Structure/function relations in Photoactive Yellow Protein

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Chapter 3

Controlled Reduction of the Humidity Induces a Short-cut Recovery Reaction in the Photocycle of Photoactive Yellow Protein

Michael A. van der Horst, Ivo H. M. van Stokkum, Norbert A. Dencher and Klaas J. Hellingwerf

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Chapter 3

3.1 Abstract

The photocycle of the blue-light photoreceptor protein Photoactive Yellow Protein (PYP) was studied at reduced relative humidity. Photocycle kinetics and spectra were measured in thin films of PYP in which the relative humidity was set at values between 5 and 98% with saturated solutions of various salts. As humidity decreased, photocycle transition rates changed, until at low humidity (< 50%) an authentic photocycle was no longer observed, and the absorption spectrum of the dark, equilibrium, state of PYP started to shift to 355 nm, i.e. to a form resembling that of pB_{dark}. At moderately reduced humidity (i.e. > 50 % r.h.), an authentic photocycle is still observed, although its characteristics differ from those in solution. As humidity decreases the rate of ground state recovery increases, while the rate of depletion of the first red-shifted intermediate pR dramatically decreases. The latter observation contrasts all so-far known modulations of the rate of the transition of the red-shifted- to the blue-shifted intermediates of PYP, which is consistently accelerated by all other modulations of the mesoscopic context of the protein. Under these same conditions the long-lived, blue-shifted intermediate was formed not only with slower kinetics than in solution, but also to a smaller extent. Global analysis of these data indicates that in this low humidity environment the photocycle can take a different route than in solution, i.e. part of pG recovers directly from pR. These experiments on wild type PYP, in combination with observations on a variant of PYP obtained by site-directed mutagenesis (the E46Q mutant protein) further document the context dependence of the photocycle transitions of PYP and are relevant for the interpretation of results obtained in both spectroscopic and diffraction studies with crystalline PYP.

3.2 Introduction

Photoactive Yellow Protein is the small, water-soluble blue-light photoreceptor protein, initially isolated from Ectothiorhodospira (now: Halorhodospira) halophila (Meyer, 1985; Meyer et al., 1987), for recent reviews, see (Cusanovich and Meyer, 2003; Hellingwerf et al., 2003). As its chromophore, the protein contains p-coumaric acid, bound to its unique cysteine side chain, via a thiol-ester linkage (Van Beeumen et al., 1993; Hoff et al., 1994a). After absorption of a blue photon, PYP enters a photocycle, in which - at room temperature - two main intermediates can be discriminated: The red-shifted intermediate pR, in which the configuration of the chromophore has been altered from trans to cis, is formed in the nanosecond time domain. Upon protonation of the phenolic oxygen of
the chromophore by the side chain of E46, the blue-shifted intermediate pB is formed on the sub-millisecond timescale. This reversible photocycle is completed by recovery of the ground state, pG, in 150 ms (Meyer et al., 1987; Hoff et al., 1994b).

The properties of PYP have been studied using a variety of (spectroscopic) techniques, under a wide range of conditions, such as in solution, in thin films, or in a crystalline lattice (Kort et al., 2003; Mano et al., 2003), and at temperatures ranging from 77K to 340K (Hoff et al., 1992; Imamoto et al., 1996). Whereas some of the properties of PYP are context-independent, some clearly differ with variations of the mesoscopic (i.e. molecular) context of the protein. At cryogenic temperature, for example, photocycle intermediates have been detected that are not observed at room temperature (i.e. PYP_B, PYP_L, PYP_BL, PYP_H (Imamoto et al., 1996)). Another context-dependent feature is the structural change that accompanies formation of the pB intermediate: Whereas crystallographic analyses have revealed that, upon light activation of PYP, only small structural changes occur close to the chromophore (Genick et al., 1997a; Genick et al., 1998; Perman et al., 1998; Ren et al., 2001b), several spectroscopic techniques indicate that large structural changes occur throughout the protein, that are so profound that they can even be described as a transient partial protein unfolding (Van Brederode et al., 1996; Hoff et al., 1999; Xie et al., 2001; Chen et al., 2003a). Xie et al. (Xie et al., 2001) showed, using time-resolved FTIR spectroscopy both for a solution of PYP and for a slurry of small crystals, that the large structural changes that occur in solution are indeed absent when PYP is confined by a crystalline lattice.

