Light responses of bacteria: site-directed mutagenesis study of PYP & photo-inactivation of E. coli and B. subtilis
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Binding of hydrogen-citrate to photoactive yellow protein is affected by the structural changes related to signaling state formation


Abstract
The tricarboxylic acid citric acid is a key intermediary metabolite in organisms from all domains of the tree of life. Surprisingly, this metabolite specifically interacts with the light-induced signaling state of the photoactive yellow protein (PYP), such that at 30 mM, it retards recovery of this state to the stable ground state of the protein with up to 30%, in the range from pH 4.5 to pH 7. We have performed a detailed UV/Vis spectroscopic study of the recovery of the signaling state of wild type (WT) PYP and two mutants, H108F and ∆25 PYP, derived from this protein, as a function of pH and the concentration of citric acid. This revealed that it is the di-anionic form of citric acid that binds to the pB state of PYP. Its binding site is located in-between the N-terminal cap and central β-sheet of PYP, which is accessible only in the signaling state of the protein. The obtained results show how changes in the distribution of sub-species of the signaling state of PYP influence the rate of ground state recovery.
Introduction

Photoactive yellow protein (PYP) from *Halorhodospira halophila* is a relatively small (125 amino acids), water soluble photoreceptor protein. For more than 10 years already PYP has been studied intensely as a model system in photoreceptor research, because it is: (i) chemically and photochemically very stable,\(^ {179, 180} \) (ii) shows considerable structural change during signaling state formation\(^ {94} \) and (iii) is the proto-type of the Per-Arnt-Sim (PAS) domain super-family of signal transduction domains.\(^ {13} \) Most of these PAS domains are involved in protein-protein interactions and function as sensors and signal transducers.\(^ {12} \) For signal transfer many bind, or interact with, a ligand or chromophore. PYP contains \( p \)-coumaric acid as a chromophore that is bound to the protein via a thiolester bond with C69.\(^ {68, 77, 81} \)

As photoreceptor PYP has a light induced photocycle. In this cycle three major intermediates can be identified, pG, pR, and pB. In the ground state of PYP, pG (also named P or PYP), the chromophore is deprotonated and has a *trans* configuration.\(^ {82} \) The absorption maximum of pG is at 446 nm.\(^ {92} \) Upon, excitation with light the pR intermediate (also named I\(_1\) or PYP\(_{L}\)) can be formed. Here the chromophore has received a *cis* configuration, but is still deprotonated.\(^ {104} \) As a result the absorption maximum (~460 nm) is red-shifted. The isomerization of the chromophore facilitates the formation of the signaling state pB (also named I\(_2\), I\(_2\)’, or PYP\(_M\)), where the chromophore is typically protonated, and where the structure of the protein has changed significantly, compared to pG.\(^ {94, 98} \) Furthermore, the absorption maximum is blue-shifted relative to pG. Presumably the structural changes that accompany pB formation lead to alteration of the interaction with a downstream, and currently poorly characterized, transducer.\(^ {111} \) pB subsequently recovers to pG, closing the photocycle.

Note that depending on the conditions, certain characteristics of these basic photocycle species may change. As such we can divide these photocycle species into several sub-species. Depending on conditions one or more of these sub-species may then be dominantly present.\(^ {105} \) A prime example is the pB species. The light-induced structural changes in PYP show pronounced pH dependence, such that at low pH the amount of structural change is decreased\(^ {205} \) compared to neutral and moderately alkaline pH. The two corresponding sub-species of pB also display different absorption spectra, with the less unfolded form (*i.e.*, the low-pH form of pB) having an absorption maximum of 368 nm and the more unfolded form having an absorption maximum of 355 nm.\(^ {105, 107, 185, 206} \) At very high pH a third form of pB can be formed, in which the solvent-exposed chromophore is deprotonated. This leads to a shift of the absorption maximum of pB to 430 nm.\(^ {105} \) Therefore, depending on the pH one or more of these sub-species may be present in significant amounts.

Recently, a photocycle intermediate (pG’) was identified that appears to catalyze the recovery from pB to pG.\(^ {105} \) This pG’ intermediate is characterized by a deprotonated chromophore in combination with a specific fold of the apo-protein. This configuration is then able to catalyze the photocycle recovery reaction, which includes
Binding of hydrogen-citrate to PYP is affected by the structural changes re-isomerization of the chromophore and refolding of the protein. The intermediates pB and pG’ are in a fast pH-independent equilibrium, while the decay of pG’ to pG is pH-dependent. Recovery via pG’ is much faster than the direct recovery of pG from pB.

The photocycle of PYP has been well-studied over the entire time-domain that is relevant for its functioning, i.e., from femtoseconds to seconds. In this study we focus on the recovery reaction of the signaling state (pB) back to the ground state (pG). Solvent conditions, such as ionic strength, temperature, pH, and buffer composition, have an effect on the rate of this recovery. E.g., the recovery rate decreases as the ionic strength is increased. For this study we have specifically studied the influence of citric acid as it has been shown that, on top of its effect on ionic strength, it slows down the recovery rate of PYP more than other organic acids do. As such it may interact with potential signal transducer interaction sites. To resolve the specific site(s) of interaction between PYP and anionic species of citric acid we have measured pG recovery of WT PYP, and the mutants H108F and Δ25 PYP, as a function of both pH and citrate concentration, keeping temperature and ionic strength constant. From these measurements we were able to determine that it is the di-anionic form of citric acid that affects the recovery rate in WT PYP. In addition, for the mutant PYPs we observed differences in the influence that citrate has on recovery. From these observations we were able to deduce that the citrate-binding surface of PYP is located in-between the N-terminal cap and the β-sheet. Subsequent NMR measurements support the assignment of this binding site for citrate.

Materials and methods

Overproduction and purification of photoactive yellow protein
WT, H108F, and Δ25 PYP were produced and isolated as described previously for WT PYP. Apo-PYP was reconstituted with the 1,1’-carbonyldiimidazole derivative of p-coumaric acid, as described previously. The reconstituted holo-proteins were purified by using a Pharmacia Äkta FPLC system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) in two subsequent steps, with Ni-affinity chromatography and anion exchange chromatography, respectively. For WT and H108F PYP, in the Ni-affinity chromatography, 20 mM NaPO₄/acetate buffer pH = 7.0, containing 150 mM NaCl, was used as the loading buffer. The proteins were eluted with a pH gradient by using the same buffer with a pH of 4.0 as the elution buffer. Immediately after Ni-affinity chromatography, the proteins were dialyzed against 50 mM Tris buffer, pH 8.0. Anion exchange chromatography was performed with 20 mM Tris, pH 8.0 as the loading buffer and a gradient of 0 to 1 M NaCl. For Δ25 PYP in the Ni-affinity chromatography 20 mM NaPO₄ pH 7.0 buffer was used with an imidazole gradient up to 0.5 M for protein elution. In anion exchange chromatography, the loading buffer was 20 mM ethanolamine pH 9.5, whereas for elution the same buffer with 1 M NaCl was used. The
purified holo-proteins were used without removal of the genetically introduced N-terminal hexa-histidine containing tag. Their purity index \( \text{OD}_{280}/\text{OD}_{446} \) was better than 0.5. A part of the samples were prepared with uniform \(^{15}\text{N}\) labeling of PYP.

