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Comparison of acid denaturation and light activation in the eubacterial blue-light receptor photoactive yellow protein

Wouter D. Hoff [a,1,b], Ivo H.M. Van Stokkum [c], Jechiam Gural [a], Klaas J. Hellingwerf [a,*]

[a] *Department of Microbiology, E.C. Slater Institute, University of Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands*

[b] *Department of Microbiology and Molecular Genetics, University of Texas Medical School, 6431 Fannin, Houston, TX 77030, USA*

[c] *Faculty of Physics and Astronomy, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands*

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Abstract

Photoactive yellow protein (PYP) is a novel type of photoreceptor containing a thiol ester-linked *p*-coumarate anion chromophore. Photoexcitation of PYP triggers a photocycle which involves at least two intermediates: an early red-shifted state and a long-lived blue-shifted state (pB). At pH values below 3 PYP is reversibly converted into a stable blue-shifted state (pB_{dark}). Here we quantify the transition from pG to pB_{dark} at reduced pH as a two-state transition with an apparent pK of 2.8 and a steepness of 1.35 and report that the formation of pB_{dark} is also induced by increased pressure (midpoint $\sim$ 1250 atm at pH 2.7). The last step in the photocycle of PYP, from pB back to pG, is strongly decelerated by acidification. By global analysis of the data we calculated the UV/Vis absorbance spectral and kinetic properties of pB_{dark} and pB, together with their pH dependencies between pH 5 and 2. Similarities between pB and pB_{dark} were found with respect to their absorbance spectra in both the UV and visible region and with respect to the effect of pH on their stability. It is proposed that an increase in acidity and/or pressure leads to the steady state partial unfolding of PYP, while photoexcitiation leads to an analogous but transient unfolding process. © 1997 Elsevier Science B.V.

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1. Introduction

The photoactive yellow protein (PYP) from *Ectothiorhodospira halophila* [1,2] is the best studied member of a new family of water soluble photoreceptors, called the Xanthopsins [3]. The protein shows photochemical activity which strongly resembles [2,4] that of the archaeal sensory rhodopsins (SR, [5]) and like those SR proteins, PYP presumably is a photoreceptor in (negative) phototaxis [6]. On the basis of X-ray crystallography at 1.4 Å resolution it was shown that the protein has an a/β fold, similar to the one observed in some eukaryotic proteins involved in signal transduction [7].

Various aspects of the PYP photocycle have been studied. The UV/Vis absorbance transients that occur during progression of PYP from *E. halophila* through its photocycle have been analyzed in detail using global analysis techniques. This analysis has

Abbreviations: pCA, *p*-coumaric acid; PYP, Photoactive yellow protein; SR, Sensory rhodopsin

* Corresponding author. Fax: 31 20 525 7056; E-mail: a417hell@chem.uva.nl

[1] Current address: Department of Microbiology and Molecular Genetics, University of Texas Medical School.

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revealed the presence, besides the ground- or initial state pG, of two photointermediates in the nsec to sec timedomain [4]. Upon flash excitation, pG is converted into a red-shifted intermediate (pR) within 2 ns. Subsequently, pR decays on a sub-msec timescale to a blue-shifted intermediate (pB), that returns to pG on a sub-second timescale.

The effects of viscosity and hydrophobicity on the PYP photocycle kinetics have been determined and indicate that during progression through the photocycle, a conformational change occurs that exposes hydrophobic sites to the solvent in pB [8]. In addition, plasmon resonance measurements indicate that illumination promotes the binding of PYP to lipid bilayers [9]. The analogy between the archaeal sensory rhodopsins [10,11] and PYP strongly suggests that pB is the signaling state of PYP. The conformational change occurring upon the formation of pB has been proposed to form the biochemical signal [6,8], leading to negative phototaxis of the cells.

Based on an analysis of the temperature dependence of the equilibrium between pG and a blue-shifted state pB_{dark} and the kinetics of the pB to pG transition, we have proposed [12,13] that partial unfolding occurs during the formation of these species from pG, resulting in the exposure of hydrophobic contact surface to the solvent, and consequently in an increase in heat capacity. This analysis revealed a striking similarity between the pB to pG transition and data that have been reported for protein folding, indicating that the formation of pB is thermodynamically equivalent to a protein unfolding process. Time-resolved X-ray crystallography experiments revealed structural changes of the chromophore and its direct surroundings, but did not indicate the occurrence of a large conformational change during the formation of pB [14].

Photoactive yellow protein contains a new type of chromophore: a *p*-coumaric acid (pCA) molecule [15], confirmed in [16], bound to Cys69 of apoPYP [17] via a thiol ester linkage [18]. From absorbance [16,18], X-ray [16], and resonance Raman [19] data it is clear that in native PYP the chromophore is present as a *p*-coumarate anion. During the photocycle this chromophore is converted from the *trans* to the *cis* conformation [20] and presumably is transiently protonated in the pB state [16], explaining the blue-shift in the absorbance spectrum of the long-lived photointermediate. The conserved residue Glu46 has been identified as the proton donor for the protonation of the pCA anion during the pR to pB transition [21]. These results imply that during the last step of the PYP photocycle, the recovery of pG from pB, three processes occur: (i) thermal pCA re-isomerization, (ii) pCA deprotonation, and (iii) resetting of the protein conformation.

The occurrence of pCA deprotonation during the pB to pG transition suggests that the bulk pH may affect the kinetics of this transition. While in the pH range from 5 to 10 the external pH does not greatly affect the kinetics of the PYP photocycle [2], small but significant effects of pH have been reported recently: the pH dependence for the kinetics of the recovery of pG shows a bell-shaped dependence, with a maximum rate near pH 8 and with apparent pK_a values of 6.4 and 9.4 [22]. At this point no explanation is available for these pK_a values. A second effect of pH on PYP is that upon acidification in the dark, pG is interconverted into a blue-shifted state, here called pB_{dark}, that absorbs maximally at approximately 345 nm [1].