The plasticity of PYP, as it has become apparent from the sensitivity of the characteristics of its photocycle transitions to the mesoscopic context of PYP, has not only come as a surprise to many protein chemists, it also complicates the detailed four-dimensional characterization of the alterations that take place in the structure of the protein following light absorption. A proper example is the translation of diffraction data of PYP, recorded during progression of the protein through its reversible photocycle in so-called Laue diffraction experiments (Genick et al., 1997a; Perman et al., 1998; Ren et al., 2001b; Schmidt et al., 2003), into alterations in the spatial structure of the protein. Before this type of analysis can be completed, a detailed analysis of the meta-stable species, and their kinetics, involved in the photocycle of PYP under those conditions, has to be completed (e.g. (Moffat, 2003; Schmidt et al., 2003)). Spectroscopic analyses of photocycle transitions in crystalline PYP, however, are technically rather challenging (see e.g. (Kort et al., 2003; Mano et al., 2003)). Furthermore, the basic structure of photocycle models, used
to analyze the spectroscopic observations made on crystalline PYP, is generally assumed to be that of PYP dissolved in aqueous buffer at neutral, to slightly alkaline pH. However, as in protein crystals the level of hydration may be sub-optimal, and because FTIR experiments provided preliminary indications that reduced hydration affects the photocycle of PYP (Xie et al., 1996; Brudler et al., 2001; Xie et al., 2001) we set out to characterize the photocycle of PYP under well-controlled conditions of reduced relative humidity. Similar studies on Bacteriorhodopsin performed earlier (reviewed in (Dencher et al., 2000)) also have revealed important aspects of the photocycle of this light-activated proton pump. For this latter photoreceptor protein it was shown that both the kinetics of the long-lived M intermediate and the efficiency of proton pumping strongly depend on the relative humidity (Korenstein and Hess, 1977; Varo and Lanyi, 1991). It was found that at a relative humidity (r.h.) of 50-60% proton pumping and light-induced conformational changes in the protein vanish, whereas a photocycle at these low humidities still is observable (Sass et al., 1997).

Future Molecular Dynamics simulations of the dynamics in the structure of PYP after light-activation, and the role of specific water molecules therein, may benefit strongly from experimental data on the effect of reduced hydration on photocycle characteristics in PYP. Our results show that photocycle models for PYP of a novel structure have to be considered in these future analyses, based on the observation that the ground state may recover directly from a red-shifted photocycle intermediate.

3.3 Experimental procedures

Sample preparation

PYP samples were prepared as described before (Hendriks et al., 2002). Protein samples had a ratio \(A_{280}/A_{446}\) of \(\leq 0.5\) and were used without removal of the N-terminal hexahistidine tag in 10 mM Tris-HCl, pH 8. Dehydrated films were prepared by spotting concentrated protein solutions (50 \(\mu\)l, \(\sim\)20 mg/ml) on a quartz slide. The protein solution was dried at room temperature, by placing the slide in a desiccator at reduced pressure (\(\sim\)0.5 bar) for \(\sim\)3 hours. The slide was then placed in a 1 x 1 cm cuvet on top of a small spacer (Figure 1). The degree of hydration was set by adding 200 \(\mu\)l of a saturated salt solution (see Table 1) to the cuvet, after which it was closed using parafilm. The films were allowed to equilibrate overnight with the different relative humidities produced by the various saturated salt solutions. Occasionally, the cuvet was not sealed, allowing the film to equilibrate to the ambient relative humidity, which was measured, and was typically 50 – 60%.
Steady-state and transient (millisecond/second) UV/Vis measurements
Steady-state UV/Vis spectra and photocycle kinetics on a millisecond to second time-scale were measured with an HP 8453 UV/Vis diode array spectrophotometer with a time resolution of 100 ms.

Laser-flash photolysis spectroscopy
To study (sub-) millisecond events we used an Edinburgh Instruments Ltd. LP900 spectrometer equipped with a photomultiplier and a CCD camera, in combination with a Continuum Surelite I-10 YAG-laser and Continuum Surelite OPO (for details, see Hendriks et al., 1999b).
Rate constants were determined by fitting the measured data using multi-exponential recovery functions in Microcal Origin. Global analysis was performed as described in (Hoff et al., 1994b; Hendriks et al., 2003).

