Hoff et al. 1997a)). 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 neglected. This latter approximation holds for the measurements presented here. When, in 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 panels a, c, and e of Figure 32. The difference between the difference-spectra obtained with 446 nm excitation (Figure 32 a and c) and those obtained with 355 nm excitation (Figure 32 e) is striking. The two sets of difference spectra obtained with 446 nm excitation (Figure 32 a and 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 Figure 32). 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 and b, and panels c and d of Figure 32, 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 \( pG \) (Figure 32 e and f). In Table 11 the results of the global analysis of the three different experiments are shown. Comparison of the rates of corresponding photocycle transitions does show minor variations, which we consider insignificant. The absolute values of these rate constants are largely in agreement with previously published values (Meyer et al. 1987; Hoff et al. 1994a; Hoff et al. 1997a). We therefore conclude that the photocycle kinetics of PYP are not significantly affected by the actinic illumination.
Figure 31. Global analysis of the data obtained with transient absorbance measurements with 355 nm laser flash excitation.

Panels a and b: Fit of the absorption spectrum obtained after 0.5 μs and 31 ms respectively. Panel c: Concentration profiles of pG (circles), pB (diamonds), and pBt (triangles), before (dashed lines, solid symbols) and after (solid lines, open symbols) the laser flash. Data points in this panel have been culled for clarity. Panel d: Estimated spectra of pG (dashed line), pB (dotted line), and pBt (solid line). The absorbance has been plotted relative to pG (εpG, 446 = 45.5 mM⁻¹·cm⁻¹ (Meyer et al. 1999)).

Table 11. Results from global analysis on the data of the tree separate data sets.
The estimated standard error has been indicated between parentheses only when it is larger than 1 in the last decimal.

<table>
<thead>
<tr>
<th>sample</th>
<th>pB/pG</th>
<th>pB/pG</th>
<th>pG</th>
</tr>
</thead>
<tbody>
<tr>
<td>λexc (nm)</td>
<td>355</td>
<td>446</td>
<td>446</td>
</tr>
<tr>
<td>excited (%)</td>
<td>12 (of pB present)</td>
<td>27 (of pG present)</td>
<td>32</td>
</tr>
<tr>
<td>kpit→pB (ms⁻¹)</td>
<td>-</td>
<td>4.2</td>
<td>3.3(3)</td>
</tr>
<tr>
<td>kpit→pG (s⁻¹)</td>
<td>0.74</td>
<td>0.60</td>
<td>0.72(7)</td>
</tr>
<tr>
<td>kpit→pBt (ms⁻¹)</td>
<td>6.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pG λmax (nm)</td>
<td>445</td>
<td>445</td>
<td></td>
</tr>
<tr>
<td>εrel</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>pR λmax (nm)</td>
<td>-</td>
<td>463 (2)</td>
<td>460 (2)</td>
</tr>
<tr>
<td>εrel</td>
<td>-</td>
<td>0.67(3)</td>
<td>0.62 (3)</td>
</tr>
<tr>
<td>pB λmax (nm)</td>
<td>363</td>
<td>363</td>
<td>363</td>
</tr>
<tr>
<td>εrel</td>
<td>0.36</td>
<td>0.34</td>
<td>0.35</td>
</tr>
<tr>
<td>pBt λmax (nm)</td>
<td>354</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>εrel</td>
<td>0.54(2)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
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. Figure 31 d and Table 11). Of all four intermediates a spectrum is shown in Figure 33 a. 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, 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.

![Figure 32. Difference spectra and singular values of the matrix of time-gated spectra.](image)

*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.*

*Panels a and b: pG sample excited with 446 nm laser flashes; Panels c and d: steady state mixture of pG and pB, excited with 446 nm laser flashes; Panels e and f: steady state mixture of pG and pB, excited with 355 nm laser flashes.*
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 on the microsecond time scale. This latter rate is approximately a thousand-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 2.1). Figure 33 shows the photocycle of PYP with this new branching reaction incorporated. The protonation state of the new pB\(^{t}\) intermediate (with a \(\lambda_{\text{max}}\) 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 \(\lambda_{\text{max}}\), determined for pB in the current data set, is at a slightly longer wavelength than previously determined values (i.e. 363 nm vs. \(\sim 340 \text{ to } 357 \text{ nm}\) (Meyer *et al.* 1987; Hoff *et al.* 1994a; Hoff *et al.* 1997a)). Nevertheless, the difference in \(\lambda_{\text{max}}\) between pB and pB\(^{t}\), measured within one data set, is significant (the standard error in the determination of \(\lambda_{\text{max}}\) is \(\sim 2 \text{ nm}\) for pB-like intermediates; see Table 11).

Figure 33. Absorption spectra of intermediates and update of the schematic representation of the photocycle of PYP.

Panel a: Scaled absorption spectra of pG (dashed line), pR (dot-dashed line), pB (dotted line), and pB\(^{t}\) (solid line). Molar extinction has been plotted relative to pG (\(\varepsilon_{\text{pG}, 446} = 45.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}\) (Meyer *et al.* 1989)). 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.

Panel b: Simplified photocycle scheme with the blue-light induced branching reaction, originating from pB, included. Approximate lifetimes have been indicated. The symbols \(h\nu_1\) and \(h\nu_2\) refer to blue (i.e. 446 nm) and UVA (i.e. 355 nm) photons, respectively.

The isomerization state of the chromophore in pG, pR and pB has been resolved with capillary electrophoresis, X-ray diffraction and NMR (Borgstahl *et al.* 1995; Kort *et al.* 1996b; Genick *et al.* 1997b; Dux *et al.* 1998; Perman *et al.* 1998). 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\(^{t}\) (Figure 33 a), 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-hydroxycinnamic acid, have an extinction coefficient that is highest in the *trans* form of these compounds (Aulin-Erdtman and Sandén 1968). 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\textsuperscript{t} to pG are a thousand 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 (Devanathan et al. 1998) and also implies that the change in exposure of a hydrophobic patch in the protein, as reported by van Brederode et al. (van Brederode et al. 1996), must be intimately linked to isomerization of the chromophore.

The kinetics of pG formation from pB\textsuperscript{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 (van Brederode et al. 1996; Hoff et al. 1999). 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 to 50 amino acids, see (Goldbeck et al. 1999)).

The intermediate(s) in the main branching pathway in sensory rhodopsin I has/have a clear biological significance (Spudich and Bogomolni 1984). For PYP this is not known yet, since phototactile 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.