Here we examine the thermodynamics of PYP, especially with respect to lowering of the pH. We find that below pH 5 the recovery of pG from pB is dramatically decelerated as a function of pH and that pB, the presumed signaling state of PYP, shows similarities to the pB_{dark} state formed at low pH and at high pressure. In combination with the results from our analysis of the effect of temperature on PYP [13], this indicates that major protein rearrangements, analogous to partial protein unfolding, occur upon both the formation of pB_{dark} and upon light-stimulation of this photoreceptor.

2. Materials and methods

2.1. Protein purification

PYP from *E. halophila* was isolated according to [1] with the modifications described in [23]. The purity of the protein was determined from its UV/Vis absorbance spectrum: the purity index of the samples (the ratio of the absorbance at 275 nm over 446 nm) used in this study was 0.5.

2.2. Absorbance spectroscopy

Absorbance spectra were recorded at room temperature with an Aminco DW-2000 spectrophotometer (SLM Instruments). In the pH titrations, small aliquots of concentrated HCl or NaOH solution were added to the cuvette under stirring and simultaneously the pH was measured with a small pH electrode (Russell), with a precision of 0.1 pH units. Especially at lower pH values care was taken to minimize excitation of PYP into the photocycle by ambient light, during the handling of the sample. Optimal results were obtained using a buffer of 2.5 mM Tris–HCl and 20 mM citrate (in a subset of the experiments a buffer consisting of 20 mM Tris–HCl was used).

For kinetic experiments, PYP was illuminated for 10 s with white light (from a 100 W halogen lamp), using a light guide. This resulted in excitation of essentially all PYP in the sample into the photocycle at lower pH values. Immediately after switching off the light, the absorbance was measured at a selected wavelength using the kinetic mode of the Aminco DW2000 spectrophotometer. This was repeated at different wavelengths and various pH values. In other experiments complete spectra (300 to 500 nm) were recorded after switching off the light. The recording of one spectrum required approximately one minute.

2.3. Absorbance measurements at high pressure

Absorbance spectra and kinetic traces of PYP at high pressure (1 to 2000 atm) were measured in a NovaSwiss high pressure system using methanol as the pressurizing fluid. The pressure was measured using a pressure gauge. The PYP sample was placed in a custom-made cylindrical cell (3 ml volume) with a small orifice on the side for filling of the cell. After complete filling of the cell with a PYP solution of various pH values (buffered with 20 mM Tris–HCl), this orifice was sealed by a small rubber tube. This allowed for changing the pressure of the PYP solution without fracturing the cell. The cell was mounted in the cavity of the high pressure vessel, which was then placed in the sample compartment of the Aminco DW-2000 spectrophotometer. In some experiments, the sample was illuminated at high pressure as described above prior to placing the high pressure vessel in the spectrophotometer.

2.4. Data analysis

Data analysis was performed essentially as described in [4]. The equilibrium and pG recovery data were analyzed, based upon results from singular value decomposition of the matrix containing absorbance spectra as a function of pH, time, or pressure, using a two-state model to fit both the dark transition from pG to pB_{dark}, as well as the last step in the photocycle (from pB to pG). The absorbance spectrum of PYP at pH 7.5 was assumed to represent the spectrum of pG only. On this basis a global analysis of the datasets, consisting of spectra as a function of pH or pressure (dark equilibrium) and time (photocycle kinetics), was performed.

2.5. Thermodynamic models

The fraction of pG as a function of pH, as determined by global analysis (see above), was analyzed using the Henderson—Hasselbalch equation in which the transition is interpreted in terms of a group whose pK corresponds to the titration midpoint:

$$\mathrm{pG} = \frac{1}{1 + 10^{\mathrm{pH} - \mathrm{p}K}} \tag{1}$$

or with a modified version in which an additional parameter n describes the steepness of the transition:

$$\mathrm{pG} = \frac{1}{1 + 10^{n(\mathrm{pH} - \mathrm{p}K)}} \tag{2}$$

The photocycle kinetics were examined by analysis of absorbance transients, recorded at different wavelengths during the last photocycle step. These kinetics were fitted, assuming either mono-exponential behaviour with a rate constant k, or a distribution of rate constants. For the distribution we chose a Gaussian distribution on a natural logarithmic scale ($\ln(k)$), which ensures that the rates are non-negative [24]. The distribution is characterized by two parameters: k_0 (average rate) and σ (width of the distribution). The decay is described by (for $t \geq 0$):

$$\int_{-\infty}^{\infty} \mathrm{e}^{\frac{-(\log k - \log k_0)}{2\sigma^2}} \mathrm{e}^{-kt} \mathrm{d}(\log k) \tag{3}$$

Assuming Arrhenius behaviour, the distribution of

rates corresponds to a distribution of activation energies:

$$\log k = -\frac{E_A}{RT} + \text{constant} \quad (4)$$

and thus the width parameter σ is equal to the width of the distribution of activation energies divided by RT.The pressure dependence of the pG to pB_{dark} transition was analyzed using [25]:

$$\Delta G = \Delta G_0 + p\Delta V \quad (5)$$

3. Results

3.1. Characterization of pB_{dark} formed at low pH

At pH values below 3.5 PYP has been reported to be converted into a blue-shifted state (pB_{dark}) in a process involving three spectral changes [1]. We have quantitatively studied this transition by global analysis of a dataset containing the absorbance spectra of PYP at different pH values between 2 and 8 at 20°C (Fig. 1(A)). The transition is not yet complete at pH