Fluorescence
For fluorescence analyses an Aminco Bowman Series 2 Luminescence Spectrometer was used. To measure emission spectra, the excitation wavelength was set at 445 nm. The slit width used was 4 nm. The samples used, both in solution or as a dehydrated film, typically had an OD at 445 nm of 0.2. To measure fluorescence at different relative humidities, saturated salt solutions were placed in the sealed cuvet house, so that it was not necessary to readjust the position of the film in between the different measurements.

Table 1: Saturated salt solutions used, with the corresponding relative humidity

<table>
<thead>
<tr>
<th>Salt</th>
<th>r.h.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄</td>
<td>98</td>
</tr>
<tr>
<td>KCl</td>
<td>86</td>
</tr>
<tr>
<td>NaCl</td>
<td>75</td>
</tr>
<tr>
<td>NaBr</td>
<td>58</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>29</td>
</tr>
</tbody>
</table>

Figure 1:
Setup showing a PYP film, deposited on quartz slide, inserted into a closed cuvet that allows modulation of the relative humidity (r.h.).
3.4 Results

Ground state spectra
To study the effect of dehydration on the photophysical properties of PYP, thin dehydrated films, made of concentrated protein solutions, were prepared on quartz slides, as described above (see also Figure 1). Using this method, PYP films with a spatially homogeneous optical density (less than 1% positional variation in transmission) were obtained. First, dark state spectra of these films at different relative humidities were recorded (see Figure 2). At the two highest humidities tested, i.e. 98% and 86%, the absorption spectrum was very similar to that of PYP in aqueous solution. However, when a lower humidity was set in the film, two changes in the spectrum were observed: (i) the main absorption peak was slightly red-shifted, from 446 nm to 449 nm, and (ii) the absorbance of the main peak decreased, and a new species was formed, with an absorption maximum at 355 nm. The latter feature can clearly be seen in difference spectra (e.g. solution spectrum minus 59% r.h.; Figure 2b). A similarly small red-shift of the main absorption peak has been seen before at low temperature in spectra of PYP in glycerol-containing buffers (Hoff et al., 1992; Imamoto et al., 1996) and in crystalline PYP (Kort et al., 2003) and may be ascribed to small changes in the structure of the protein.
that may modulate the length of the hydrogen bond between E46 and the phenolate chromophore (Kroon et al., 1996)). For convenience we refer to the blue-shifted intermediate as pB_{dark}, the species that is formed in solution in the dark at low pH, because of protonation of the chromophore. By recording the formation of pB_{dark} in time, the equilibration could be traced as a function of time, as can be seen in Figure 2c. In the selected geometry of a closed cuvet this equilibration turns out to be bi-exponential, with a large part of the conversion being completed within 5 minutes. Nevertheless, because of the slow component involved, equilibration was always allowed to occur overnight before measurements were started. The nature of the two components is not known; it might arise from bulk water and protein-bound water that evaporate with different rates.

**Photocycle kinetics**

First, the rate of recovery of the ground state pG, after blue-light excitation, was measured in partially dehydrated films. Transient absorption changes in the main absorption peak (445 nm) were recorded after excitation with 445 nm laser light. In moderately dehydrated films, *i.e.* with a relative humidity above 50%, an authentic photocycle was observed. Strikingly, the rate of ground state recovery (*i.e.* pB → pG) in dehydrated films increases as compared to the ground state recovery in solution. Whereas at a relative humidity of 98%, the recovery rate is comparable to the rate in solution, relative humidities of 86% and 75% yield rate constants that have increased up to five-fold (see Table 2). Closer examination of these data and fitting the traces to a multi-exponential recovery function shows that the recovery in these dehydrated films is bi-exponential, whereas the recovery in solution is close to mono-exponential (see *e.g.* (Meyer et al., 1987; Hoff et al., 1994b)).