**Sample/buffer preparation**

Buffer solutions with defined ionic strength were prepared by mixing 1.0 M Acetic acid, 0.2 M \( \text{Na}_2\text{HPO}_4 \), 1.0 M Trizma\textsuperscript{®} base, 0.5 M Boric acid, 4.0 M \( \text{NaCl} \), 1.0 M HCl, and 1.0 M NaOH. The required amount of these stock solutions was calculated\textsuperscript{105} Where necessary, minor pH adjustments were made with either 1.0 M HCl or NaOH.

**Transient ms/s UV/Vis spectroscopy**

UV/Vis spectra were recorded using an HP8453 UV/Vis spectrophotometer. Time resolved spectra were recorded from 250 to 600 nm, using an integration time of 0.1 s. A ‘Kraayenhof’-cuvette\textsuperscript{193} was used to thermostat the sample at 25°C, and allowed measurement of the sample pH in the measurement set-up. A Schott KL1500 LCD lamp was used to illuminate the sample. Actual sample illumination was computer controlled via a shutter system, allowing synchronization of sample illumination with the spectroscopic measurement. A 2 s delay was used between the start of the measurement, and a typical 5 s illumination period of the sample.

**Data analysis: Transient UV/Vis data**

Datasets obtained for each condition were analyzed as described previously\textsuperscript{105} From this analysis both recovery rate constants and pB spectra were extracted. A typical analysis is described below (see Data analysis walkthrough).

**Data analysis: Spectral**

The pH and citrate-concentration dependence of the pB spectra obtained from the transient UV/Vis data was analyzed using the model depicted inside the ‘pB balloon’ in Figure 4.8. The rationale behind this model is further explained in the Results section. Equation 4.1 is a mathematical description of the fraction of each species in this model as function of pH and citrate concentration. Here the subscripts A though E represent the different pB species: A, pB\textsubscript{l}; B, pB\textsubscript{m}/pG’ mixture; C, pB\textsubscript{h1}; D, pB\textsubscript{h2}; and E, pB\textsubscript{citr}. \( K_{\text{citr}} \) represents the binding constant of HCitrate\textsuperscript{2−} to pB\textsubscript{m} (for a detailed explanation of this nomenclature: see legend to Figure 4.8). Note that [HCitrate\textsuperscript{2−}] is pH dependent; the concentration of HCitrate\textsuperscript{2−} was calculated on the basis of the pH and the total amount of citrate in the sample. We used the following pK values for transition between the different forms of citrate: 3.15, 4.77 and 6.40\textsuperscript{208}.
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\[ f_A = \frac{1}{1 + X(1,1) + X(1,2) + X(1,3) + K \cdot X(1,1)} \]
\[ f_B = \frac{1}{Y(1,1) + 1 + X(2,2) + X(2,3) + K} \]
\[ f_C = \frac{1}{Y(1,2) + Y(2,2) + 1 + X(3,3) + K \cdot Y(2,2)} \]
\[ f_D = \frac{1}{Y(1,3) + Y(2,3) + Y(3,3) + 1 + K \cdot Y(2,3)} \]
\[ f_E = \frac{K}{Y(1,1) + 1 + X(2,2) + X(2,3) + K} \]
\[ X(v, w) = 10^{\sum_{n, (pK, -pH)} -n \cdot (pK, -pH)} \]
\[ Y(v, w) = 10^{\sum_{n, (pK, -pH)} n \cdot (pK, -pH)} \]
\[ K = \frac{\text{HCitrate}^{2-}}{K_\text{citr}} \]

**Equation 4.1**

**Data analysis: Kinetic**

To analyze the pH dependence of the recovery rate at different citrate concentrations we assumed that this rate is directly related to the fraction of each species of pB. Therefore we assigned a rate constant to each species of pB as indicated by Equation 4.2. Here the fraction of each species \( f_A \) through \( f_E \) is obtained from Equation 4.1. Furthermore, the rate constant for the pB\textsubscript{m}/pG'-mixture \( k_B \) is pH dependent.\textsuperscript{105}

\[ k = k_A \cdot f_A + k_B \cdot f_B + k_C \cdot f_C + k_D \cdot f_D + k_E \cdot f_E \]
\[ k_B = \frac{k_{B,\text{high}} - k_{B,\text{low}}}{1 + 10^n \cdot (pK, -pH)} + k_{B,\text{low}} \]

**Equation 4.2**

**Data analysis: Parameter confidence**

Confidence intervals were determined, for the parameters used in the spectral and kinetic analysis, according to a method described elsewhere.\textsuperscript{209} In short, confidence intervals were determined by varying the value of the parameter around the calculated optimum, and subsequently re-optimizing the other parameters. The values of the parameter were subsequently plotted against the Sum Square Error (SSE) of the re-optimized analysis. Obtained plots generally have a parabola-like shape, with the optimum value at the minimum. The confidence interval was subsequently determined by setting a threshold SSE-value (using a Fisher's F distribution).

**Guanidine HCl denaturation**

Measurements were performed at 25°C and pH 6.0 using the previously described buffer (see sample/buffer preparation). Buffer with varying amounts of Guanidine HCl were prepared by mixing buffers containing 0 M and 5.5 M Guanidine HCl. The
Guanidine HCl concentration was determined via the refractive index of the buffer solution. The same spectrophotometer setup as described above was used to record spectra. For each condition first a dark spectrum was recorded, followed by a spectrum of the sample in a light induced steady state.