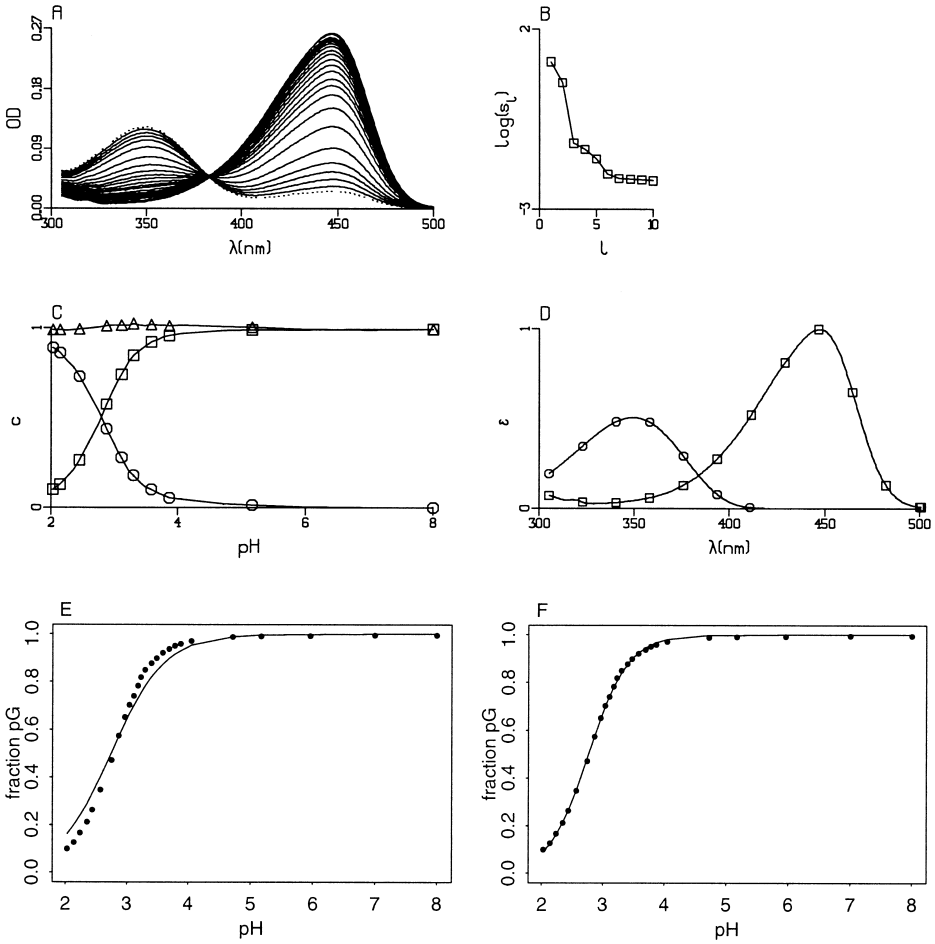

Fig. 1. Global analysis of the low pH-induced absorbance changes in PYP. The absorbance spectrum of PYP was determined at pH values between pH 2 (dotted) and 8 (A). The first ten singular values of the matrix of spectra from (A) are presented in (B) on a logarithmic scale. The dataset in (A) was decomposed by global analysis using a two-state model, leading to the concentration profiles and absorbance spectra shown in (C) and (D), respectively. Squares indicate pG, circles indicate pB_{dark}, and triangles indicate the sum of these two concentrations. The calculated concentration profile of pG was fitted with a Henderson—Hasselbalch model with $pK = 2.76$ (E), and with an augmented model with $pK = 2.77$ and $n = 1.35$ (F).

2, but at lower pH values technical problems in decreased reversibility of the transition occurred, hampering thermodynamically interpretable measurements below pH 2. Singular value decomposition of the dataset indicated the presence of two states (Fig. 1(B)). Application of a two state model yielded the pure spectra of the two states (Fig. 1(D)) and the pH dependence of the concentration of these two states (Fig. 1(C)). The residuals of this fit showed some structure, especially around 470 nm (not shown). This has also been observed in a fit of the temperature dependence of the pG to pB_{dark} equilibrium [13] and was interpreted as the presence of multiple, nearly isoenergetic and spectrally similar species. The absorbance maximum of pB_{dark} was found to be 349 nm and its extinction coefficient was determined to be 0.51 ± 0.01 relative to pG, using the constraint that the sum of the concentrations of pG and pB_{dark} must be constant. These characteristics of pB_{dark} are similar, but not identical, to those reported for pB ($\lambda_{max} = 355$ nm, $\epsilon_{max} = 0.40$; [4]).

When the fraction of pG as a function of pH was fitted with the Henderson–Hasselbalch equation, the residuals were unacceptably large (Fig. 1(E)). Therefore, we introduced an additional parameter n to describe the steepness of the transition. The fit now was satisfactory and the estimated parameters are a pK of 2.77 and $n = 1.35$ (Fig. 1(F)). The pH titration