<table>
<thead>
<tr>
<th></th>
<th>pG decay (s⁻¹)</th>
<th>pB formation (s⁻¹)</th>
<th>pB decay (s⁻¹)</th>
<th>pG formation (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>ND</td>
<td>1.9*10⁻⁷</td>
<td>ND</td>
<td>20</td>
</tr>
<tr>
<td>E46Q</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.7 (91%)</td>
</tr>
<tr>
<td>E46Q</td>
<td>417 (67%)</td>
<td>417 (67%)</td>
<td>14 (68%)</td>
<td>417 (67%)</td>
</tr>
<tr>
<td>E46Q</td>
<td>72 (33%)</td>
<td>72 (14%)</td>
<td>1.7 (13%)</td>
<td>72 (19%)</td>
</tr>
<tr>
<td>E46Q</td>
<td>180 (53%)</td>
<td>180 (53%)</td>
<td>22 (42%)</td>
<td>180 (53%)</td>
</tr>
<tr>
<td>E46Q</td>
<td>52 (47%)</td>
<td>52 (47%)</td>
<td>13 (11%)</td>
<td>52 (47%)</td>
</tr>
</tbody>
</table>

Table 2: WT and E46Q PYP photocycle rate constants, in solution and at reduced humidity.
ND: not determined

As can be seen in Table 2, the recovery traces in the dehydrated films show a clear second exponent, although no trend can be observed
regarding the relative amplitude of this component. Surprisingly, at reduced humidity, the rate of pB formation has slowed down when compared to the rate observed in PYP in solution (see Table 2). This is the first protein (environment) modification in which pB formation is slowed down; in all other cases studied so far (e.g. point mutations, truncations, addition of salts and/or organic solvents, etc.) the rate of pB formation increases when compared to wildtype, whereas the rate of pG recovery decreases (see e.g. references (Genick et al., 1997b; Devanathan et al., 1998; van Aalten et al., 2002b)); note, however, that the E46Q mutant is an exception to this latter rule: The pG recovery rate has increased in this mutant). Furthermore, traces recorded at 495 nm show that - compared to solution - the rate constant of depletion of this intermediate in partially dehydrated films is extremely large (417 s\(^{-1}\) and 72 s\(^{-1}\), see Table 2). The long-lived presence of the pR intermediate was confirmed by measuring spectra at different timepoints after excitation, using a CCD camera (result not shown). As can be seen in these difference spectra, pR absorption is indeed present until at least 10 microseconds after excitation. At lower relative humidities, the fractional amount of PYP protein that enters the photocycle upon a single laser pulse decreases, until at values below 50% r.h., no photocycle transitions are observed anymore. These findings show that the photocycle of PYP in solution and in (partially) dehydrated films proceeds differently. To derive a kinetic scheme of the photocycle at these reduced humidities, we analyzed the photocycle kinetics at one relative humidity using global analysis, as is described below.

The photocycle kinetics at a relative humidity of 86% were studied in detail at five wavelengths: 355, 400, 447, 370 and 490 nm (Figure 3). Note that a small positive difference absorption is instantaneously present at 355 nm, which is attributed to pR – pG. Note further that the rate of decay of pR – pG difference absorption at 470 and 490 nm differs from the rise of pB – pG difference absorption at 355 nm. Also a small fraction of pR decays with ~100 ms. These data were globally analyzed using five exponential decays with lifetimes of 0.1, 1.9, 14, 90 ms and 0.6 s. The Decay Associated Difference Spectra (DADS) are depicted in Figure 4A, whereas the normalized DADS are shown in Figure 4B. These DADS can be interpreted as follows: the first DADS (solid, 0.1 ms) possesses very small amplitudes and represents a small evolution of the pR – pG difference absorption spectrum. The second DADS (dot, 2 ms) is typical for the formation of pB from pR: a loss of absorption at 447 - 490 nm concomitant with a rise at 355 nm. The third DADS (dash, 14 ms) is atypical: a loss of absorption at 470 and 490 nm and a rise at 355 - 447 nm. This can be attributed to a decay of pR to both pB and the ground state.
The fourth DADS (dotdash, 90 ms) represents the decay of pB to the ground state, but from the small positive amplitude at 490 nm it is clear that this state is still in equilibrium with its pR precursor. Finally, the fifth DADS (chaindash, 0.6 s) is the pB – pG difference absorption spectrum; its amplitude is about five times smaller than that of the fourth DADS. It is interpreted as a small fraction of pB that decays to the ground state more slowly.