For the analysis spectra at 0 M Guanidine HCl were taken as native species reference spectra. Fully denatured species spectra were determined by averaging the spectra at the highest concentration Guanidine HCl (~3.3 M). These reference spectra were used in combination with Equation 4.3 to determine protein stability. In Equation 4.3 \( f_{\text{native}} \) and \( f_{\text{denatured}} \) represent the fraction native and denatured protein respectively; \( m \) is the m-value; \([\text{Gnd}]_{1/2}\) the Guanidine HCl concentration for which \( f_{\text{native}} = 0.5\); \( R \) the gas constant (8.314 J K\(^{-1}\) mol\(^{-1}\)); \( T \) temperature (293 K); \( \Delta G_0 \) is an estimate of the conformational stability of the protein in absence of denaturant. Parameter confidence was determined as described earlier for the spectral and kinetic analysis of the transient UV/Vis data (see above).

\[
\begin{align*}
\Delta G_0 &= m \cdot \left[ \text{Gnd} \right]_{1/2} \\
\Delta G_0 &= m \cdot \left[ \text{Gnd} \right]_{1/2} \\
\end{align*}
\]

**NMR measurements**

Solution-NMR spectra were recorded on a Bruker AvanceII 500 MHz spectrometer operating at a \(^1\)H frequency of 500 MHz. NMR samples of uniformly \([^{15}\text{N}]\)-labeled WT PYP were prepared in Shigemi tubes, containing 200 µl 0.6 mM protein in 20 mM buffer solution (90 % H\(_2\)O/10 % D\(_2\)O, 150 mM NaCl, pH 6.2), both in absence and presence of 20 mM or 50 mM sodium citrate. DSS (0.2 mM) was used as internal chemical shift reference. 1D proton spectra and sensitivity enhanced 2D \(^{15}\text{N}^-^{1}\text{H} \) HSQC spectra were recorded at 38 and 53.5°C. Typically, 256 and 8 scans were collected for 1D and 2D spectra, respectively, with 160 increments used in the 2D HSQC spectra. To measure NMR spectra of the pB state of PYP, laser light was sent from a Spectra Physics Stabilite 2017 Argon Ion Laser (5 Watt) through an optical fiber attached to a Shigemi glass insert into the NMR sample. The laser was operating in multiline emission mode, delivering blue-green light at maximum amplitudes between 447 to 515 nm. The duration of laser illumination was controlled by a mechanical shutter that was controlled from the pulse program of the spectrometer. Each scan of the 1D and 2D spectra was started with a 400 ms pre-illumination period at a power of 2.7 Watt. The sample was illuminated during the pulse sequence and the acquisition of the FID and kept in the dark during the 3.6 s relaxation delay.
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**Autodock calculations**

Docking of HCitrate\(^{2-}\) on PYP was carried out using the Autodock 4.0\(^{212}\) routine that has been implemented in Yasara Structure version 10.11.8. The high-resolution 0.82 Å crystal structure 1nwz.pdb\(^{71}\) was used for pG. A simplified model for the structure of pB was generated by deleting amino acids 1-18 of the atomic coordinates of 1nwz.pdb. This choice is based on the high-temperature solution NMR data of pB that show a lack of secondary structure and solvent protection for amides in this region. In addition, the ensemble structure 2kx6.pdb\(^{213}\) was used for pB. HCitrate\(^{2-}\) was built in Yasara with idealized geometry using optimized AM1 atom charges at pH 6.2 and a final -2 charge distribution, which leads to one terminal protonated carboxyl group.\(^{214}\) The atom charges of the protein together with its pCA chromophore were calculated at pH 6.2 in Yasara and the final pG and pB structure both contain a protonated H108 imidazole ring with charge +1. Global docking with a fully flexible citrate ligand was performed over 200 runs for both pG and pB using standard Autodock parameters. Grid size was 0.4 Å and the clustering criterion was set to 5 Å. The six clusters that had favorable binding energies were further analyzed. The lowest-energy global conformer per cluster was solvated in Yasara, equilibrated in 200 mM NaCl at 53.5°C and energy-minimized by simulated annealing in a free Molecular Dynamics run (Amber03 force field, periodic boundary conditions, 7.86 Å non-bonded cutoff, Ewald electrostatics). Details of molecular binding interactions were calculated and visualized by Ligplot.\(^{215}\)

**Data analysis walkthrough**

This chapter focuses on the analysis of the results from a series of transient ms/s UV/Vis datasets, where the results from analyses of single datasets are used. Here we would like to walk through a typical analysis of a single raw dataset. This analysis consists of the following three steps, baseline correction, Singular Value Decomposition (SVD) analysis, and a Non-linear Least-Squares Fit (NLSF) of the data. The mathematical details of the analysis are described elsewhere,\(^{105}\) as such for this walkthrough we are mostly limiting ourselves to a graphical depiction of the analysis. For this walkthrough we have taken a WT PYP dataset recorded at pH 6.0 in the absence of citrate.

A single raw dataset typically consists of a time series (30 seconds in length) of absorption spectra (250-600 nm) recorded with a 100 ms time interval. Baseline correction is performed on a per absorption spectrum basis, where the average of the OD values between 550 and 600 nm is subtracted from the absorption spectrum. Though this correction is generally a minor one, it does improve the quality of the subsequent analysis. Figure 4.1 depicts two representations of the baseline corrected data. In both the influence of the 5 s light pulse (from 2 to 7 s) can clearly be seen. First the sample is bleached by the light pulse. This is followed by dark recovery of the sample. For subsequent analysis only the recovery part of the dataset is used, *i.e.*, the data to the right of the white line in Figure 4.1B.
The second analysis step is mainly used to check if the model we use for analysis is consistent with the dataset. This is done by performing an SVD-analysis on the recovery part of the baseline corrected dataset. In the SVD-analysis the dataset is split into three components, a scaling matrix (*i.e.*, a diagonal matrix with scaling factors; S-matrix), a matrix with spectral components (U-matrix), and a matrix with temporal components (V-matrix). From the obtained scaling matrix alone it is clear that there are only two significant components in our dataset (Figure 4.2). A closer look at the spectral-matrix multiplied by the scaling-matrix, confirms this (Figure 4.3). Note that there is a minor third component that has some structure, all other components are basically noise. However, since this third component is so small we can safely ignore it for our subsequent analyses. A closer look at the temporal multiplied by the scaling-matrix, paints a similar picture (Figure 4.4). 

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**Figure 4.1.** Baseline corrected dataset. Note grey scale in panel A and B are not identical (grey scale in panel B from low to high intensity is from black to white). B) Data to the right of the white line was used for subsequent analyses.

**Figure 4.2.** SVD-analysis -scaling matrix. The diagonal of the SVD-scaling matrix is plotted as a function of the SVD-rank. Plots A and B only differ in the way the Y-axis is scaled.
Binding of hydrogen-citrate to PYP is affected by the structural changes

In the third analysis step the data is generated that is used for the analysis described in this chapter. The model we use in the NLSF analysis of the recovery part of our dataset assumes the dataset contains only two significant components. This is in line with the SVD-analysis described above.

The result of the NLSF-analysis is basically two rate constants, the fraction the first rate constant contributes, and two spectra. Of these two spectra one represents the ground state spectrum. The other represents the light induced steady state spectrum, which is mostly a mixture of the pG and pB species. In Figure 4.5 the temporal part of the analysis is shown, and in Figure 4.6 the spectral part. From the residuals of the
analysis a temporal and spectral standard deviation was determined (see Figure 4.5B and 4.6B). The described analysis was performed for three datasets at each measured condition.