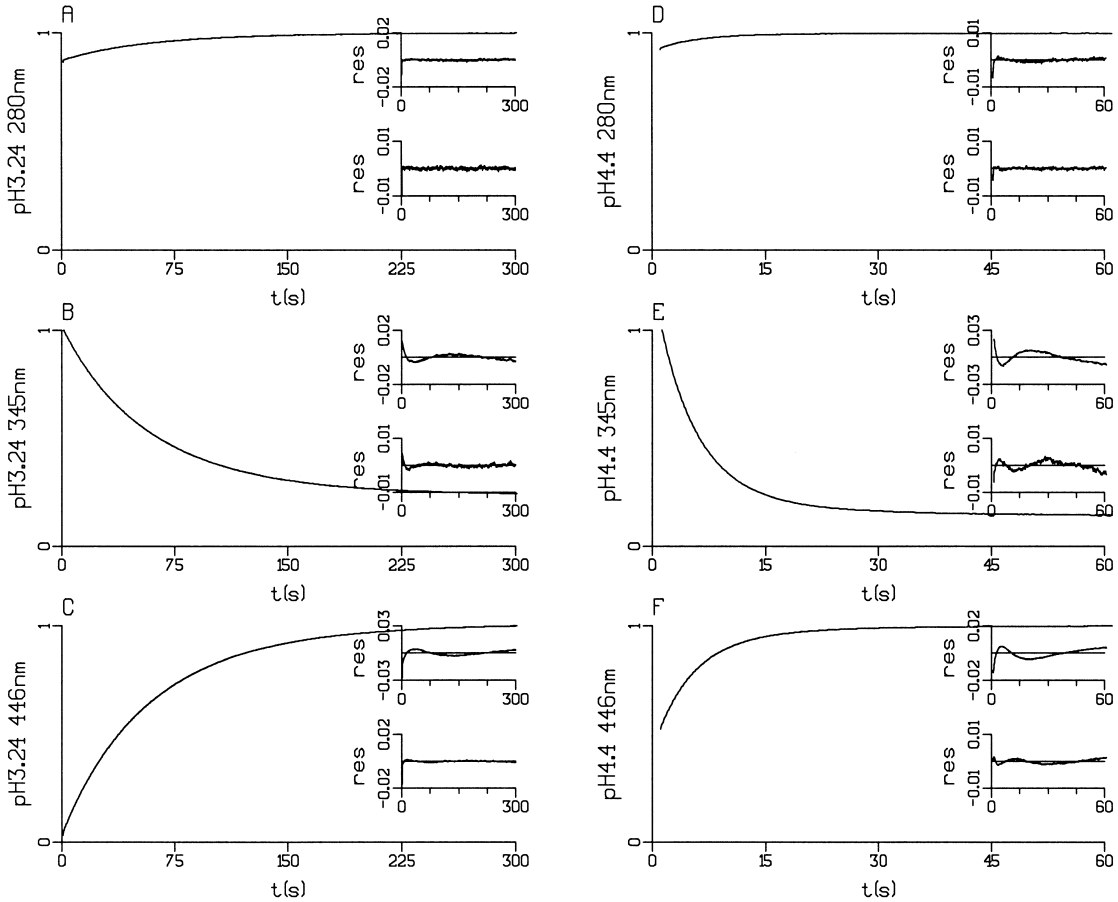

Fig. 2. The PYP photocycle is strongly decelerated at low pH and is associated with absorbance changes in the aromatic absorbance band. Absorbance traces at 446 (C,F), 345 (B,E), and 280 (A,D) nm were determined during the recovery of pG (immediately after the illumination of PYP) at pH 4.40 (D,E,F) and 3.24 (A,B,C). The photocycle kinetics at these wavelengths were fitted using either a single exponent or a distribution of rate constants of Gaussian shape in the energy domain. Insets show residuals with a single decay rate constant (top) and a distribution of decay rate constants (bottom), scaled to the maximum of the absorbance. The original extrema of the traces A to F (before scaling) are: 0.486, 0.292, 0.90, 0.502, 0.148, and 1.02, respectively.

was repeated four times. The pK values found varied between 2.70 and 2.86, while the n values ranged from 1.31 to 1.63. These differences are ascribed to the uncertainty in the pH measurement (0.1 unit), batch to batch variability, and small differences in the buffer used. However, this level of accuracy allows the conclusion that the n parameter deviates from an integer value.

3.2. Recovery of pG is dramatically slowed down at low pH

We determined the kinetics of pG recovery from pB at room temperature at various pH values between 2.4 and 4.5, by monitoring the absorbance changes at 446, 345, and 280 nm. At pH values below 5 this process is slowed down dramatically (see Figs. 2 and 3). Global analysis of the absorbance transients at these 3 wavelengths, assuming mono-exponential behaviour, yielded clearly structured residuals (Fig. 2, upper insets; Fig. 3(B)). To find a more appropriate kinetic model for the pB to pG transition, we tested the applicability of two additional models: (a) two independent exponentials, and (b) a Gaussian distribution of rate constants. The bi-exponential model strongly reduced the structure in the residuals, leading to good fits (not shown). However, we found that the fits assuming a Gaussian distribution of rate constants (which have one parameter less than the bi-exponential model) yielded approximately equally good fits (Fig. 2, lower insets; Fig. 3(B)). At essentially all pH values studied here, the application of this latter model significantly decreased the residuals, as shown quantitatively by the root mean square error of the fits, which are reduced by almost one order of magnitude (Fig. 3(B)). The resulting pH-dependence of the kinetics of pG recovery, as determined with the mono-exponential decay model and with the maximum of the Gaussian distribution of decays, are essentially identical (Fig. 3(A)). Clearly, below pH 5 the PYP photocycle is strongly decelerated as a function of pH. A slight reduction in the recovery rate constant at pH 4.6 has already been reported [26]. The width of the Gaussian distribution of rate constants is pH-independent in the range of pH 2.5 to 4.4 (Fig. 3(C)).

We have also applied this model to a large dataset of flash-induced difference spectra, recorded at pH 7.5 and at various temperatures [4,13]. It was observed that at this pH value the recovery can be described well with a sum of two exponentials. For example, at 18°C 93% of pB decays with a τ of 150 ms, while the remaining 7% showed a τ of 2 s [4]. These recoveries could *not* be fitted with a single Gaussian distribution of rate constants. Thus, the width of the distribution of rate constants of the faster phase of 93% at pH 7 is apparently reduced with respect to the width below pH 4.4 while the origin of the slower phase of 7% remains unclear.