### 2.4 Concluding remarks

In this study we have characterized the photocycle branching reaction, discovering a new intermediate in the progress. Furthermore, we have shown that re-isomerization of the chromophore is at least one of the rate determining steps in dark photocycle recovery. Further study of this photon induced branching reaction may prove important for a better understanding of photocycle recovery in the dark.
Deuterium isotope effect

In the Photoactive Yellow Protein (PYP), as in all proteins, there are many exchangeable protons that can be exchanged for deuterium atoms easily and rapidly. PYP contains 235 exchangeable hydrogen atoms, 42 of which are from (de)protonatable groups. As such, for any characteristic of PYP, in which exchangeable protons play a significant role, it might be possible to observe a deuterium isotope effect (DIE) upon deuteration of the exchangeable protons. In this study we will be mostly concerned with the kinetic deuterium isotope effect (KDIE) of the different photocycle transitions. Both normal and inverse KDIEs can be observed, i.e. in deuterium oxide the reactions can be slower and faster, respectively. For H/D exchange, often a factor of 1.41 (square root of 2) is observed in KDIE experiments, which is caused by the difference in mass of the hydrogen and the deuterium atom. Nevertheless, larger factors are also frequently measured, reflecting hydrogen-tunneling mechanisms (Kohen and Klinman 1999). The KDIE will not only help to determine reaction mechanisms within the photocycle, but by analyzing it in combination with the pH dependence that most PYP photocycle reactions display, it is possible to determine whether these reactions are dependent on the hydronium ion or on the hydroxide ion concentration. This is of crucial importance when comparing data obtained in H₂O and D₂O at different pH or pD values.

In this study we have been able to confirm known reaction mechanisms in the photocycle of PYP, elaborate on certain photocycle transitions, and obtain more information about the details of the third basic photocycle step. Combining the pOH (or pH) dependence and the KDIE has been crucial in our approach. Furthermore, the results from this study will aid in choosing optimal conditions in future studies of specific photocycle events and/or intermediates.

3.1 Materials & Methods

3.1.1 Sample preparation

In this study only wild type PYP with its His-tag removed was used. The production and purification of PYP is described in Chapter 2 section 1. Samples for the time trace experiments were prepared by mixing the following four solutions. 1) Water or deuterium oxide (1.8-2.0 ml). 2) A buffer mixture in water or deuterium oxide (176 μl), consisting of citric acid, 1,3-bis[tris(Hydroxymethyl)methylamino]propane, and 3-[Cyclohexylamino]-1-propanesulfonic acid (250 mM each). 3) Wild type PYP in deuterium oxide buffer mixture (4.8 μl). 4) A 1 M sodium hydroxide or sodium deuteroxide solution (0-160 μl). After mixing, the pH was measured with a Mettler Toledo InLab®423 pH-electrode. The pD was obtained by adding 0.4 to the electrode reading (Glasoe and Long 1960). Samples ranging in pH from 5.1 to 10.6 and ranging in pD from 5.7 to 11.5 were obtained. The optical density at the absorption maximum of wild type PYP of the samples was approximately 0.5. Samples for the time gated spectra experiments were prepared by making a batch solution of wild type PYP in 20 mM of the buffer mixture in water described above at pH 8.10 and 9.55.

3.1.2 Transient (ms/s) UV/Vis measurements

An HP 8453 UV/Vis diode array spectrophotometer was used with a time-resolution of 100 ms. Spectra were collected from 210 to 600 nm. Samples were flashed with a 500 μs photoflash. Samples were measured at room temperature, just before the start of the accompanying laser-flash photolysis measurements.

3.1.3 Laser-flash photolysis spectroscopy

We used an Edinburgh Instruments Ltd. LP9000 spectrometer, equipped with a photomultiplier and a CCD camera, in combination with a Continuum Surelite OPO laser (for further details see section 1). The PYP sample was excited with 446 nm laser flashes of ~5-6 mJ/pulse (pulse width 6 ns). During the measurements the sample was kept at 20°C in a Peltier controlled cuvette holder. Time-traces were recorded at 500, 450, and 360 nm with the slow-board option of the photomultiplier (time-resolution ~2 μs). Optical interference filters were used before the sample to minimize measurement artifacts induced by probe light. The following time-windows were measured for all three wavelengths, −10 to 190 μs (0.2 μs resolution), and −1 to 19 ms (20 μs resolution). In addition, for traces at 450 and 360 nm one of the following time-windows was used, depending on recovery rate, −0.25 to 4.75 s (5 ms resolution), −0.5 to 9.5 s (10 ms resolution), or −1 to 19 s (20 ms resolution).
Time gated spectra were recorded pseudo-randomly with the CCD camera in order to prevent long-term trends in the data. 80 time points were chosen, evenly spaced on a logarithmic time-scale between 30 ns and 2 s. A gate of 5% of the delay time was chosen with 10 ns as minimum and 10 ms as maximum.

### 3.1.4 Data analysis

Data from the different time-windows were merged, correcting for any intensity differences between the traces. Merged traces obtained at a single pH or pD were analyzed simultaneously. For the initial analysis with a simple sequential model, the program Origin 6.0 (Microcal Software, Inc.) was used. For analysis with more complex models the merged traces were analyzed with a global fitting program described elsewhere (van Stokkum et al. 1994), making use of the Target Analysis method (van Stokkum and Lozier 2002). For the Target Analysis to succeed, several spectral constraints needed to be introduced for the model depicted in Figure 37a. The intermediate pR has no contribution at 360 nm. The intermediates pB and pB' only have a contribution at 360 nm and do not contribute to the 450 and 500 nm traces. The intermediate pB_deprot only contributes to the 450 nm trace. Due to a discrepancy between the recovery kinetics of the 360 and 450 nm trace, which is caused by a probe light induced branching reaction in the 360 nm trace, a correction needed to be introduced in the model. Two corrections were tried, which are described in the Results section.

For the analysis of the CCD data, the complex model used for the analysis of the time traces was found to be too complex with regard to ground state recovery. As such the part of the complex model describing pB formation was used in conjunction with a simple one-step recovery from pB to pG (see Figure 39a). Two constraints were used in the Target Analysis, i.e. pB' and pB are zero above 425 nm. These constraints were found valid by also testing the constraints that pB' and pB are zero above 460 nm, which resulted in approximately zero absorption between 425 and 460 nm for both pB' and pB. Also, as a reference a calculated absorption spectrum of the ground state was used (see section 3.2).