Figure 4.5. NLSF-analysis -temporal. A) Fraction ground state (solid line) and fraction light induced steady-state (dashed line, is mostly a mixture of pG and pB). B) standard deviation based on fit residuals.

Figure 4.6. NLSF-Analysis -Spectral. A) spectra of groundstate (solid line) and light induced steady-state (dashed line, is mostly a mixture of pG and pB). B) Standard deviation based on fit residuals.

Results
We have extended our previous work on WT PYP, by measuring the pH dependency of the photocycle recovery rate over a large pH range (4.5 to 10.5) in the presence of citrate. As before, we have kept temperature, ionic strength, and buffer composition constant and varied only pH and citrate concentration (0, 10, 20, and 30 mM). It should be noted that with 30 mM citrate we could only measure up to pH 7.5, as it was not possible to keep the ionic strength at 250 mM above this pH in the presence of 30 mM citrate. Furthermore, we have carried out the same set of measurements with the mutant derivatives H108F and Δ25.
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Figure 4.7. Initial analysis of WT PYP. A) kinetic analysis of pH dependent recovery rate in the presence of varying amounts of citrate. 0 mM citrate (●), 10 mM (▲), 20 mM (▼), and 30 mM (■). Fitted lines are best fits through the data, i.e., no parameter constraints were used. B) Left-axis, difference curve (solid line) of two fit curves from panel A (0-20 mM citrate). Right axis, fraction of citrate species H\textsubscript{3}Citrate (dashed line), H\textsubscript{2}Citrate\textsuperscript{−} (dash single-dotted line), HCitrate\textsuperscript{2−} (dash double-dotted line), Citrate\textsuperscript{3−} (dash triple-dotted line). C) Spectral analysis of pH dependent pH spectra at 0 mM citrate. From low to high pH the spectral line styles are dotted, solid, dashed, and dash-dotted.

WT PYP - initial analysis
We confirmed the observation for WT PYP that at low pH the presence of citrate retards the recovery reaction of PYP\textsuperscript{207} When we look at the pH dependence of the recovery kinetics, the pH transition of these kinetics appears to shift towards higher pH with increasing citrate concentration (Fig. 4.7). We fitted the data-points using the model from our previous work\textsuperscript{105} To visualize the citrate induced change we subtracted the fitted curve from Figure 4.7A at 20 mM from the one at 0 mM citrate. Comparison with citrate titration curves (see Fig. 4.7B) revealed that the citrate induced retardation seems
to correlate specifically to the presence of hydrogen-citrate (i.e., HCitrate$^{2−}$). It is therefore likely that this species retards the photocycle recovery rate. The slight discrepancy between the maxima for HCitrate$^{2−}$ and the retardation effect in Figure 4.7B is likely caused by a difference in influence of HCitrate$^{2−}$ on the different pB sub-species.

At higher pH (>7.5), i.e. the pH range in which Citrate$^{3−}$ is most abundant, the addition of citric acid has the opposite effect: consistently a small acceleration of the recovery reaction is observed. Because of its small size this effect has not been further analyzed.

In our previous work we linked the pH transition of the recovery rate to the pH transition of pB spectra.$^{105}$ In this previous study we obtained a low pH pB spectrum (pB$_l$; $\lambda_{\text{max}}$ ~368 nm), a medium pH pB spectrum (mixture of pB$_m$ with $\lambda_{\text{max}}$ ~355 nm, and pG’ $\lambda_{\text{max}}$ ~450 nm), and a high pH pB spectrum (pB$_h$; $\lambda_{\text{max}}$ ~430 nm). One might therefore expect the transition of the low to medium pH pB spectra to shift to higher pH as well. However, in our initial analysis the spectral pH transition appeared to shift to lower pH, i.e., in the opposite direction of the shift of the kinetic pH transition. It was therefore clear to us that our relatively simple photocycle scheme no longer sufficed to analyze pH dependent photocycle events in the presence of citrate.

In our previous work we noted that in all likelihood there is an additional spectral transition for pB at high pH, caused by deprotonation of tyrosine residues. Improvements in our analysis protocol have allowed us to confirm the presence of this transition. As a result we now include two high pH pB sub-species instead of just one (i.e., pB$_h$ now becomes pB$_{h1}$ and pB$_{h2}$). The resulting spectra for each sub-species of pB are shown in Figure 4.7C.

**WT PYP: incorporation of the influence of citrate into the photocycle model**

For the incorporation of the influence of citrate into our photocycle model, we have used the following criteria and assumptions, which are based on our initial analysis. The influence of citrate on PYP is caused by HCitrate$^{2−}$. HCitrate$^{2−}$ only influences PYP when it can bind to a relatively unfolded form of PYP, i.e., the pB form that dominates at medium pH (pB$_m$). When HCitrate$^{2−}$ is bound to pB, the latter can no longer form pG’. Effectively, this leads to the addition of a HCitrate$^{2−}$ bound pB intermediate (pB$_{citr}$), which is in equilibrium with pB$_m$ (Fig. 4.8). As, such our pH dependency model for pB has changed from a basic single species with 2 pH transitions model,$^{105}$ to one that has 3 pH transitions plus a HCitrate$^{2−}$ dependent equilibrium. Here we should be able to distinguish spectra for, pB$_l$, (pB$_m$ + pG’), pB$_{h1}$, pB$_{h2}$, and pB$_{citr}$. Note, that pB$_{citr}$ and pB$_m$ are expected to have similar spectral characteristics, with one difference, the pB$_m$ intermediate cannot be distinguished from pG’. Furthermore, by introducing an additional pB species at high pH we can no longer assume that only the pB$_m$ + pG’ mixture contributes significantly to the recovery rate. As such, the recovery rate is determined by summing the fraction of each sub-species of pB multiplied by its
Binding of hydrogen-citrate to PYP is affected by the structural changes representative recovery rate. Note, that only the recovery rate linked to the pB\textsubscript{m} + pG’ mixture is pH dependent (see Eq. 4.2). Also, this pH dependent rate should have a significantly higher value than the other rate constants. Simulations with this model (Eq. 4.1 and 4.2) confirm that with increasing citrate concentration the pH dependence of the presence of pG’ shifts to higher pH, while the transition from (pB\textsubscript{m} + pB\textsubscript{citr}) to pB\textsubscript{l} shifts to lower pH.