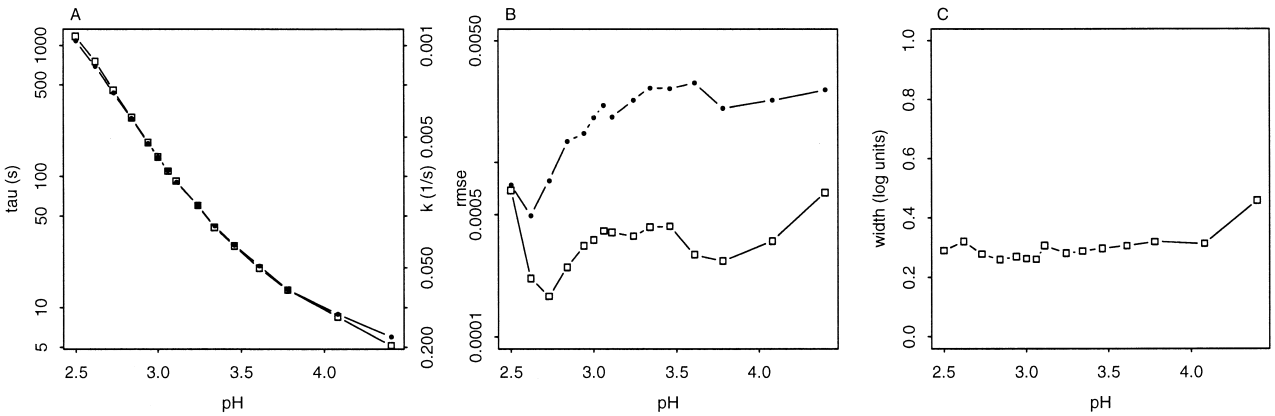

Fig. 3. The kinetics of the pB to pG transition as a function of pH. The time constants of the pB to pG transition at a range of pH values between 2.5 and 4.5 were measured at 446 nm and were fitted using either a single exponent (filled circles) or a distribution of rate constants of Gaussian shape in the energy domain (open squares). Depicted are (A) the lifetime τ (reciprocal of the decay rate) on the left ordinate and the decay rate k on the right ordinate, (B) the root mean square error of the fits relative to the maximum of the absorbance, and (C) the width of the Gaussian distribution of decay rates.

At pH 2.5 approximately 70% of pG is converted to pB_{dark}. However, the remaining pG can still be excited into the photocycle (see Fig. 4 for an analysis of a dataset at pH 2.84). Apparently, the deceleration of groundstate recovery at low pH is a process which occurs *independently* from the transition of pG to pB_{dark}. Fig. 4(A) shows the recovery from pB to a pG/pB_{dark} mixture after illumination of PYP at pH 2.84 and demonstrates that the absorbance spectra of pB and pB_{dark} are similar but not identical. These data were analyzed using the model shown in Fig. 4(C), assuming that the transition between pG and pB_{dark} occurs significantly faster than the transition from pB to pG. Thus, 10 s of illumination with white light results in the accumulation of PYP in the pB state. This model is supported by singular value decomposition of the dataset, revealing the presence of two intermediates, instead of three (Fig. 4(B)). We