Figure 3: PYP photocycle kinetics at 86% reduced humidity.
Numbers on the ordinate indicate the five monitoring wavelengths. The dashed lines represent a global five-exponential fit to the measured data. Note that the time axis is linear up to 0.2 ms, and logarithmic thereafter.

Figure 4: Estimated Decay Associated Difference Spectra
A: Estimated Decay Associated Difference Spectra (DADS) from the data presented in Figure 3. B: Normalized DADS. Key: solid, 0.1 ms; dot, 2 ms; dash, 14 ms; dotdash, 90 ms; chaindash, 0.6 s.
Based upon these observations a target analysis was performed using the kinetic scheme depicted in Figure 5A. This is a minimal scheme that can account for the observed multiexponential decay of both pR and pB. The initial pR relaxes in 0.1 ms to two different types called pR' and pR'', which are assumed to possess identical Species Associated Difference Spectra (SADS), but decay differently: one can decay directly to the ground state, and to pB, whereas the other decays only to pB, with a small back reaction. Finally, to describe the slow decay of pB a branching to pB' and the ground state is invoked. Again, pB and pB' possess identical SADS. Because of these spectral constraints the three branching ratios and equilibrium (which require four parameters) can be estimated. The estimated SADS which are depicted in Figure 5B, are typical for pR, relaxed pR and pB. Thus instead of five DADS and five lifetimes, three SADS and nine kinetic parameters (see Table 2) are sufficient to describe these measurements.

About 35% of pR relaxes to a state which can decay directly to the ground state in 14 ms.

Thus 19% of the photocycling states takes this short cut, i.e. recovers to pG without formation of the pB intermediate.

The dependence of the photocycle transition rates on the hydration of the protein was also studied in the PYP variant E46Q. In this variant, Glu46, that is hydrogen-bonded to the chromophore and donates a proton to this chromophore during pB formation (Xie et al., 1996), has been replaced by a Gln residue.

**Figure 5: PYP photocycle at 86% relative humidity.**

(A) Photocycle scheme used for the target analysis of the 86% humidity data. Note the short-cut reaction from pR'' to pB. Branching fractions and rate constants are collated in Table 2. The steps before pR is formed, are drawn here for completeness, they are not included in the analysis.

The dark spectra of this variant showed the same dependence on humidity as the wild-type protein, \textit{i.e.} a small red-shift of the main absorption peak and pB\textsubscript{dark} formation at low relative humidities. Using this variant in kinetic studies, the same trend was found as in wild-type PYP; after blue-light excitation, the rate of pG recovery increased, whereas the rate of pB formation has decreased (see Figure 6).

![Figure 6: Normalized E46Q PYP photocycle kinetics in solution and at 86\% reduced humidity.](image)

Kinetic traces are shown for solution (outer curve) and 86\% \textit{r.h.} (inner curve). The dashed lines represent the fit to the measured data using a slightly modified scheme from Figure 5A and the parameters collated in Table 2. In order to compare the groundstate recovery with the pB decay, the 445 nm trace has been inverted. Note that the time axis is linear up to 0.2 ms, and logarithmic thereafter.

\textit{Fluorescence emission}

Fluorescence excitation and emission spectra were recorded for PYP films at different relative humidities. Both the excitation and emission spectra (see Figure 7A) of the (dehydrated) PYP film have the same shape and maximum as PYP in solution. However, the quantum yield of fluorescence $\Phi_f$ was found to be dependent on the relative humidity. This was concluded from the results displayed in Figure 7a, where the intensities of the emission signals are directly compared, and in Figure 7b, where the equilibration of a dehydrated film from 59\% \textit{r.h.} to 86\% \textit{r.h.} is followed by recording the intensity of the emission signal as a function of time. At these relative humidities, no pB\textsubscript{dark} formation is observed. At lower relative humidity the effect of humidity on the quantum yield of fluorescence is less pronounced. At 29\% \textit{r.h.}, where pB\textsubscript{dark} is formed, the film was also excited at 360 nm. This resulted in a small emission signal at $\sim$ 430 nm (result not shown). All changes were fully reversible upon subsequent increases of the humidity.