**Figure 4.8.** Photocycle scheme of PYP indicating pH and citrate dependence of pB species. In this scheme the three basic photocycle species pG, pR, and pB are indicated by boxes. Inside the box of the pB species a scheme is shown for the distribution of pB sub-species as function of pH and citrate, where pB\textsubscript{m}, pB\textsubscript{h1}, and pB\textsubscript{h2} represent sub-species of pB that dominate at low-, medium-, and high pH, respectively. As pK\textsubscript{BC} and pK\textsubscript{CD} differ only modestly (~1 pH unit; see Table 4.1), we introduced two sub-species with subscript h for high pH (i.e., h1 and h2). pB\textsubscript{w} represents a sub-species of pB that has HCitrate\textsuperscript{2−} bound to it, and is in exclusively formed via an equilibrium with pB\textsubscript{m}. The thick black arrows between the boxes of the basic photocycle species indicate the route of the photocycle. Intermediates that may be involved in the transition from one basic species to the other are indicated inside these arrows. Note that depending on the conditions these intermediates may or may not be involved. *E.g.*, pG’ is only involved if the transition of pB to pG goes through the pB\textsubscript{m} sub-species of pB. If the transition of pB to pG goes through any of the other sub-species of pB, pG’ is not involved.

Analysis of the data with the model incorporating citrate would benefit from a global approach that includes all datasets in a single analysis and combines kinetic and spectral analysis. However, at this time this is not possible due to the size of the complete dataset, as well as minor differences in scaling between datasets recorded under a multitude of conditions. As such, we were forced to apply the model on sub-sets of the data. To retain the required global aspect in the analysis we made the following assumptions: (i) The pKs of the transitions between the different sub-species of pB are constant between sub-sets of data, (ii) The pK-values for transition between the different forms of citrate are 3.15, 4.77 and 6.40,\textsuperscript{208} and have a cooperativity constant of 1, (iii) The binding constant for the binding of HCitrate\textsuperscript{2−} to pB\textsubscript{m} is constant between sub-sets of data, (iv) The pH dependence of the recovery rate, which is linked to the fraction
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pB\textsubscript{in}/pG', is not dependent on the citrate concentration, and (v) the model parameters should allow for a good fit through both kinetic and spectral aspects of the dataset.

Table 4.1. Analysis results with pH and citrate dependent model for the rate of PYP recovery

<table>
<thead>
<tr>
<th>Transition\textsuperscript{a}</th>
<th>Kinetic\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT PYP</strong></td>
<td></td>
</tr>
<tr>
<td>( pK_{A,B} )</td>
<td>5.9551</td>
</tr>
<tr>
<td>( pK_{B,C} )</td>
<td>9.869</td>
</tr>
<tr>
<td>( pK_{C,D} )</td>
<td>10.564</td>
</tr>
<tr>
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</tr>
<tr>
<td>( n_{B,C} )</td>
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</tr>
<tr>
<td>( n_{C,D} )</td>
<td>1.60</td>
</tr>
<tr>
<td>( K_{\text{citr}} )</td>
<td>0.063</td>
</tr>
<tr>
<td>( k_{A} )</td>
<td>0.082</td>
</tr>
<tr>
<td>( pK_{B} )</td>
<td>7.00</td>
</tr>
<tr>
<td>( n_{B} )</td>
<td>1.01</td>
</tr>
<tr>
<td>( k_{B,\text{low}} )</td>
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</tr>
<tr>
<td>( k_{B,\text{high}} )</td>
<td>1.620</td>
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<tr>
<td>( k_{D} )</td>
<td>0.00</td>
</tr>
<tr>
<td>( k_{E} )</td>
<td>0.00</td>
</tr>
</tbody>
</table>

| **H108F PYP**                |                             |
| \( pK_{A,B} \)               | 5.406                       |
| \( pK_{B,C} \)               | 9.5086                      |
| \( pK_{C,D} \)               | 10.564                      |
| \( n_{A,B} \)                | 0.743                       |
| \( n_{B,C} \)                | 0.8654                      |
| \( n_{C,D} \)                | 1.60                        |
| \( K_{\text{citr}} \)        | 100                         |
| \( k_{A} \)                  | 0.0000                      |
| \( pK_{B} \)                 | 6.89                        |
| \( n_{B} \)                  | 0.94                        |
| \( k_{B,\text{low}} \)       | 0.694                       |
| \( k_{B,\text{high}} \)      | 0.466                       |
| \( k_{C} \)                  | 0.047                       |
| \( k_{D} \)                  | 0.0000                      |
| \( k_{E} \)                  | 0                           |

| **Δ25 PYP**                  |                             |
| \( pK_{A,B} \)               | 1                           |
| \( pK_{B,C} \)               | 9.270                       |
| \( pK_{C,D} \)               | 10.174                      |
| \( n_{A,B} \)                | 1                           |
| \( n_{B,C} \)                | 0.917                       |
| \( n_{C,D} \)                | 1.51                        |
| \( K_{\text{citr}} \)        | 100                         |
| \( k_{A} \)                  | 0                           |
| \( pK_{B} \)                 | 7.1                         |
| \( n_{B} \)                  | 1.05                        |
| \( k_{B,\text{low}} \)       | 0.00425                     |
| \( k_{B,\text{high}} \)      | 0.00612                     |
| \( k_{C} \)                  | 0.02017                     |
| \( k_{D} \)                  | 0.01759                     |
| \( k_{E} \)                  | 0                           |

Values that were forced are depicted in Italic. Unit of \( K_{\text{citr}} \) is M. Unit of the rate constants (\( k \)) is s\(^{-1}\).

\( ^{a} \) Parameters in the Transition-column were determined via pH dependent spectral analysis in the absence of citrate, with the exception of the \( K_{\text{citr}} \)-parameter, which was based on global analysis of kinetic data.

\( ^{b} \) Parameters in the Kinetic-column were determined via a global analysis of kinetic data.
Binding of hydrogen-citrate to PYP is affected by the structural changes

PYP: Analysis with citrate-incorporated model
As a starting point we used the spectral analysis in the absence of citrate. The parameters obtained from this analysis cannot be influenced by citrate and therefore conform to our constraint that these parameters are not citrate dependent. Subsequently, a kinetic analysis was performed, which allowed us to fit data measured as function of both pH and citrate concentration in a single global fit. In this analysis all parameters related to the transition between the different sub-species of pB and citrate were fixed, with the exception of the binding constant of HCitrate$^{2-}$ to pB$_{in}$. The result of this analysis is summarized in Table 4.1. When these values for the model parameters were applied to the pH dependent spectral data in the presence of citrate, a reasonably good description of the data was obtained (data not shown). The resulting spectrum for pB$_{cit}$ is indeed similar (not identical) to pB$_{in}$, only without the contribution of pG’ (data not shown).