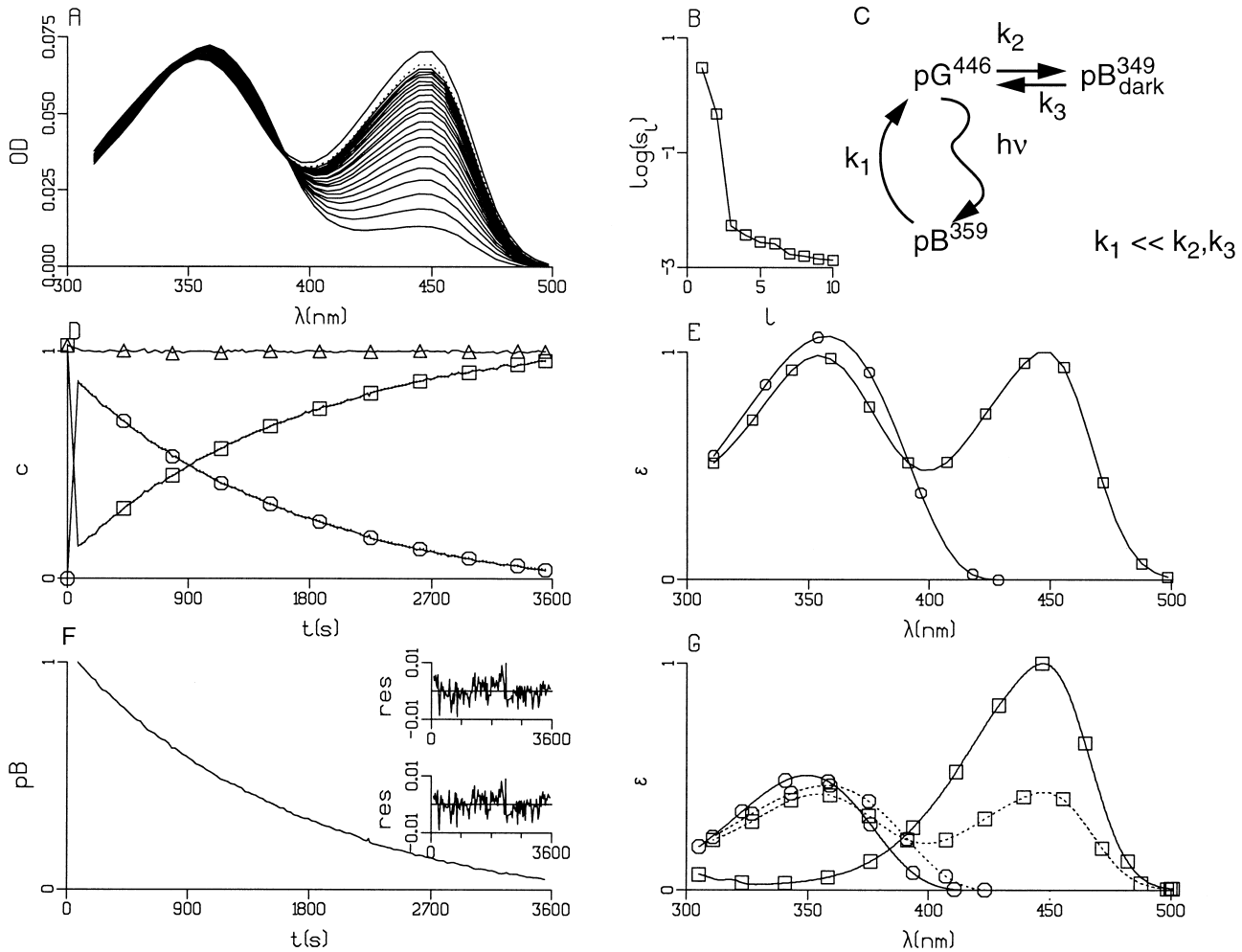

Fig. 4. Measurement and global analysis of the PYP photocycle at pH 2.84. (A) Absorbance spectrum of PYP measured at times between zero (spectrum with greatest amplitude at 446 nm), i.e. just before illumination, and recovery of this spectrum after 10 s of illumination, followed up to 3551 s (dotted spectrum). (B) The first ten singular values of the matrix of the spectra from (a) on a logarithmic scale. (C) Model used to analyze the data. The dataset in (A) was globally analyzed using a two-state model, yielding the fits shown in (D) and (E). (D) Relative concentration of pG plus pB_{dark} (squares) and pB (circles). Triangles correspond to the sum of these two concentrations. (E) Spectra of pG plus pB_{dark} (spectrum at $t = 0$, squares) and pB (circles). (F) Fit of the decay of the concentration of pB from (D) with a single decay and a distribution of decay rates (see legend of Fig. 2). (G) spectra from (E) (dotted) plotted to the scale of the spectra from Fig. 1(D), assuming a relative concentration of pG = 0.43. Note the difference in spectrum between pB_{dark} (solid, circles) and pB (dotted, circles).

have used this model to fit the data in Fig. 4(A), resulting in the fit shown in Fig. 4(D) and (E). The kinetics of pG recovery can in this case be described with a single exponential (Fig. 4(F)). This is probably caused by the fact that the dataset consists of whole spectra at various time intervals, which possess a lower signal to noise ratio as a function of time than the kinetic traces at selected wavelengths. According to the model shown in Fig. 4(C), pB will recover to pG, which is in fast equilibrium with pB_{dark}. A decomposition of the data into pB, pB_{dark}, and pG is shown in Fig. 4(G). The spectral parameters found for pB are: $\lambda_{max} = 359$ nm and $\epsilon_{max} = 0.46$, very similar, but slightly different from the values found for pB in the photocycle at room temperature and neutral pH ([4]; i.e. at pH = 2.84 the λ_{max} of pB appears to be slightly red-shifted) and clearly red-shifted with respect to pB_{dark} ($\lambda_{max} = 349$ nm). About 43% of the PYP was found to be in the pG state in this fit; a somewhat higher value was expected on the basis of the results shown in Fig. 1. We attribute this to the uncertainty in the pH measurement (0.1 pH unit) and to batch variability.

3.3. Acidification and illumination induce UV absorbance changes in PYP

Earlier work indicated the occurrence of a large conformational change in PYP upon illumination and/or acidification [8,9,12,13]. To further examine these conformational changes, we studied the absorbance changes upon illumination and acidification in the spectral region of the absorbance caused by aromatic amino acids.

First, we probed the spectral changes during the pB to pG transition at 280 nm, in parallel with measurements of pCA absorbance at 345 and 446 nm (Fig. 2). At 280 nm a bleaching signal was observed, that is approximately 10% of the bleaching observed at 446 nm. The time constants for the absorbance transients occurring at 280, 345, and 446 nm, are indistinguishable (not shown). This indicates that the changes in chromophore absorbance (345 and 446 nm) are coupled to changes in the absorbance at 280 nm (the aromatic region).

Then, to spectrally resolve the changes in the region of absorbance by aromatic amino acids, we measured and analyzed the UV absorbance difference spectra of PYP, as induced by acidification and illumination. As can be seen in Fig. 5, the difference spectra caused by these two treatments are very similar. In both cases, the absorbance at 270 nm bleaches approximately 12% with respect to the bleaching at 446 nm. The difference spectra have a maximum at approximately 270 nm and their shape indicates a reduction in extinction coefficient rather than a spectral shift in the absorbance band of PYP at 275 nm.

3.4. Effects of high pressure on PYP

In view of the expected large protein conformational change during the pR to pB photocycle transition, experiments were performed to examine the possibility of trapping the pR or pB intermediate at room temperature (at various pH values) by an increase in pressure. Such trapping was not observed, and the pB intermediate that formed at 1000 atm and pH 2.7 converted back to the pG state at a rate similar to that observed at 1 atm (data not shown). However, at this low pH value an increase in pressure has a strong effect on the pG to pB_{dark} equilibrium (Fig. 6). Upon a pressure increase from 1 to 1250 atm approximately half of the pG present at this pH was

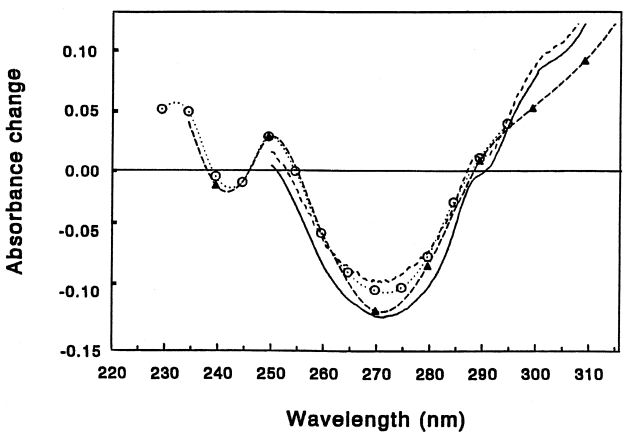

Fig. 5. Low pH- and light-induced UV absorbance changes in PYP. Absorbance difference spectra in the aromatic region are shown, caused by illumination of PYP at pH 4.4 (dashed line with closed triangles) and pH 3.5 (dotted line with open triangles) and by a decrease in pH from 5.8 to 2.8 (solid line) and from 3.5 to 2.5 (dashed line), using different batches of PYP to check the reproducibility of the signals. The difference spectra were scaled to 1 with respect to the bleaching at 446 nm.