### 3.2 Results

Since the photocycle kinetics of PYP are pH dependent (Genick et al. 1997a), a kinetic analysis was carried out in a large pH range (5.1 to 10.6) and pD range (5.7 to 11.5), to distinguish between pH and deuterium isotope effects on the photocycle kinetics. At 20°C the dissociation constant of water $pK_w$ (with $K_w$ in mol·l$^{-1}$) is 14.1669 for H$_2$O and 15.049 for D$_2$O (Weast 1988). Due to this difference in $pK_w$, a clear distinction can be made in plotting data as function of pH / pD or pOH / pOD, when comparing data obtained in H$_2$O and D$_2$O (Weast 1988). Due to this difference in $pK_w$, a clear distinction can be made in plotting data as function of pH / pD or pOH / pOD, when comparing data obtained in H$_2$O and D$_2$O. In fact, data obtained in D$_2$O appears to shift 0.68 units with respect to the same data obtained in H$_2$O when plotted as function of pOH / pOD instead of pH / pD. For a property with a clear pH dependence it is then possible to determine if this dependence is indeed a pH (hydronium ion) or in fact a pOH (hydroxide ion) dependence. Both types of dependence can be observed in PYP and data is plotted accordingly in this study. The choice between pH and pOH dependence is mainly based on an alignment of the shape of the curves. In some cases the underlying chemistry of the used photocycle model was also used to base a choice on.

![Figure 34. Spectral deuterium isotope effect.](image)

*In panel a the fitted spectra of the species involved in ground state recovery are shown for pH 5.1, 7.9, 9.2, 9.8, 10.1 and 10.6. The transition between the two peaks has a $pK_a$ of 10. In panel b the absorption maximum of pG (circles) and pB with a protonated chromophore (triangles) is plotted as function of pOH/pOD for the samples in H$_2$O (filled symbols) and D$_2$O (open symbols).*
Besides kinetic effects, there may also be some spectral effects due to the substitution of hydrogen atoms for deuterium atoms. Therefore transient spectra were recorded with a time resolution of 100 ms for each sample and the difference spectra, representing the recovery component, were determined. After subtracting the contribution of the pG spectrum (using a calculated pG spectrum) from these difference spectra, the absorption bands of species other than pG were obtained (see Figure 34). At high pH the chromophore in pB becomes deprotonated, forming a species which is red shifted with respect to pB and appears with an apparent 

\[ pK_a \]

of 10 and a cooperativity constant, \( n \), in the Henderson-Hasselbalch equation of 0.74. This is in line with earlier observations (see Chapter 2 section 2). From these spectra the absorption maxima of the pB state were determined. In the case where pB is present with both a protonated and deprotonated chromophore the peak maximum of pB with a protonated chromophore was determined via deconvolution of the spectrum. In Figure 34 b the peak maxima of pG and pB are compared for the protein in H2O and in D2O. As these absorption maxima appear to be dependent on the hydroxide / deuteroxide ion concentration of the solution, they are plotted as function of pOH / pOD. For pG a red shift of 2 nm is observed for the deuterated samples. For pB the deuterated samples show a blue shift of ~2 nm over a large range, which changes to a red shift above pOH ~8.5. In addition, pB shows a clear pOH dependence of the peak maximum, which shifts over a range of 5 nm in H2O. Contrary to pB, pG shows a negligible pOH dependence of the peak maximum. As a control we also carried out a titration of the chromophore in a protein denatured with 8 M guanidine hydrochloride. In this titration the chromophore has a \( pK_a \) of 8.7 and an \( n \) of 1 (data not shown).

To determine the photocycle kinetics with microsecond time resolution, traces were recorded at 360, 450 and 500 nm (see Figure 36 for representative traces at pH 7.06). To start with, the data was analyzed using a simple 2 exponent sequential model (see Figure 35 a). This model was also used in an earlier study on the pH dependence of the photocycle of PYP (Genick et al. 1997a). In Figure 35 the pOH dependence obtained with this simple model is shown. As a result of the measurement conditions, the recovery rate measured at 450 nm is slower than that measured at 360 nm (see Figure 35 c). This is caused by the 360 nm probe light, which invokes the photocycle branching reaction, leading to an accelerated recovery (see section 2 and (Miller et al. 1993)). At low pOH the rates at 360 and 450 nm are almost identical. This is probably caused by the presence of pB with either a protonated or a deprotonated chromophore at these pOH values (see Figure 34 a and below). Because of this, both 360 and 450 nm light is able to initiate the branching reaction via pB with a protonated or a deprotonated chromophore, respectively. The rate of formation of pB (see Figure 35 b) shows a normal KDIE (i.e. rate in deuterium oxide is slower) over the entire measured pH range. For ground state recovery (see Figure 35 c and d) an inverse KDIE (i.e. rate in deuterium oxide is faster) can be observed. However, at low pOH this effect diminishes. Interestingly, when measured at 360 nm (see Figure 35 c) the KDIE disappears below pOH ~6, whereas when it is measured at 450 nm it disappears below pOH 4. Presumably this difference is caused by the photocycle branching reaction. The shape of the pOH dependence, both of pB formation and of pG recovery, is similar to the previously determined pH dependency for these reactions (Genick et al. 1997a). The simple model (see Figure 35 a) only provides an acceptable fit at the pH extremes, and is not able to properly describe the data over the entire measured pH range. This is illustrated in Figure 36 d where the root mean square deviation (rmsd) of the fits with the simple model (circles) are plotted as function of pH. The increased rmsd values between pH 6 and 9.5, together with residuals from the fit of the traces (see Figure 36 a-c for representative fits at pH 7.06) clearly show the inadequacy of this simple model.

A more complex model was subsequently constructed on the basis of what is known from literature and what was inferred from the KDIE observed with the simple model. On the basis of FTIR measurements it was shown that an additional intermediate pB' is formed from pR, which preceeds pB (Xie et al. 2001). This pB' has very similar, or possibly the same, spectral characteristics as pB. The pB' intermediate differs from pR only in one important respect, i.e. where in pR a buried negative charge resides on the chromophore where it can be effectively neutralized via delocalization of the charge, a hydrogen bonding network and the vicinity of a positive charge from Arg52, in pB' this buried negative charge resides on Glu46 where it no longer can be effectively neutralized. This is a stressful situation for the protein that potentially can be resolved in two different ways. One, Glu46 could be protonated by the chromophore, i.e. a return to the pR state. Two, formation of pB. Indeed, only when the formation of pB' from pR is considered to be reversible, does the incorporation of this intermediate significantly improve the fit. This is illustrated by a fit on the data at pH 7.06 (see Figure 36). Here the rmsd obtained with the simple model represented in Figure 35 a is 1.32 mOD. The addition of the pB' intermediate in a unidirectional reaction results in an rmsd of 1.29 mOD, which is only a very small decrease. However, by making the pR to pB' reaction reversible an rmsd of 0.62 mOD is
obtained, which is a significant reduction. With respect to pB formation the addition of the pB' intermediate in equilibrium with pR, was sufficient to obtain an acceptable fit of the part of the data representing pB formation.