![Figure 4.9. pH dependent recovery rate. Recovery rate of WT PYP at an ionic strength of 1.0 M in the presence of 0 (●), 20 (▼), and 100 (♦) mM citrate. Lines are fitted curves from Figure 4.7A and are from WT PYP at an ionic strength of 250 mM in the presence of 0 (solid line), 10 (dashed line), 20 (dotted line), and 30 (dash-dotted line) mM citrate.](image)

Ionic strength choice
When it became clear the binding constant of citrate was likely higher than 30 mM, we decided to see if we could measure with citrate concentrations up to 100 mM. However, this required an increase of the solvent ionic strength that we had been using up till that point. As ionic strength also influences photocycle kinetics, we did a quick test over a limited pH range before starting an extensive series of new measurements. This test revealed that at an ionic strength of 1.0 M we were no longer able to clearly distinguish the citrate induced retardation of the recovery rate (Fig. 4.9).

H108F-PYP analysis
Since citrate appears to bind only to PYP’s pB$_{in}$ sub-species, we decided to determine the effect of citrate on the H108F mutant of PYP. This mutant seems to undergo less structural change upon formation of pB. Therefore, it may not be able to bind citrate, or behave differently in the presence of citrate.

The analysis of the data obtained for H108F PYP was done in the same way as for WT PYP. However, the analysis proved to be less straightforward than that of WT PYP. The confidence intervals for the pK$_{CD}$, n$_{CD}$, and K$_{cit}$ parameters turned out to cover a very large range, indicating they could not be determined from the current dataset. Therefore, for the pK$_{CD}$, and n$_{CD}$ parameters, values obtained for WT PYP were
used. The $K_{citr}$ parameter was fixed to a value (100 M) that basically removes the influence of citrate from the model. As a result the parameter $k_E$ could also be fixed (to 0 s$^{-1}$) as it should have no influence in the model anymore. The results from this analysis are collected in Figure 4.10 and Table 4.1. We were not able to perform these measurements at citrate concentrations above 30 mM. It was therefore extra important to determine the confidence limits of the fitted value for the binding constant of citrate. To determine the lower limit of the $K_{citr}$ parameter for H108F the parameter $k_E$ was not fixed. The determination of the confidence limit for $K_{citr}$, as shown in Figure 4.11 for WT and H108F PYP, enabled us to determine that our limited citrate concentration range made determination of $K_{citr}$ not very precise and for the H108F mutant essentially impossible (Fig. 4.11B), though we were able to determine a lower limit for $K_{citr}$ in the H108F mutant. Notably, the lower limit for $K_{citr}$ (160 mM) is still significantly higher than the WT value for $K_{citr}$ (47-90 mM).

![Figure 4.10. Analysis of H108F PYP. A) Kinetic analysis of pH dependent recovery rate in the presence of varying amounts of citrate. 0 mM citrate (●), 10 mM (▲), 20 mM (▼), and 30 mM (■). Fitted line is global fit at 0 mM citrate using our photocycle model that includes the influence of citrate (see Table 4.1 for model parameters). Fitted lines at 10, 20, and 30 mM are highly similar and are therefore not shown. B) Spectral analysis of pH dependent pB spectra at 0 mM citrate. From low to high pH the spectral line styles are dotted, solid, dashed, and dash-dotted.](image-url)
Binding of hydrogen-citrate to PYP is affected by the structural changes

Figure 4.11. Determination of parameter confidence for $K_{\text{cit}}$ for WT PYP (A) and H108F PYP (B). Horizontal line indicates SSE value that was used as limit to determine confidence range for the parameter.

Other major differences with the WT data are that the pK transition between sub-species of pB at low pH has shifted to a lower pK by ~0.5 pH unit. A similar shift was observed for this mutant when monitoring the pH dependent proton uptake and release upon illumination. Furthermore, the contribution of pG' in the mixed pB$_m$/pG' spectrum is significantly less compared to that in WT PYP, as we noted previously. Lastly, from the obtained rate constants for H108F PYP (see Table 4.1) it is clear that recovery of H108F is significantly slower than WT PYP.

**Δ25 PYP analysis**

Since there is a good chance that the N-terminus is involved in citrate binding, we decided to also determine the effect of citrate on the Δ25 mutant of PYP. In this mutant the N-terminus is absent. Due to the very slow recovery kinetics of this mutant we added a neutral density filter between the light source of the spectrophotometer and the sample, to minimize probe light induced photocycle activation. As a result we were no longer able to reliably record spectral information below 300 nm.

The analysis of the data obtained for Δ25 PYP ground state recovery shows no sign of being influenced by citrate. For the analysis of Δ25 PYP recovery, we were only able to detect pH induced transitions at high pH; the low pH transition around pH 6 appears to be absent in Δ25 PYP (Fig. 4.12). As a result we had to adjust the analysis models. The spectral analysis at 0 mM citrate was performed with a simple 2 pH transition model. For the subsequent kinetic analysis, the low pH transition was fixed at a pK of 1, effectively removing the influence of the pB$_l$-species from the model in the pH domain we analyzed. Also $K_{\text{cit}}$ was set to 100 M to remove the influence of citrate from the model. Additionally the rates $k_A$ and $k_E$ were fixed to 0 s$^{-1}$ as they should have no influence in the model. Again, the parameters obtained from the spectral analysis at 0 mM citrate were used (fixed) in the global analysis of the pH-dependent kinetic data (see Fig. 4.12 and Table 4.1). Note, that the low pH spectrum for pB of Δ25 PYP is similar to the pB$_m$ spectra of WT and H108F PYP. Also, recovery is slower for this pB$_m$ species than pB$_{h1}$. The contribution of pG’ in the pB$_m$ spectra is very low. Furthermore, the pH dependence of $k_B$ remains, although in contradiction to the WT and H108F data,
the rate is slower at low pH. Removing, the pH dependence of \( k_B \) results in a poor fit (data not shown), indicating this pH dependence is not destroyed when removing the N-terminus from PYP.

**Figure 4.12.** Analysis of \( \Delta 25 \) PYP. Kinetic analysis of pH dependent recovery rate in the presence of varying amounts of citrate. 0 mM citrate (●), 20 mM (▼), and 30 mM (■). Fitted line is global fit at 0 mM citrate using our photocycle model that includes the influence of citrate (see Table 4.1 for model parameters). Inset: spectral analysis of pH dependent pB spectra at 0 mM citrate. From low to high pH the spectral line styles are solid, dashed, and dash-dotted.

**Protein stability**

The citrate induced slower recovery of WT PYP can be interpreted as a stabilization of the pB structure by citrate. Therefore we determined the relative stability of WT, H108F and \( \Delta 25 \) PYP (both in their pG- and pB-form) in the absence and presence of citrate via Guanidine HCl titrations. The results are summarized in Table 4.2. Interestingly, based on \( \Delta G_0 \) alone, citrate only has a clear stabilizing impact on the protein’s conformational stability in pG of \( \Delta 25 \) PYP and destabilizes WT in pG. For pB no significant stabilization by citrate is observed. Therefore, the retardation of pG recovery in the presence of citrate is likely not caused by a stabilizing effect of citrate.

