Controlled Reduction of the Humidity Induces a Shortcut Recovery Reaction in the Photocycle of Photoactive Yellow Protein

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ABSTRACT: The photocycle of the blue-light photoreceptor protein Photoactive Yellow Protein (PYP) was studied at reduced relative humidity (RH). Photocycle kinetics and spectra were measured in thin films of PYP in which the relative humidity was set at values between 29 and 98% RH with saturated solutions of various salts. We show that in this range, approximately 200 water molecules per PYP molecule are released from the film. As humidity decreased, photocycle transition rates changed, until at low humidity (RH < 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, that is, to a form resembling that of pB_{dark} . At moderately reduced humidity (i.e., >50% RH), 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, that is, 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.

Photoactive Yellow Protein (PYP¹) is the small, watersoluble blue-light photoreceptor protein, initially isolated from Ectothiorhodospira (now: Halorhodospira) halophila (1, 2); for recent reviews, see refs 3, 4. As its chromophore, the protein contains p-coumaric acid, bound to its unique cysteine side chain, via a thiol-ester linkage (5, 6). 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 submillisecond time scale. This reversible

photocycle is completed by recovery of the ground state, pG, in 150 ms (2, 7).

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 (8, 9), and at temperatures ranging from 77 to 340 K (10, 11). Whereas some of the properties of PYP are contextindependent, 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}, and PYP_H (11)). Another contextdependent 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 (12-15), 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 (16-19). Xie et al. (18) 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.

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¹ Darmstadt University of Technology. ¹ Abbreviations: DADS, decay-associated difference spectrum; PYP, Photoactive Yellow Protein; pG, stable ground (receptor) state of PYP; pR (or I_1 , or PYP_L), red-shifted intermediate of PYP; pB (or I_2 , or PYP_M), blue-shifted (signaling) intermediate of PYP; pBdark, blueshifted (protonated, low pH) form of PYP; RH, relative humidity; SADS, species-associated difference spectrum.

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 but 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 (12, 14, 15, 20) 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 (for example, see refs 20, 21). Currently, optical spectroscopy offers the best time resolution and signal-to-noise ratio for such studies (22). Spectroscopic analyses of photocycle transitions in crystalline PYP, however, are technically rather challenging (for example, see refs 8, 9). 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 protein may be confined in conformational space, and because FTIR experiments provided preliminary indications that reduced hydration affects the photocycle of PYP (18, 23, 24), 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 ref 25) 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 (26, 27). It was found that at a RH of 50-60% proton pumping and light-induced conformational changes in the protein vanish, whereas a photocycle at these low humidities still is observable (28).

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.

EXPERIMENTAL PROCEDURES

Sample Preparation. PYP samples were prepared as described before (29). Protein samples had a ratio A_{280}/A_{446} of ≤ 0.5 and were used without removal of the N-terminal hexa-histidine tag in 10 mM Tris-HCl, pH 8. Dehydrated films were prepared by spotting concentrated protein solutions (~50 μ L, ~20 mg/mL) on a clean quartz slide. The protein solution was dried at room temperature by placing the slide in a desiccator at reduced pressure (~0.5 bar) for ~3 h. The slide was then placed in a 1 cm × 1 cm cuvette on top of a small spacer. The degree of hydration was set by adding 200 μ L of a saturated salt solution (see Table 1) to the cuvette, after which it was closed using Parafilm. The films were allowed to equilibrate overnight with the different

Table 1: Saturated Salt Solutions Used, with the Corresponding Relative Humidity

salt	RH
CuSO ₄	98
KCl	86
NaCl	75
NaBr	58
$CaCl_2$	29

relative humidities produced by the various saturated salt solutions. Occasionally, the cuvette was not sealed, allowing the film to equilibrate to the ambient relative humidity, which was measured, and was typically 50–60%. The number of water molecules involved in a transition between two different relative humidities was determined using a Sartorius M2P microbalance; small differences in total weight of a quartz slide with a protein film were determined during equilibration to a specific relative humidity within the setup of the microbalance.

Steady-State and Transient UV/Vis Measurements. Steadystate 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. 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 ref 30). Global and target analysis was performed as described in refs 7, 31, and 32. Spectra shown in Figures 4 and 5b were smoothed using spline functions.