Figure 35. KDIE obtained with the simple photocycle model.
In panel a the photocycle model, used to analyze the data presented in panels b - d, is shown. Data obtained in H₂O is represented with closed symbols; data obtained in D₂O with open symbols. All data is plotted as function of pOH / pOD. In panel b the rate representing pB formation (k₁) is shown. In panel c the ground state recovery measured at 360 nm (k₂) is shown. The dashed line represents recovery at 450 nm in H₂O. In panel d the ground state recovery measured at 450 nm (k₃) is shown.

On the basis of the inverse KDIE of the recovery rate obtained with the simple model, we conclude that deprotonation of the chromophore by a hydroxide ion from solution is likely involved before isomerization of the chromophore takes place (see Discussion). Therefore we included a new photocycle intermediate, which we have named pB_{deprot}, that is formed from pB. As this intermediate is formed through a (de)protonation reaction, it is most likely that this reaction is reversible. As a result of the addition of this equilibrium between pB and pB_{deprot}, the rmsd of the fit at pH 7.06 was further lowered from 0.62 to 0.50 mOD. Note that the incorporation of the pB_{deprot} intermediate in the recovery step is purely based on the observed KDIE, not on the UV/Vis traces themselves. The resulting model as depicted in Figure 37 a was then used to fit the data. As indicated by the analysis with the simple model, it is necessary to correct for the fact that the recovery kinetics differ between the 360 and 450 nm traces. Two types of correction were tried. For the first a branching reaction from pB directly to pG, only present in the 360 nm data, was incorporated. In the second the pB_{deprot} to pG reaction is considered separately in the 360 and 450 nm traces. The results obtained with both types of correction were very similar, with the exception of the pB to pB_{deprot} equilibrium. The rate constants obtained for this equilibrium showed relatively large errors with the first branching correction, while the second branching correction did not display this problem. Therefore, the more complex model was used with the incorporation of the second branching correction. With the described more complex model an acceptable fit over the entire measured pH range was obtained (see Figure 36 d).
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Figure 36. Comparison between the simple and the complex model.
Data recorded at 360 (panel a), 450 (panel b) and 500 nm (panel c) is shown for pH 7.06. The data is represented by dots. The solid line gives the fit with the complex model and the dashed line with the simple model. Accompanying residuals are shown in the lower part of the panels. Note the different scales. In panel d the rmsd obtained for the fits of the data in water with the simple (circles) and complex (stars) model are shown as function of pH.

For the pR to pB’ reaction (see Figure 37 b) a normal KDIE can be observed over the entire pOH range. The reverse reaction (see Figure 37 c) shows a normal KDIE between pOH 5.7 and 6.5, no KDIE at higher pOH values, and an inverse KDIE at lower pOH values. Formation of pB from pB’ (see Figure 37 d) shows no clear KDIE at low pOH values, a normal KDIE at intermediate pOH values going to a normal or inverse KDIE for higher pOH values. It is good to note here that at the pH extremes the simple photocycle model was able to describe the data equally well (see Figure 36 d) and showed a normal KDIE at both pH extremes for the formation of pB (see Figure 35 b).

For the formation of pB deprot from pB (see Figure 38 a) no KDIE is observed at lower pOH values and an inverse KDIE at pOH values above ~5. The reverse reaction (see Figure 38 b) shows a pH dependence with no KDIE at lower pH values and an inverse KDIE at pH values above ~8. For the last step in the photocycle, i.e. formation of pG from pB deprot, rates were obtained specific for the 450 nm data set (see Figure 38 c) and the 360 nm data set (see Figure 38 d). In the data from the 450 nm data set a pH dependence is observed with no KDIE below pOH ~6.5, and an inverse KDIE for pOH values above ~6.5. In the data from the 360 nm data set no clear KDIE could be observed due to the relatively large error in the rate constants. Interestingly, we have been able to dissect the observed KDIE of the simple photocycle model into reactions with different dependencies (pOH and pH), and with different KDIEs using the more complex model depicted in Figure 37 a. This supports the application of this more complex model.
In addition to the kinetic traces at specific wavelengths with microsecond time resolution, spectral data with nanosecond time resolution were collected at pH 8.10 and 9.55. For the analysis of these data it is necessary to use the absorption spectrum of the ground state as a reference. However, we have noticed that the measured ground state spectrum is possibly not suitable. This is indicated by the following: When the measured ground state spectrum is subtracted from spectra obtained with 100 ms time resolution or through accumulation of the pB intermediate with continuous wave irradiation, we find a pR-like residue. In these spectra the presence of a significant amount of pR is not expected. Also, when the measured ground state spectrum is used as a reference in the analysis of time-gated spectra on a nanosecond to second timescale, the spectrum of pR contains a long, offset like, tail on the blue side of the absorption peak (Hoff et al. 1994a). Lastly, when the complex model depicted in Figure 37 is used, the pR intermediate has a clear fast decay component similar to that found in the absorption traces, but also a slow decay component similar to the decay rate of pB, which is not observed in the absorption traces. In an attempt to simulate a ground state spectrum we found that the ground state spectrum can be simulated very well by two skewed Gaussians (Fraser and Suzuki 1969; Sevilla et al. 1989) above 385 nm. The maxima of these two skewed Gaussians were selected to be at 425 and 452.4 nm, values that have been observed in a low temperature study (Masciangioli et al. 2000). We determined an appropriate calculated ground state spectrum via a global analysis of five time-gated difference spectra that did not contain a pR signal, using the Origin 6.0 program. The resulting calculated ground state spectrum is very similar to the measured ground state spectrum, but has a little less absorption on the red side of absorption spectrum and has no residual absorption on the blue side of the absorption spectrum (see Figure 39). This calculated ground state spectrum was then used as reference in the Target Analysis. The calculated ground state spectrum improved the analysis significantly, which is demonstrated by the absence of the offset like blue absorption in the absorption spectra of pR that were determined with the Target Analysis (see Figure 39).
Figure 38. KDIE obtained for recovery with the complex model.
In Figure 37a the photocycle model used to analyze the data presented in this figure is shown. Data obtained in H\textsubscript{2}O is represented with closed symbols; data obtained in D\textsubscript{2}O by open symbols. Error bars are shown for data points where the error is larger than the size of the symbol. Data is plotted as function of pOH / pOD with the exception of the data in panel b, which is plotted as function of pH / pD. In panel a the rate representing pB\textsuperscript{deprot} formation (k\textsubscript{3}) is shown. The dashed line represents the values for the reverse reaction in H\textsubscript{2}O. The KDIE of this reverse reaction (k\textsubscript{-3}) is illustrated in panel b. In panels c and d the formation of pG from pB\textsuperscript{deprot} is plotted for data recorded at 450 nm (k\textsubscript{4}) and 360 nm (k\textsubscript{5}) respectively.