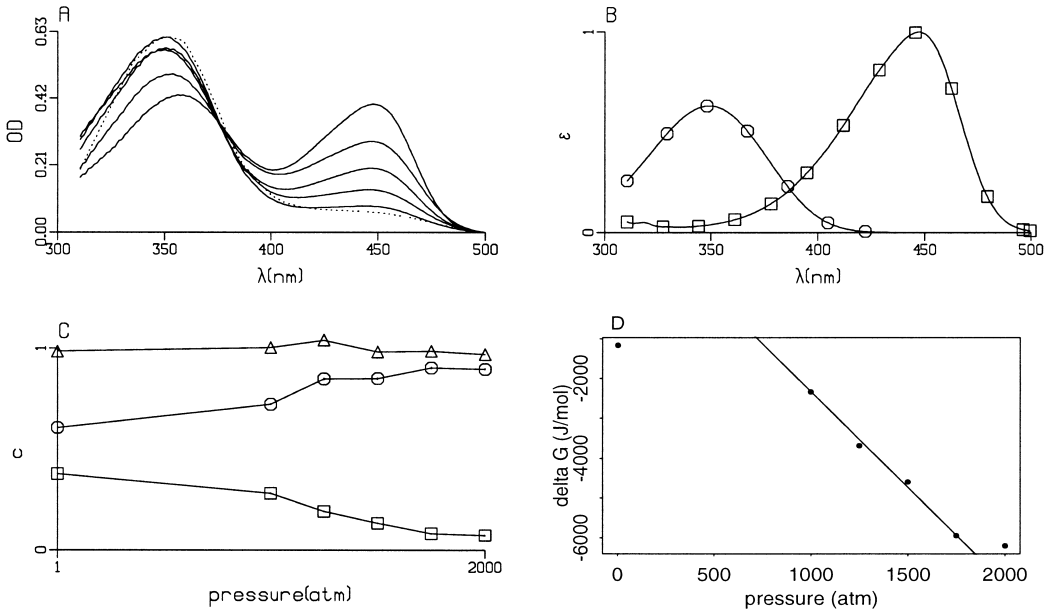

Fig. 6. The effect of pressure on the pG to pB_{dark} equilibrium. The absorbance spectrum of PYP at pH 2.7 was determined at various pressures between 1 and 2000 atm (dotted) (A). Application of a two-state model yielded the absorbance spectrum of the pB_{dark} species formed (B) and the pressure dependence of the pG concentration (C). Squares indicate pG, circles indicate pB_{dark}, and triangles indicate the sum of these two concentrations. This was used to calculate the pressure dependence of the ΔG between pG and pB_{dark} (D).

reversibly converted to a species resembling pB_{dark}. This transition could be fitted with a two-state model, yielding the spectral parameters of pB_{dark} ($\lambda_{max} = 349$ nm, $\epsilon_{max} = 0.60 \pm 0.06$; Fig. 6(B)) and the pressure dependence of the pG concentration (Fig. 6(C)). Due to leakage of small amounts of methanol at high pressure, the baseline in the absorbance measurements reported in Fig. 6(A) is less stable than that of the other data described here. Therefore, within the error limits, the spectral parameters of the pB_{dark} species formed at high pressure are identical to those of the species formed at low pH.

The pressure dependence of the pG concentration (Fig. 6(C)) allows the calculation of the pressure dependence of the ΔG between pG and pB_{dark} (Fig. 6(D)). We assume a linear dependence of the ΔG on pressure (Eq. (5); see [25]). The slope of this dependence is directly correlated with the difference in volume (ΔV) between pG and pB_{dark}. The total change in ΔG between 1 and 2000 atm (5.5 kJ/mol) would correspond to ΔV of approximately -27 ml/mol, while the middle four datapoints, which are well described by a linear model, yield the higher value of -48 ml/mol. Further correction of the data (see [27,28]) was not performed, since their effect on the estimated value of ΔV falls within this range. The technical limitations of the experimental set-up used here hamper a more precise estimation of the ΔV between pG and pB_{dark}, but the measurements reported in Fig. 6 clearly show that an increase in pressure at low pH destabilizes pG.

4. Discussion

The pB_{dark} state. We show here that the transition from pG to pB_{dark} caused by acidification can be described as a two-state transition. Global analysis of the spectral changes induced by low pH yield the absorbance spectrum of the pB_{dark} state and its population as a function of pH. The spectral parameters of the pB_{dark} species found in this way are virtually identical to those of the pB_{dark} species that is populated at elevated temperatures [13]. The latter species was proposed to be partially unfolded, based on the increase in heat capacity associated with its formation. The absorbance maximum of pB_{dark} close to 350 nm indicates that the pCA chromophore is in a

specific environment in the pB_{dark} state which causes its spectral tuning, since the absorbance maximum for thiol ester-linked pCA in model compounds and in fully denatured PYP is positioned at 335 nm [16,18,29]. Circular dichroism measurements show that pB_{dark} formed at low pH still has strong peptide and aromatic circular dichroism signals (W.D. Hoff and K.J. Hellingwerf, unpublished results). These results indicate that this species is *partially* unfolded. The pressure dependence of the pG to pB_{dark} equilibrium can be used to estimate the ΔV associated with this transition. The negative value found for this parameter in PYP corresponds well with those reported for the pressure-induced unfolding of various small water soluble globular proteins [25,27,28,30]. This leads to the conclusion that the pB_{dark} state formed at low pH is a partially unfolded state of PYP formed by acid denaturation.

The quantitative analysis of the transition from pG to pB_{dark} at low pH reveals a complicating factor: an additional parameter *n* is required to obtain satisfactory fits of the data. Such non-integer *n* values have been observed previously, often without a satisfactory explanation. In the case of bacteriorhodopsin, non-integer values of *n* in pH-titrations were interpreted to be caused by multiple interacting p*K*'s [31]. The origin of the non-integer *n* value in the case of PYP is unclear at this point and may indicate the necessity of a different model to analyze the acid denaturation process.