When we look at the \([\text{Gnd}]_h\) parameter, a consistently higher value is observed in the presence of citrate, with the single exception of the pG form of WT PYP. This indicates that the denaturant and citrate compete, at least in part, for the same binding sites. Furthermore, in pB of WT and H108F the shift is larger (~0.3-0.4 mM) compared to the shift under the other conditions (~0.1 mM). This is consistent with the disruption of the N-terminus in pB.

Finally, the m-value is interesting in the sense that it represents the change in solvent accessible surface area (SASA) upon denaturation.216 Here a higher m-value represents a larger change in SASA. As might be expected, between pG and pB the m-value is consistently smaller in pB, even in \( \Delta 25 \) PYP. Furthermore, citrate seems to
Binding of hydrogen-citrate to PYP is affected by the structural changes consistently decrease the m-value in WT and H108F, while no significant change is observed for \( \Delta 25 \) PYP. This indicates that citrate may have a disruptive effect on the N-terminus of PYP.

### Table 4.2. PYP conformational stability based on guanidine denaturation

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<th>WT PYP a</th>
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<tr>
<td></td>
<td>pG</td>
<td>pB</td>
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<tr>
<td>[Citrate]</td>
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<tr>
<td>[Gnd]₅/₂</td>
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<td>(25.6-27.7)</td>
<td>(24.6-32.7)</td>
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<table>
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<tr>
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<td>pG</td>
<td>pB</td>
<td></td>
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</tr>
<tr>
<td>[Citrate]</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>[Gnd]₅/₂</td>
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<td>2.626</td>
<td>2.253</td>
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<td>(6.74-7.37)</td>
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<tr>
<td>( \Delta G_0 )</td>
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<td>40.6</td>
<td>17.3</td>
<td>18.2</td>
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<tr>
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<td>pB</td>
<td></td>
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</tr>
<tr>
<td>[Citrate]</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>[Gnd]₅/₂</td>
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<td>2.120</td>
<td>2.015</td>
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<td>7.45</td>
</tr>
<tr>
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<td>(14.24-14.49)</td>
<td>(7.09-8.04)</td>
<td>(6.98-7.91)</td>
</tr>
<tr>
<td>( \Delta G_0 )</td>
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<td>15.2</td>
<td>16.1</td>
</tr>
<tr>
<td>kJ·mol⁻¹</td>
<td>(29.2-29.9)</td>
<td>(30.2-30.7)</td>
<td>(14.1-16.4)</td>
<td>(14.9-17.2)</td>
</tr>
</tbody>
</table>

\( ^a \) Conformational stability was determined for WT, H108F, and \( \Delta 25 \) PYP at pH 6.0 for both the pG and pB form at 0 and 52 mM citrate. Values in brackets indicate the 95 % confidence interval for the fit-parameters.

### Solution-NMR analysis of citrate binding

To get more detailed information about a possible binding site for citrate we performed several solution-NMR measurements on WT PYP. Experiments were performed in the presence and absence of citrate at pH 6.2. Assignment of individual amides in the ground state (pG) follow from 3D NOESY spectra, while corresponding assignments in the pB state were made at a higher temperature than in previous studies\(^{132}\) to eliminate the conformational line broadening that hampered resonance assignments of the pB state of WT PYP. Details of the assignments for pB at high temperature will be described elsewhere.
Figure 4.13. Chemical shift perturbations of amide proton pairs of WT PYP in the pG (panel A) and the pB state (panel B) in the presence of 50 mM citrate. Absolute chemical differences $\Delta^{1}H + ^{15}N$ are calculated as root-mean-square differences weighted according to $\sqrt{(\delta^{1}H)^2 + (\delta^{15}N/6.515)^2}$. Missing data points due to high-solvent exchange rates or exchange broadening are indicated by small circles, triangles correspond to proline positions. Yellow bars in panel D show amides for which less accurate delta values are calculated, mostly due to partial overlap of amide protons in the compared NMR spectra, or by low sensitivity of peaks in the non-citrate reference spectrum. The horizontal dotted lines give the estimated average limit for which significant chemical shifts differences can still be measured accurately (>0.003 and >0.007 ppm for pG and pB, respectively). The secondary structure cartoon displayed at the top of the diagrams in panel A and B is derived from the crystal structure of pG (1nwz.pdb). C) Overlay of the $^{15}N$-$^1$H spectrum of pG recorded at 53.5 °C. Inserts of HN-N F6 and the amino HD22-ND2 Q32 cross peaks actually show the maximal chemical shifts changes that have been observed in pG (<0.04 ppm). D) Overlay of the $^{15}N$-$^1$H spectrum of pB recorded at 53.5 °C. The largest chemical shift perturbations of amide proton signals are labeled in the plot.
Binding of hydrogen-citrate to PYP is affected by the structural changes

Titration up to 20 mM citrate does not reveal any chemical shift perturbation of amide protons in the spectra of the pG state at 38°C. Only at 50 mM citrate and a higher temperature (53.5°C) small perturbations are seen just above the detection limit of 0.003 ppm (Fig. 4.13A and C). The largest changes (up to 0.04 ppm) are observed for amino proton HE22 of Q32, amides F6, F28, R124. The amides in loop regions around L88-N89 and G112-S117 are perturbed up to 0.02 ppm. In conclusion, citrate displays very weak binding towards the pG state of PYP.

In contrast, a clear citrate-induced difference can be observed in the HSQC spectrum of the pB intermediate, recorded under similar conditions as for pG (see Fig. 4.13B and D). Chemical shift changes of pB in the presence of citrate amount up to 0.06 ppm and are spread over the entire protein sequence. The most pronounced perturbations, however, are observed for amides in the important helical region (44-51), and the β-strand regions (90-99) and (103-110). Also the N-terminal region at positions G25 and G29 is disturbed relatively strongly in the pB state after binding of citrate. Figure 4.13B shows that the first 19 amino acids of the N-terminus remain largely invisible despite the much improved spectral quality of the HSQC spectra of pB at elevated temperature. Possibly, the loss of secondary structure in the N-terminus of pB causes an increased solvent-exchange rate of these amides that result in diminished proton signals. From the titration data on pB it is concluded that HCitrate\(^{2-}\) may bind to either the junction between the N-terminal side and helix (44-51) of PYP and/or the exterior side of the two long β-sheets (88-97) and (103-111) that are bridged by a hairpin loop. Either way, the NMR perturbation data indicate that almost the whole structure of the pB molecule is affected by binding of citrate.