As the time resolution of the CCD dataset was higher than the one of the time traces, we were able to see a relaxation of the pR intermediate. This relaxation has also been observed recently in transient grating experiments (Takeshita et al. 2002a; Takeshita et al. 2002b). Therefore an additional step in the photocycle was introduced that reflects this, i.e. a unidirectional reaction from pR\textsubscript{1} to pR\textsubscript{2} (see Figure 39a). With the time traces we saw that the rmsd of the analysis improved tremendously upon introduction of the reversible character of the pR to pB\textsuperscript{'} transition. In the CCD data the improvement in rmsd is not as dramatic with a change from an rmsd of 4.59 mOD for the simple model (see Figure 35a) to an rmsd of 3.86 mOD for the more complex model depicted in Figure 39a (compare the rmsd change of 1.32 mOD to 0.62 for the time traces at pH 7.06). Though we also tried the complex model depicted in Figure 37a, the quality of the data was not sufficient to use this model confidently. As such we present the results obtained with the somewhat less complex model as depicted in Figure 39a. As Figure 39c indicates the absorption spectra of both pR species are very similar where pR\textsubscript{1} has a slightly higher extinction coefficient than pR\textsubscript{2}. Note that the offset like blue absorption of the pR spectra observed before (Hoff et al. 1994a), has disappeared as a result of using the calculated ground state spectrum as a reference. Also, the pB\textsuperscript{'} intermediate seems to be slightly red shifted with respect to the pB intermediate (see Figure 39d). The spectra of the intermediates obtained at pH 9.55 (not shown) are very similar to those found at pH 8.10. The rate constants obtained in the analysis of the CCD data at pH 8.10 and pH 9.55 (see Table 12) agree with those found with the UV/Vis traces.
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**Figure 39. Spectral analysis of the photocycle at pH 8.1.**

In panel a the photocycle model used to analyze the CCD data is shown. Panel b shows the measured ground state spectrum as dots (1 out of every 6 data points is shown). The calculated ground state spectrum is shown as a solid line with the contribution of the 425 nm and 452.4 nm skewed Gaussian to this fit, shown as dashed lines. In panel c the absorption spectrum of pR1 (dashed line) and pR2 (solid line) obtained in the analysis are shown. Panel d presents the absorption spectrum of pB’ (dashed line) and pB (solid line) obtained in the analysis.

The effect of acid denaturation of PYP was also studied. The \( pK_a \) for pB\textsubscript{dark} formation in H\textsubscript{2}O and D\textsubscript{2}O were determined as 2.7 and 3.2 respectively. In both solvents the cooperativity constant \( n \) in the Henderson-Hasselbalch equation is 1.5, which is within the previously reported range for this value (Hoff \textit{et al.} 1997a). This indicates that PYP is less stable in D\textsubscript{2}O. Ionic hydrogen bonds could play an important role in the stability of PYP, as these bonds are less strong in D\textsubscript{2}O.

**Table 12. Rate constants obtained from the CCD data sets.**

Rate constants obtained from the CCD data sets, using the model depicted in Figure 39 a. The estimated standard error in the last digit is indicated in parentheses.

<table>
<thead>
<tr>
<th>pH</th>
<th>( k_1 (s^{-1}) )</th>
<th>( k_2 (s^{-1}) )</th>
<th>( k_{-2} (s^{-1}) )</th>
<th>( k_3 (s^{-1}) )</th>
<th>( k_4 (s^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.10</td>
<td>59(7) \times 10\textsuperscript{4}</td>
<td>2400(130)</td>
<td>2700(400)</td>
<td>1840(160)</td>
<td>4.65(4)</td>
</tr>
<tr>
<td>9.55</td>
<td>20(4) \times 10\textsuperscript{5}</td>
<td>2350(80)</td>
<td>710(100)</td>
<td>440(70)</td>
<td>4.49(6)</td>
</tr>
</tbody>
</table>

### 3.3 Discussion

As a result of changing the solvent from H\textsubscript{2}O to D\textsubscript{2}O, exchangeable hydrogen atoms in PYP are exchanged for deuterium atoms. Consequently, any property of the protein in which an exchangeable hydrogen atom is involved may be influenced. Additionally, there are several (de)protonatable groups that may induce a pH or pOH dependence in any property of PYP. It is possible to distinguish between a pH and pOH dependence via the DIE. We have mainly investigated effects on photocycle kinetics, but also \textit{e.g.} information concerning the absorption maximum of the ground and signaling state was obtained.

Spectral tuning of the 4-hydroxy cinnamic acid chromophore in the PYP ground state can be divided into three contributions (Yoda \textit{et al.} 2001), \textit{i.e.} counter ion effect (5300 cm\textsuperscript{-1}), medium effect of the protein matrix...
(700 cm\(^{-1}\)), and hydrogen-bonding effect (~1600 cm\(^{-1}\)). This latter contribution is likely the cause of the observed 2 nm red shift of the ground state spectrum in D\(_2\)O (see Figure 34 b). In the ground state the chromophore is involved in two ionic hydrogen bonds (Borgstahl et al. 1995). Such hydrogen bonds become weaker when deuterium is the bridging atom (Scheiner 2000). As such, in D\(_2\)O the contribution of the hydrogen-bonding effect to the spectral tuning is less, i.e. a smaller blue shift, which effectively results in a red shift of the main band in the visible absorption spectrum. For the signaling state, pB, a spectral deuterium isotope effect was also observed. Interestingly, the isotope effect changes from a blue to a red shift as function of pOH. When the absorption maxima are plotted as function of pH the change from a blue to a red shift occurs around pH 7.5. No property with an apparent pK\(_a\) of 7.5 has been observed for PYP as of yet. When plotted as a function of pOH (see Figure 34 b) the change from a blue to red shift occurs around pOH 8.5, which is equal to a pH of 5.5. In earlier studies a pK\(_b\) of 5.5 has been correlated with a change in folding state of pB (see Chapter 2 sections 2, 3, and 4). A pOH dependence of the absorption maximum of pB is therefore likely. Especially since a change in folding state of pB could also explain the change from a blue to a red shift. As in pB the chromophore is protonated, it is likely that any effect on tuning in pB involving exchangeable hydrogen atoms is mainly caused by hydrogen-bond(s) formed by the hydroxy group of the chromophore. Here a distinction can be made between neutral and ionic hydrogen bonds. Whereas ionic hydrogen-bonds become weaker when deuterium becomes the bridging atom, in neutral hydrogen bonds, the bond becomes stronger when deuterium is the bridging atom (Scheiner 2000). Consequently, a blue shift would indicate involvement of (a) neutral hydrogen bond(s) and a red shift involvement of ionic hydrogen bonds. It is unclear whether the change from neutral- to ionic hydrogen bonding is due to a change in hydrogen-bonding partner or a change of the protonation state of the hydrogen-bonding partner. Interestingly, the possible ionic hydrogen bond is formed in the pB state that is supposedly more folded (see Chapter 2 sections 3). Furthermore, it has now been shown for the first time that the absorption spectrum of pB is pOH dependent. The interpretation that in pB the phenolic hydroxy group is involved in (a) hydrogen bond(s) with the protein is supported by the fact that the pK\(_a\) of this hydroxy group in pB is about 10, as is shown in Figure 34 a (see also Chapter 2 section 2). Without such an interaction one would expect the pK\(_a\) to be 8.7 as indicated by pH titration of the denatured protein. It is assumed here that the pK\(_a\) of the chromophore in the cis configuration is very similar to that of the chromophore in the trans configuration.