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

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. 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 1). At the two highest humidities tested, that is, 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, the following two changes in the spectrum were observed: (i) the main absorption peak was slightly red-shifted, from 446 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% RH; Figure 1b). A similarly small red-shift of the main absorption peak has been seen before at low temperature in spectra of PYP in



FIGURE 1: UV/vis spectra of PYP at reduced humidity: (A) solution spectrum (solid line) and the spectra in the range from 86% to 50% RH (dashed lines); (B) difference spectrum (solution minus 50% RH); and (C) equilibration of the film from 86-50% RH, as a function of time.

glycerol-containing buffers (10, 11) and in crystalline PYP (9) 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 (33)). For convenience we refer to the blue-shifted intermediate as pB_{dark} , the species that is formed in solution in the dark at



FIGURE 2: Number of water molecules per protein molecule that are released from the film at low humidity. Changes are shown relative to 0% RH. Note that the value at 0% should be regarded an offset; it is unlikely that there are actually no water molecules left inside the PYP molecule at this humidity. Vertical lines indicate the standard deviation in these values.

low pH, because of protonation of the chromophore. By recording the formation of pB_{dark} in time, the equilibration could be recorded as a function of time, as can be seen in Figure 1c. In the selected geometry of a closed cuvette, this equilibration turns out to be biexponential, with a large part of the conversion being completed within 5 min. 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. The number of water molecules involved in the transition between two relative humidities could be determined from the small differences in total weight during equilibration to a specific relative humidity. Weight changes in the order of $10-100 \ \mu g$ were observed (see Figure 2). They occurred on a time scale similar to the time scale of the spectral changes (i.e., within \sim 5 min; result not shown). For these determinations, slides with different concentrations of protein were used; the average value for two measurements is shown in the figure. For example, in the transition from 86% to 100% RH, approximately 50 water molecules were taken up per protein molecule. All changes were shown to be fully reversible and linearly dependent on the amount of protein on the slide.

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, that is, with a relative humidity above 50%, an authentic photocycle was observed. Strikingly, the rate of ground-state recovery (i.e., $pB \rightarrow pG$) 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 5-fold (see Table 2). Closer examination of these data and fitting of the traces to a multiexponential recovery function show that the recovery in these dehydrated films is multiexponential. The recovery in solution is close to

Table 2:	Estimated Ra	te Constants and	l Branching R	atios. Obtained	Using the	Kinetic Scheme	of Figure 5a ^{<i>a,b</i>}

	U	, U	U	
sample _{humidity}	pR decay (s ⁻¹)	pB formation (s ⁻¹)	pB decay (s ⁻¹)	pG formation (s ⁻¹)
WT _{sol}	740 (72%)	740 (72%)	1.6 (54%)	1.6 (54%)
	5000 (28%)	5000 (28%)	7 (46%)	7 (46%)
$WT_{98\%}$	ND^{c}	ND^{c}	ND^{c}	2.7 (91%)
				9.0 (9%)
$WT_{86\%}$	417 (67%)	417 (67%)	14 (68%)	14 (68%)
	72 (33%)	72 (14%)	1.7 (13%)	1.7 (13%)
				72 (19%)
$WT_{75\%}$	180 (53%)	180 (53%)	22 (42%)	22 (42%)
	52 (47%)		1.3 (11%)	1.3 (11%)
				52 (47%)
E46Q _{sol}	ND^{c}	$19 \times 10^3 (97.4\%)$	21 (94%)	21 (94%)
			6 (3.4%)	6 (3.4%)
				$10^3 (2.6\%)$
E46Q _{86%}	ND^{c}	$6.2 \times 10^3 (52\%)$	54 (49.8%)	54 (49.8%)
			4.8 (2.2%)	4.8 (2.2%)
				131 (48%)

^{*a*} The sum of the fractions of pB formation and of pB decay does not add up to 100% because part of pR decays directly to pG. This part is indicated by the third line in the cells of the pG formation column at reduced humidity. ^{*b*} The estimated relative error in the parameters is 10%, except for WT_{sol}, where it is 20%. ^{*c*} ND = Not determined.