The PYP photocycle at low pH. While acidification does not inhibit the formation of pB, it hampers the second half of the PYP photocycle: Below pH 5 the pB to pG photocycle transition is dramatically decelerated as a function of pH. Since the pCA deprotonates during this transition, in general this process is expected to be decelerated by an increase in H^+ concentration, as is indeed observed (Figs. 3 and 4). This proposal is in line with the situation in bacteriorhodopsin, where a pH *increase* leads to a deceleration of groundstate recovery [32], the opposite of what is observed for PYP. This can be rationalized in terms of the chromophore protonation state, since in bacteriorhodopsin the Schiff base linkage that links the retinal chromophore to the protein becomes *protonated* during groundstate recovery, while in PYP the pCA chromophore becomes *deprotonated* during groundstate recovery. During the formation of pB from pG, the pCA chromophore is protonated and FTIR spectroscopy has indicated Glu46 as the proton donor for this process [21]. A precise interpretation of the effect of pH on pG recovery is hampered by the fact that the proton *acceptor* which extracts the proton from the pCA during the pB to pG transition has not yet been identified. The site of proton release may be the external medium, or an amino acid that functions as the proton acceptor and that may become protonated at low pH. In this respect it is interesting to note that the transient uptake of one proton from the medium during the PYP photocycle has been described [33].

The pB to pG photocycle kinetics at pH values between 4.5 and 2.4 reported here are not well described by a single rate constant. It has been observed before that the recovery of the groundstate is not a simple mono-exponential process, neither in solution [4], nor in single crystals of PYP [34]. The physical basis for this observation, however, was not resolved. Here we find that satisfactory fits can be obtained for the pB to pG photocycle transition using a rather narrow distribution of rate constants of Gaussian shape in the energy domain. Such a model is physically interpretable in terms of a distribution of pB conformations (substates; see [35]). The presence of substates in pG was also suggested by the presence of residuals around 470 nm in fits of the temperature- and pH-dependence of the pG to pB_{dark} equilibrium (see above).

The strong deceleration of the pB to pG photocycle transition reported here offers a powerful tool for detailed study and comparison of the pB_{dark} and pB states. This approach has already been used (i) to demonstrate the occurrence of pCA *trans* to *cis* isomerization during the PYP photocyle through the extraction of *cis*-pCA from PYP after illumination [20] and (ii) to study the formation of pB by FTIR absorbance difference spectroscopy [21].

Thermodynamics of the PYP photocycle and its modulation at low pH. We have analyzed two parallel effects of acidification on PYP: (i) the (dark) transition to an acid-denatured state of PYP, pB_{dark}, and (ii) the deceleration of the last photocycle step. On the basis of the data presented in Figs. 1 and 3, and Fig. 4 we propose a model that results in a coherent view on the pH dependence of the PYP photocycle thermodynamics (Fig. 7). The core of the

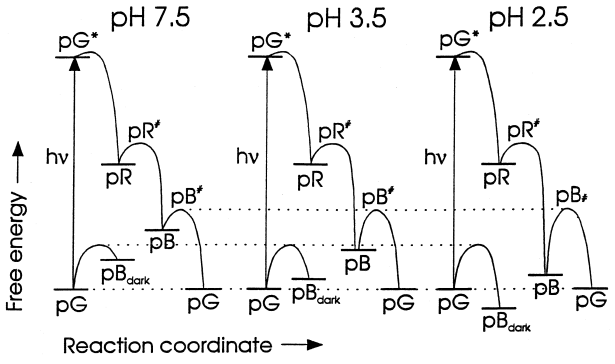

Fig. 7. Summary of the effect of pH on PYP thermodynamics. The relative free energy content of pG, pR, pB, pB_{dark}, and the transition states connecting the different states is schematically depicted at pH 7.5, 3.5, and 2.5.

proposal is that pB and pB_{dark} are similar to each other. This is supported by a number of findings: (a) The formation of these species has been reported to result in an increase in heat capacity of similar size [13]. (b) The UV absorbance changes in the aromatic region associated with the formation of these two species from pG are very similar (Fig. 5). This suggests that the protein conformational changes, upon illumination and upon acid denaturation, are similar. At this stage it cannot be excluded, however, that pCA itself also contributes to the absorbance change at 280 nm. (c) The species have very similar chromophore absorbance maxima, somewhat red-shifted with respect to 335 nm. (d) The circular dichroism signals of pB and pB_{dark} in the aromatic absorbance region are very similar, both somewhat reduced with respect to the signals of pG (W.D. Hoff and K.J. Hellingwerf, unpublished results).

The model in Fig. 7 proposes that: (a) Both pB and pB_{dark} are stabilized with respect to pG by a decrease in pH. (b) The stabilization of pB results in an increase in the $\Delta G^{\#}$ of the last step in the photocycle and thus in a deceleration of this process. (c) The stabilization of pB_{dark} results in the transition of PYP from pG into this state of the protein at low pH. In this model the major difference between the two blue-shifted states is the isomerization state of the chromophore, where the pCA is *cis* in pB and *trans* in pB_{dark}, leading to a higher energy content of pB with respect to pB_{dark} (see Fig. 7) and consistent with the slight blue-shift of pB with respect to pB_{dark}. Thus, the model can qualitatively explain all observed effects of low pH on PYP.

High concentrations of the denaturing agent urea also decelerate the pB to pG transition [2]. This is in line with the proposal that pB is a partially denatured state: a change in ΔG between pG and pB_{dark} in favor of the latter species appears to have a decelerating effect on pG recovery, irrespective of the nature of the denaturing agent (see Fig. 7).

In conclusion, the results reported here indicate that the acid denatured state of PYP (pB_{dark}) is both spectrally and thermodynamically similar to the photocycle intermediate pB. This is in line with the conclusions drawn from studies on the temperature dependence of PYP stability and its photocycle [13]. Thus, studies of the behaviour of PYP as a function of two independent parameters (pH and temperature) *both* indicate the similarity between protein unfolding and the PYP photocycle.

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