To determine the KDIE for the photocycle of PYP, the progress of the photocycle was monitored at three representative wavelengths. The resulting traces were first analyzed with a simple photocycle model (see Figure 35 a). Though the shape of the pOH dependence (see Figure 35 b - d) compares well with the previously published pH dependence for pB formation and pG recovery (Genick et al. 1997a), our rate constants are about a factor of 3 lower for both transitions. The reason for this discrepancy is unclear, but may lie in the fact that different buffers were used in the two studies. Also different illumination conditions may have had an influence. E.g. a difference in intensity of light that can induce the photocycle branching reaction, can lead to significant differences in the rate of pG recovery (Miller et al. 1993). Note, that in previous, non pH dependent, studies, recovery rates similar to those found in this study were reported (see Chapter 2 section 3, and (Meyer et al. 1989; Hoff et al. 1994a)). As the simple photocycle model is not able to accurately fit the data over the entire measured pH range (only at the pH extremes is it able to accurately describe the data), a more complex model (see Figure 37 a) was designed. With respect to pB formation, the different photocycle reactions in this complex model reflect the following reactions. The formation of pB' from pR is characterized by the protonation of the chromophore by Glu46. This results in a shift of the buried negative charge from the chromophore to Glu46, where it can no longer be effectively stabilized. This introduces a stress situation for the protein that can be resolved by either returning to pR or forming pB. During the formation of pB from pB' the protein undergoes a structural change. We have shown that formation of pB, as described by the complex model, is able to accurately describe pB formation over a large pH range. As such, it also explains the previously observed bi-exponential behavior of the pR to pB transition (Meyer et al. 1987; Hoff et al. 1994a). The large improvement in rmsd shown in Figure 36 d for the fit with the complex model (see Figure 37 a), compared to the simple model (see Figure 35 a), is mainly caused by the improvement of the model regarding pB formation.

For the recovery step a reasonable fit was obtained with the simple model. However, an inverse KDIE was observed for pG recovery (see Figure 35 d). An inverse KDIE could indicate that the breaking of an ionic hydrogen bond is part of a rate determining step, as ionic hydrogen bonds are weaker when deuterium is the bridging atom (Scheiner 2000). In light of the spectral deuterium isotope effect described above, this is unlikely. The spectral deuterium isotope effect suggests that the chromophore is most likely involved in a neutral hydrogen bond for most of the measured pH range. Alternatively, the inverse KDIE could suggest that
deprotonation of the chromophore by a hydroxide ion from solution is a rate determining step. As the deuteroxide ion is a stronger base than the hydroxide ion (Scheiner 2000), it is a more potent proton extractor. This also explains the apparent pOH dependence of this reaction. Furthermore, it has already been shown that when the chromophore is deprotonated it isomerizes more easily (Sergi et al. 2001). This makes it likely that the chromophore needs to be deprotonated before re-isomerization of the chromophore can take place. A mechanism also proposed on the basis of an in depth analysis (Demchuk et al. 2000) of previously reported pH-dependent kinetics (Genick et al. 1997a). Therefore, an additional intermediate representing the pB intermediate in a deprotonated form pBdeprot was introduced in the model. As formation of pBdeprot is a (de)protonation event it is likely that this reaction is reversible. The model incorporating these characteristics is presented in Figure 37 a. This complex model provided an accurate fit of the data over the entire measured pH range. However, at the pH extremes the model was too complex for the data, resulting in an increased inaccuracy of the obtained rate constants.

\( pR \) to \( pB' \)

Formation of \( pB' \) from \( pR \) (Figure 37 b) shows a pOH dependence. A normal KDIE is observed over the whole measured pH range. The \( pR \) to \( pB' \) reaction describes a proton transfer reaction, for which a normal KDIE is expected (Scheiner 2000). It has been suggested in the past that upon \( pB \) formation the chromophore is deprotonated by the solvent (Genick et al. 1997b). A normal KDIE would then only be observed if protonation of the chromophore is achieved via water, as \( D_2O \) is a weaker acid than \( H_2O \). Protonation via the hydronium ion would lead to an inverse KDIE as the deuterated form of the hydronium ion is a stronger acid, like the deuterium oxide ion is a stronger base than the hydroxide ion (Scheiner 2000). Though, protonation of the chromophore by water is also compatible with the obtained data, we favor the interpretation that the chromophore is protonated via Glu46, as suggested on the basis of FTIR results (Xie et al. 1996; Xie et al. 2001).

\( pB' \) to \( pR \)

Formation of \( pR \) from \( pB' \) shows a different pOH dependence as \( pB' \) formation from \( pR \) (compare Figure 37 b and c). From this pOH dependence it is clear that at the pH extremes the equilibrium between \( pR \) and \( pB' \) shifts toward \( pB' \). This explains why the simple model is able to accurately describe \( pB \) formation at the pH extremes. Though, the transition from \( pB' \) to \( pB \) is spectrally silent at 360 nm (see Figure 39 d), it is made visible through the reversible character of the \( pR \) to \( pB' \) reaction which causes the bi-exponential character of the formation of the spectral species representing both \( pB' \) and \( pB \). With a shift of the equilibrium towards \( pB' \) it is no longer possible to make a clear distinction between \( pB' \) and \( pB \) on a kinetic basis.