monoexponential (see, for example, refs 2, 7), depending on the exact measuring conditions. As can be seen in Table 2, the recovery traces in the dehydrated films show a clear third exponent. Surprisingly, at reduced humidity, the rate of pB formation has slowed 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; 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 wild-type, whereas the rate of pG recovery decreases (see, for example, refs 34-36; note, however, that the E46O 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 pR intermediate in partially dehydrated films is extremely small (180 s⁻¹ and 52 s⁻¹ at 75% RH, see Table 2). The long-lived presence of the pR intermediate was confirmed by measuring spectra at different time points 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 ms 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% RH, 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, 470, and 490 nm (Figure 3). Note that a small positive difference absorption is instantaneously present at 355 nm, which is attributed to pR – pG (see ref 7 for the pR spectrum). Note further that the rate of decay of pR – pG difference absorption at 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 0.2, 2, 14, and 90 ms and 0.6 s (7). Each trace can be well-described



FIGURE 3: PYP photocycle kinetics at 86% reduced humidity at five wavelengths, as indicated on the ordinate. 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.

by a sum of exponential decays with positive (decay) or negative (rise) amplitudes. The residuals of the fits in this figure are presented in supplementary Figure 1 (Supporting Information). The root-mean-square error of the fit (0.3 mOD) corresponds to a signal-to-noise ratio better than 100. Representative traces from soluble WT PYP are presented in supplementary Figure 2 (Supporting Information). The global analysis amplitudes comprise the decay-associated



FIGURE 4: (A) Estimated decay-associated difference spectra (DADS) from the data presented in Figure 3. (B) Normalized DADS. Key: solid, 0.2 ms; dot, 2 ms; dash, 14 ms; dotdash, 90 ms; chaindash, 0.6 s.

difference spectra (DADS) which are depicted in Figure 4a, whereas the normalized DADS are shown in Figure 4b. The DADS can be interpreted as follows: the first DADS (solid, 0.2 ms) possesses very small amplitudes and represents a small evolution of the pR - pG difference absorption spectrum, indicating the presence of more than one pR intermediate. 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 5 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. On the basis of these observations, a target analysis was performed using the kinetic scheme depicted in Figure 5a (31). 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 speciesassociated 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 (note that here, pR' and pB' are only used to discriminate between species with the same spectrum; however, they are not related to species pR' and pB' that are found elsewhere in the literature, e.g., see ref 31). 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 (identical SADS for pR' and pR" and for pB and pB'), the three branching ratios and equilibrium (which require four parameters) can be estimated from the data of Figure 3. The estimated SADS which are depicted



FIGURE 5: (A) Photocycle scheme used for the target analysis of the 86% humidity data. Note the shortcut reaction from pR'' to pB. Branching ratios and rate constants are collated in Table 2. The steps that take place before pR is formed, on a nanosecond time scale, are drawn here for completeness; they are not included in the analysis. (B) Estimated species-associated difference spectra (SADS). Key: solid, pR; dot, relaxed pR; dash, pB.

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 33% 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, that is, 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 (23), has been replaced by a Gln residue. In this case, the proton required for chromophore protonation has been shown to originate from a solvent water molecule (see, for example, refs 18, 37). The dark spectra of this variant showed the same dependence on humidity as the wild-type protein, that is, a small red-shift of the main absorption peak and pB_{dark} formation at low relative humidities. Using this variant in kinetic studies, we found the same trend as in wild-type PYP; at reduced humidity, after bluelight excitation, the rate of pG recovery increased, whereas the rate of pB formation has decreased (see Figure 6).