3.4 Discussion

We were able to prepare thin films of PYP that allowed us to study the photochemical properties of this protein as a function of the degree of hydration. This system has advantages when compared to spectroscopy in crystals for two reasons: first, the practical reason that these samples are not only easier to work with than the crystalline samples, but also can be made with a relatively low optical density, simplifying spectroscopic studies.
Secondly, any effects we see here are a result of protein hydration alone, whereas in crystals effects in principle can also be due to the high protein concentration and inter-protein contacts in the crystal (Van Aalten et al., 2000). However, the latter experiments obviously remain highly interesting because of the opportunity to simultaneously obtain structural and spectroscopic information.

Since most differences we describe here are found in proportion with the reduction in humidity, they must indeed be a result of the low hydration, and not of the high protein concentration in the film.

We found that variation in the degree of hydration ultimately results in changes in the dark, steady state spectrum of PYP. Most notably, at low humidity (< 50%) we see that a pB\textsubscript{dark}-like state is formed. The formation of a blue-shifted species, similar to the photocycle intermediate pB and the low-pH induced pB\textsubscript{dark}, indicates protonation of the chromophore. Presumably, this occurs due to (partial) unfolding of the protein at these low humidities or because of direct protonation of the chromophore. Here, pB\textsubscript{dark} formation starts at a relative humidity of \(~50\%\). The clear isosbestic point at 380 nm indicates that a two-state transition is involved. The conversion is fully reversible, as can be seen when (partially) rehydrating the film (not shown).

At low relative humidities, \textit{i.e.} below 50\% r.h., a photocycle is not observed after excitation with a short pulse of blue light. However, prolonged illumination with continuous blue light, does yield (small) spectroscopic changes at all humidities tested. Interestingly, however, at relative humidities above 50\%, the protein does show an authentic
photocycle using a single-turnover flash, albeit with characteristics that differ substantially from the photocycle in solution. Most importantly, the rate of pG recovery has increased, whereas the rates of pB formation and pR depletion have decreased. The small amount of pB that is formed, together with the exceptionally long lifetime of pR, and the fast recovery of pG, show that at reduced humidity, the photocycle can proceed through a different route than in solution. Detailed global analysis leads to the conclusion that at reduced hydration, from pR, a part of the population can directly return to the ground state, *i.e.* without formation of the blue-shifted intermediate pB and the accompanying structural changes. Recently, the possibility of a branching reaction in the photocycle of PYP was proposed (Borucki *et al.*, 2003). It was shown for E46Q and E46A variants of PYP that at high pH values, a precursor of pB can directly relax to the groundstate pG.

By measuring the intensity of fluorescence emission, the early events in the PYP photocycle are probed. We found that in (partly) dehydrated films, the quantum yield of fluorescence has increased compared to solution. Whereas at 86% r.h. the quantum yield is not significantly different from the quantum yield of 0.22% that is found in solution, decreasing the humidity to 59% leads to a quantum yield of fluorescence that has increased by ~25%. The change in relative humidity could also be followed in time using the fluorescence signal, resulting in a time trace with kinetics comparable to those found when following the change in humidity by UV/Vis spectroscopy. These results show that the hydration state of the protein is also important during the early events of the photocycle. Probably the longer lifetime of the excited state is accompanied by slower formation of the first photocycle intermediate $l_0$.

Summarizing, we propose a photocycle model where at low hydration, pR can directly relax to pG without pB formation and the accompanying large structural changes, as depicted in Figure 5A. These results stress the importance of the mesoscopic context of Photoactive Yellow Protein in general, and the hydration state in particular, *i.e.* they show the extreme plasticity of the photocycle transitions of PYP. This is especially of great importance when using techniques to study PYP characteristics that make use of samples that are not in aqueous solution. For example, when structurally characterizing photocycle intermediates using Laue crystallography, it is extremely important to use a photocycle model that correctly describes the phenomena taking place in those specific conditions. Furthermore, Molecular Dynamics simulations that study the dynamics in the structure of PYP after light-activation, and the role of specific water molecules therein, are underway; the experimental data on the effect of reduced hydration on photocycle...
characteristics in PYP we present here will be of great benefit to these types of studies.

Acknowledgements:
We thank I. Peset for help with the initial characterization of PYP films, R. Cordfunke for expert technical assistance and drs. S. Yeremenko for his help in maintenance and improvement of the set-up for ns time-resolved UV/Vis absorption spectroscopy.