One would expect the \( pB' \) to \( pR \) reaction to be a proton transfer reaction like the \( pR \) to \( pB' \) reaction. As such a normal KDIE is expected for the whole measured pH range. However, a normal KDIE is only observed for a small pOH range (pOH 5.7 - 6.5). At low pOH values an inverse KDIE and at higher pOH values no KDIE is observed. It is therefore likely that the \( pB' \) to \( pR \) reaction may have more than one reaction mechanism. Between pOH 5.7 - 6.5 a proton transfer mechanism, \textit{i.e.} one in which Glu46 is protonated by the chromophore, may dominate. At low pOH values, a mechanism in which a hydroxide ion extracts a proton from the chromophore and then protonates Glu46 could explain the observed KDIE at low pOH values. In such a mechanism it is likely the protein is already starting to change its fold, allowing a hydroxide ion to enter the chromophore pocket. Then after the proton is transferred via the hydroxide ion, the protein may return to the \( pR \) fold. Here the proton extraction by the hydroxide ion is the rate determining step. If the rate determining step would be protonation of Glu46 by the newly formed water molecule a normal KDIE would be expected. Likewise, at higher pOH values, indirect proton transfer via a hydronium ion could explain the absence of a KDIE. Only here, protonation of Glu46 by the hydronium ion and deprotonation of the chromophore via the newly formed water molecule are both rate determining steps, effectively canceling each others inverse and normal KDIEs. Again, for this mechanism to work it is likely that the protein has already started to change its fold, before returning to \( pR \). Alternatively, a water molecule could first deprotonate the chromophore after which the newly formed hydronium ion protonates Glu46. In a recent molecular dynamics study (Groenhof et al. 2002b) it was calculated that after Glu46 donates its proton to the chromophore, the hydrogen bonding network is very quickly lost and structural changes start to occur, which is in line with the above hypotheses.
Formation of pB from pB’ shows no clear KDIE at low pOH and a normal going to no KDIE at higher pOH values (see Figure 37 d). Basically the protein changes its fold in this step, exposing the chromophore to solvent. The normal KDIE can be explained by the need to break neutral backbone hydrogen bonds. As neutral hydrogen bonds are stronger when deuterium is the bridging atom, it is more difficult to drastically change the protein fold in D₂O. At low pOH values, either it is not necessary to break the backbone hydrogen bonds, or breaking them is no longer rate determining, e.g. due to the presence of a buried charge (Xie et al. 2001) which would then drive the structural change. As at very high pOH values the data are not reliable enough, it is not possible to draw conclusions about the KDIE at those pOH values. However, it is clear that the normal KDIE diminishes in going to higher pOH values. This could be explained by a decrease in the extent of structural change accompanying pB formation, as is also suggested to occur at low pH values (see Chapter 2 sections 2,3, and 4), and thus at high pOH values. Less structural change means less backbone hydrogen bonds need to be broken, which would lead to a smaller KDIE. The diminished need for structural change can be explained by protonation of Glu46 by either water or a hydronium ion, effectively removing the buried charge on Glu46, which has been suggested to be the driving force for structural change in the pB’ to pB reaction (Xie et al. 2001). Coincidentally, protonation of Glu46 by a hydronium ion is also the first step in one of the suggested mechanisms for pR formation from pB’ at high pOH.

As suggested by the analysis with the simple model, the pB to pB² deprot reaction represents deprotonation of the chromophore by a hydroxy ion. As such, the reaction must show a pOH dependence. Interestingly, the inverse KDIE expected for deprotonation of the chromophore via a hydroxide ion is only observed above pOH ~5 (see Figure 38 a). The absence of a KDIE at lower pOH values could be explained by the fact that at low pOH the concentration of hydroxide ions is high and as such the small difference in basicity between hydroxide and deuteroxide ions no longer makes a significant difference for the rate of deprotonation.

In the reverse reaction the chromophore is most likely protonated by a hydronium ion. As such, this reaction is presumably pH dependent. This results in a similar picture as observed for the pB to pB² deprot reaction (see Figure 38 b). A KDIE is only observed for higher pH values, at low pH (below pH ~8) the concentration of hydronium ions is sufficient to mask the difference in acidity between the deuterated and protonated form of the hydronium ion.

The mechanisms described for the reversible reaction of the pB to pB² deprot transition imply that the obtained rate constants contain a contribution from the hydroxide or hydronium ion concentration, i.e. \( k_3 = k_{3c} [\text{OH}^-] + k_{3d} [\text{H}_3\text{O}^+] \). However, after correction of the rate constants, the new rate constants predict that the equilibrium between pB and pB² deprot is reached within 100 ns and has a \( pK_a \) of ~7.3. This is in contradiction with the \( pK_a \) of 10 that is found experimentally for the equilibrium between pB and pB² deprot (see above and Figure 34 a). Therefore, the pB² deprot in the complex model not only represents deprotonation of the chromophore, but also implies a certain protein fold, that allows isomerization to take place. Here, deprotonation of the chromophore by hydroxide ions is a rate-determining step only at higher pOH values. Otherwise, structural change is the rate-determining step. Here the structural change leads to no KDIE at lower pOH values either due to the small amount of structural change necessary or due to cancellation of different isotope effects. Similarly, protonation of the chromophore in the return reaction is only a rate determining step at higher pH values and structural change is rate determining where no KDIE is observed.

The inverse KDIE for the pB to pB² deprot reaction disappears below pOH ~5. A \( pK_a \) of 4 (\( pK_a \) 10) was determined for the equilibrium between pB and pB² deprot, with a cooperativity constant \( n \) in the Henderson-Hasselbalch equation of 0.74. As such at pOH ~5 the equilibrium contains approximately 15% pB² deprot and 85% pB. Assuming that in pB² deprot the phenolate part of the chromophore no longer is involved in hydrogen bonds with the protein and is exposed to solvent, the \( pK_a \) of the chromophore would be expected to be 8.7 with \( n \) is 1, as suggested by pH titration of the chromophore in the denatured protein. It is assumed here that the \( pK_a \) of the chromophore in the cis configuration is very similar to that of the chromophore in the trans configuration. In this case at the point the KDIE disappears (pH ~8) in the pB² deprot to pB reaction an equilibrium between these two intermediates is expected to have 85% pB² deprot and 15% pB. These values are the inverse of the values found in the pB to pB² deprot reaction. Note that the true equilibrium between pB and pB² deprot has a \( pK_a \) of 10, but that the \( pK_a \) that is felt by the pB² deprot species in the reaction from pB² deprot to pB is 8.7 in the above assumption. The observation that the implied pB² deprot to pB ratios coincide inversely with the
disappearance of the observed KDIE is a sign that the introduction of the pB to pB\textsuperscript{deprot} equilibrium reaction, in the recovery step of PYP, is justified.