Fluorescence Emission. Fluorescence excitation and emission spectra were recorded for PYP films at different relative humidities. Both the excitation and emission spectra (see Figure 7) of the (dehydrated) PYP film have the same shape and maximum as PYP in solution. However, the quantum yield of fluorescence Φ_f was found to be dependent on the relative humidity. This was concluded from the results



FIGURE 6: Normalized E46Q PYP photocycle kinetics in solution and at 86% reduced humidity. Kinetic traces are shown for solution (outer curve) and 86% RH (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. To compare the ground-state 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.

displayed in panel a, where the intensities of the emission signals are directly compared, and in panel b, where the equilibration of a dehydrated film from 59% to 86% RH is followed by recording the intensity of the emission signal as a function of time. At these relative humidities, no pB_{dark} formation is observed. At lower relative humidity, the effect of humidity on the quantum yield of fluorescence is less pronounced. At 29% RH, where pB_{dark} is formed, the film was also excited at 360 nm. This resulted in a small emission signal at ~430 nm (result not shown). All changes were fully reversible upon subsequent increases of the humidity.

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 the following 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. Second, 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 interprotein contacts in the crystal (38). However, the latter experiments obviously remain highly interesting because of the opportunity to simultaneously obtain structural and spectroscopic information (22, 39). 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_{dark}-like state is formed. The formation of a blue-shifted species, similar to the photocycle intermediate pB and the low-pH induced pB_{dark}, indicates protonation of the chromophore. Presumably, this occurs due to (partial) unfolding of the protein at these low humidities followed by protonation of the chromophore. Here, pB_{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).



FIGURE 7: PYP fluorescence at reduced humidity. (A) Fluorescence spectra in solution and in dehydrated films. Solid line, solution; dash, 86% RH; dot, 58% RH; dash-dot, 29% RH. (B) Changes of fluorescence quantum yield as a function of time, during equilibration from 58–86% RH. The excitation wavelength was set at 445 nm.

At low relative humidities, that is, below 50% RH, 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 singleturnover 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, part of the population can directly return to the ground state, that is, without formation of the blue-shifted intermediate pB (and presumably also without the accompanying structural changes).

We showed that a comparable effect of reduced humidity on the photocycle is observed in the E46O variant of PYP. This variant, in which the proton-donor glutamic acid has been replaced by a glutamine residue, shows, in solution, a ground-state recovery that is faster than in WT PYP. However, also in this protein, a moderate reduction of the hydration results in an increased recovery rate. Figure 6 shows this effect (compare the outer (solution) curve with the inner (dehydrated film) curve) but also emphasizes the difference between pB depletion and pG formation; traces collected at 447 nm (inverted sign) and 355 nm are presented, showing that they overlap in solution, whereas they do not in the dehydrated film as a result of the branching from pR to pG. Recently, the possibility of a branching reaction in the photocycle of PYP was proposed (37). It was shown for E46Q and E46A variants of PYP that at high-pH values, a precursor of pB can directly relax to the ground-state 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% RH 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 $\sim 25\%$ to 0.28%. 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 increased fluorescence is due to a longer lifetime of the excited state and accompanied by slower formation of the first photocycle intermediate I₀.

In the crystal structure of PYP, 92 water molecules have been assigned. They are mostly, however, found only in areas near the surface, and in particular, no water molecules are found near the chromophore. The two water molecules closest to the chromophore are H₂O-243 and H₂O-244 near Arg-52 (40). Another water molecule that has been described in more detail is H_2O-200 , which is the only water molecule that is present in a hydrophobic pocket in the protein, located between the N-terminal cap and PAS core of the protein. This water molecule is supposed to be exposed to the solution upon pB formation, (41). Although it is tempting to speculate that one of these water molecules plays a functional role during the photocycle of PYP and thus might be responsible for the effects in this study, the technique presented in this work cannot be used to pinpoint the involved water molecule-(s). Furthermore, our results show that a relatively large number of water molecules is involved in the transitions between the different relative humidities tested (Figure 2). This suggests that the water molecules responsible for the effects we describe are not buried, since they are likely to be released from the protein film later (i.e., at lower humidities) than the water molecules on the protein surface.

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; that is, 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 photocycle intermediates are structurally characterized using Laue crystallography, it is extremely important to use a photocycle model that correctly describes the phenomena taking place in those specific conditions (22). 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 (J. Vreede et al., unpublished); 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.

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SUPPORTING INFORMATION AVAILABLE

Figure S1, residuals from the global five-exponential fit presented in Figure 3 and Figure S2, comparable time traces from solublized WT PYP. This material is available free of charge via the Internet at http://pubs.acs.org.

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