The precise characteristics of the structural change in the pB to pB\textsuperscript{deprot} step are unclear. It is likely though that the protonation state of the Glu46 residue has an influence on this step. This is based on information about the recovery rate of the Glu46Gln mutant, which displays a faster recovery of the ground state than the wild type protein (Genick \textit{et al.} 1997a). In the Glu46Gln an amide group effectively replaces the carboxyl group of residue 46. As this is a neutral group, it is likely that Glu46 needs to be protonated in pB\textsuperscript{deprot} in order for the final recovery step to take place. This is in line with an earlier observation where it was proposed that for recovery to take place, the chromophore has to be in a deprotonated state and the Glu46 needs to be in protonated state (Demchuk \textit{et al.} 2000).

\[ \text{pB}^{\text{deprot}} \rightarrow \text{pG} \]

In the final reaction from pB\textsuperscript{deprot} to pG (see Figure 38c) it is likely that isomerization is the rate-determining step. As isomerization is not likely to involve any exchangeable protons, no KDIE is expected for this reaction. However, this only seems true below pOH \textasciitilde 6.5. Above pOH \textasciitilde 6.5 an inverse KDIE is observed. Interestingly, this is very similar to what was observed in the analysis with the simple model of the 360 nm data (see Figure 35c), in which isomerization occurs, at least in part, photoactively. It would therefore seem that the observed KDIE is characteristic of the refolding event in the recovery after isomerization has taken place. However, any structural changes following isomerization will likely occur at least 2 orders of magnitude faster than the rates observed for the pB\textsuperscript{deprot} to pG photocycle step. This conclusion is based on experiments in which acid denatured PYP is re-natured (Lee \textit{et al.} 2001b) in a pH range overlapping with the pH range showing the inverse KDIE. An alternative explanation is that due to the unfavorable $pK_a$ of 10 for deprotonation of the chromophore in pB that at high pOH (low pH) the presence of hydroxide ions may be important for the dark isomerization step in order to keep the chromophore deprotonated long enough for isomerization to take place.

When ground state recovery is compared between the simple and complex model, it is worth noting that an inverse KDIE was observed over almost the entire measured pH range with the simple model, where the kinetics showed a pOH dependence. With the complex model, the obtained rates showed an inverse KDIE for only part of the measured pH range for the pB to pB\textsuperscript{deprot} equilibrium and the pB\textsuperscript{deprot} to pG step. Also, not only reactions that showed a pOH dependence were observed but also a reaction that showed a pH dependence. Additionally, through plausible assumptions we were able to show that the point at which the KDIE disappears in both reactions of the pB to pB\textsuperscript{deprot} equilibrium occur inversely at the same implied pB to pB\textsuperscript{deprot} ratios. All this supports the relevance of the introduction of the pB\textsuperscript{deprot} intermediate.

**Spectral kinetic analysis**

With the insight we have obtained through the analysis of the time traces, we have applied the new photocycle model to spectral data of the photocycle recorded at pH 8.10 and 9.55. As these data had nanosecond time resolution, we were also able to observe a transition from one pR-like intermediate to another. Such a transition was recently observed with transient grating (Takeshita \textit{et al.} 2002a; Takeshita \textit{et al.} 2002b), and reflects structural relaxation of the protein far from the chromophore. Though, it is claimed that this transition is not observable via UV/Vis spectroscopy, an earlier UV/Vis study (Hoff \textit{et al.} 1994a) already noted an additional pR-like component could be observed. However, it was ignored as the quality of the data was deemed not good enough to confidently make the distinction between the two pR components. Inclusion of the transition of pR1 to pR2 shows that the two pR intermediates have very similar absorption spectra differing mainly in their extinction coefficient. The reversible character of the pR to pB\textsuperscript{-} reaction is clearly observed in the dataset recorded at both pH 8.10 and 9.55. This enabled us to determine an absorption spectrum for both pB\textsuperscript{-} and pB. It is notable that these two spectra are very similar but not identical. The absorption spectrum of pB\textsuperscript{-} is slightly red-shifted with respect to pB. As the quality of the CCD data was not good enough, we were not able to obtain reliable information with regard to the pB\textsuperscript{deprot} intermediate introduced on the basis of the KDIE of the photocycle recovery step. This lack of quality of the data is evident from the absence of an additional small shoulder around 430 nm in the pB spectrum at pH 9.55 (data not shown) as would be expected on the basis of the spectra shown in Figure 34a. It is likely that the absorption spectrum of the pB\textsuperscript{deprot} intermediate is very similar to that of pG. This is also suggested by the fact that not only deprotonation of the chromophore but also the folding state of PYP is an important feature of this proposed intermediate. As such the absorption spectrum is not necessarily the same as that of the shoulder observed in the pB spectrum at high pH (see Figure 34a), which is also caused by a pB species with
a deprotonated chromophore. The different fold of \( \text{pB}^{\text{deprot}} \) may cause an additional red shift. In fact, an analysis incorporating \( \text{pB}^{\text{deprot}} \) resulted in a pG like spectrum for \( \text{pB}^{\text{deprot}} \) (data not shown). However, as mentioned before we feel the data is not of sufficient quality to confidently draw such a conclusion. It would however explain why this intermediate has not been observed before. Also, it is in line with the observation that refolding, as measured via the Nile Red fluorescent probe (see Chapter 2 section 3) seems to be slightly slower than recovery monitored with UV/Vis spectroscopy at 468 nm.

**Acid denaturation**

When acid denaturation is compared in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) it appears that PYP is less stable in \( \text{D}_2\text{O} \). As ionic hydrogen bonds are less strong in \( \text{D}_2\text{O} \), it is likely that such (a) bond(s) causes the decreased stability. The prime candidates for this are the hydrogen bonds between the chromophore, and Glu46 and Tyr42. These two hydrogen bonds then have a greater influence than the neutral hydrogen bonds which are much more abundant and are stronger when deuterium is the bridging atom.

### 3.4 Concluding remarks

By comparing pH effects using both \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) as solvent, we have been able to show that most characteristics in PYP are not pH, but rather pOH dependent; an essential distinction when comparing data obtained in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \). Furthermore, we have shown that the pB’ intermediate, which was introduced on the bases of FTIR experiments and contains a protonated chromophore (Xie et al. 2001), is in equilibrium with pR. Its absorption spectrum is slightly red-shifted with respect to pB. On the basis of the KDIE of the photocycle-recovery reaction we were able to show that deprotonation of the chromophore is an essential step before re-isomerization can take place. This necessitates the introduction of a new photocycle intermediate, \( \text{pB}^{\text{deprot}} \), to represent this step. This intermediate is in equilibrium with pB. We have shown for the first time that the absorption maximum of pB (containing a protonated chromophore) is pH dependent. Furthermore, the stability of PYP is mainly governed by ionic hydrogen bonds.