**Docking studies of citrate binding**

In order to independently search for possible binding positions of HCitrate\(^{2-}\) (see Fig. 4.14E) on the surface of the pG and pB states of PYP, we performed a docking study at pH 6.2 using Autodock. The docking results on pG are schematically displayed in Figure 4.14A. The four main clusters indicated by roman numerals point to binding sites that are complementary to the negatively charged citrate molecule. In summary, the preferred binding positions are located at (I) in the loop pocket surrounded by K17, W119, L113, S114 and the side-chain of Q32, (II) near F6, and the side-chains of K106 and K123, (III) near side-chains of K123 and R124, and (IV) in the loop region adjacent to N87 and L88. In fact, the amide protons that are predicted to ligate to citrate correspond exactly to the slight perturbations observed in the HSQC spectra. Thus, despite the low-affinity binding of citrate, these calculations are in full agreement with the experimental observations.
Figure 4.14. Autodock results of docked divalent citrate (see also the structural formula in E) on the pG and pB structure. Citrate docking orientations of the best 200 solutions are shown as clusters. Circles indicate the position of the most favorable docking solutions, sequentially ordered with roman numerals going from high to low binding energy. The chromophore pCA covalently attached to Cys69 is indicated in green. The N-terminus (residues 1-28) is colored magenta, the backbone in red (29-43, 75-125), blue (44-51) and yellow (52-74) shows the corresponding structure of both pG and pB. A) front view of pG (1nwz.pdb, 0.82 Å crystal structure). B and C) front and side view, respectively, for a simplified pB model that is compatible with the NMR structure in solution (a truncated Δ(1-18) pG). D) ensemble pB structure (2kx6.pdb, based on various structural probes)\\textsuperscript{213}. E) Structural formula of sodium citrate in its H\textit{Citrate}\textsuperscript{2−} state. Note that the molecule has symmetric carboxylate end groups. For a schematic representation of the possible binding sites indicated by roman numerals in A-D see Figure 4.15.

Figure 4.15 (next page). Proposed binding modes of trivalent citrate to PYP pG (1nwz.pdb)\\textsuperscript{71} (A), truncated Δ(1-18) pG derived from the same crystal structure (B), and chosen as most simplified pB model that is compatible with the NMR structure in solution, and an ensemble structure of pB (2kx6.pdb)\\textsuperscript{213} that is based on data from a multitude of techniques and PYP mutants (C). Several docking positions have been found for pG and pB. The best representative, lowest energy conformer of each docking ensemble was immersed in a water box, annealed and energy-minimized by means of a short free Molecular Dynamics simulation. The detailed docking interactions between citrate and the protein for these refined structures are schematically represented by Ligplot\\textsuperscript{215} maps, showing favorable hydrogen bonds and non-bonding molecular interactions in the complex. Ligand plots are numbered according to Autodock\\textsuperscript{212} clustering analysis that sequentially order the best solutions from highest to lowest binding energy. The Ligplot interaction details agree well with the most significant shifts of amide atom pairs that are observed experimentally in the NMR spectrum of pG and pB.
Binding of hydrogen-citrate to PYP is affected by the structural changes

Figure 4.15.
Docking studies of citrate binding

In order to independently search for possible binding positions of HCitrte$^{2-}$ (see Fig. 4.14E) on the surface of the pG and pB states of PYP, we performed a docking study at pH 6.2 using Autodock. The docking results on pG are schematically displayed in Figure 4.14A. The four main clusters indicated by roman numerals point to binding sites that are complementary to the negatively charged citrate molecule. In summary, the preferred binding positions are located at (I) in the loop pocket surrounded by K17, W119, L113, S114 and the side-chain of Q32, (II) near F6, and the side-chains of K106 and K123, (III) near side-chains of K123 and R124, and (IV) in the loop region adjacent to N87 and L88. In fact, the amide protons that are predicted to ligate to citrate correspond exactly to the slight perturbations observed in the HSQC spectra. Thus, despite the low-affinity binding of citrate, these calculations are in full agreement with the experimental observations.

Docking results on the pB structure are difficult to obtain, as at the time of this writing no solution structure of the pB state of WT PYP at atomic resolution is available. The major problem for pB is that it is very dynamic. It basically is an ensemble of structures that may be divided in sub-ensembles. Depending on the used conditions the distribution of sub-ensembles may differ. It is therefore not surprising that a structure that perfectly suits our needs is not available. What we do know is that the structure of the pB state is less ordered than that of pG, especially in the N-terminus. To get an impression of the situation in WT PYP pB we opted to test two structures, one a simplified model based on the pG structure, the other an ensemble structure for pB based on various structural probes. For the simplified model of pB we use a truncated model ($\Delta 1-18$) derived from the pG crystal structure, in which the coordinates of 18 amino acids of the N-terminus are removed. This is based on the lack of amide proton signals from these 18 amino acids in the HSQC spectra at high temperature. With this model we basically test for possible binding sites that are protected by the N-terminus in pG. This model does not take into account any other structural changes that may occur in pB. The docking results for this simplified model of pB are shown in Figure 4.14B and 4.14C. The removal of the N-terminal residues leads to a newly available positively charged surface patch (I) that is normally occluded in the pG state by the aromatic ring of F6. The amino acids involved in this pocket are located on the beta sheet surface and include protonated H108, K106, K110, and K123. Cluster (I) is the dominant docking solution (74 %) found for the simplified pB model, other docking solutions of citrate are less favorable in energy and converge to similar surface positions already seen for the non-truncated structure of pG. Interestingly, the Cluster I binding position can be made accessible in pG by simply rotating the ring and backbone of F6 out of the way (see below).

A structure for pB that recently became available (2kx6.pdb) is an ensemble structure that is based on data from a multitude of techniques and PYP mutants, that were recorded under varying conditions. Also for this structure multiple binding
Binding of hydrogen-citrate to PYP is affected by the structural changes possibilities for citrate were found (Fig. 4.14D). Clusters I (27 %) and II (30 %) are the dominant docking solutions. Docking cluster I is located in an open type loop in the N-terminal domain consisting mostly of hydrogen bonds to the amide backbone involving residues K17, L23, L26-F28. Docking cluster II involves H108, a residue also found in the simplified model of pB, and is located on the N-terminal side of the central β-sheet.

The problem with this ensemble of structures is that the presence of different sub-ensembles of pB structures was not really taken into account. Also, the structures in the ensemble are all relatively similar, and therefore may only reflect one of the sub-ensembles of pB, or worse could represent a hybrid structure for pB that may not be representable for any of the major sub-ensembles of pB. In other words, the results from the docking studies of citrate binding to pB should be considered indications of possible citrate binding sites, and do not necessarily reflect reality. That said, the obtained results are in line with our measurements.

![Figure 4.16. Plot of the energy-minimized Δ(1-18) pG model containing a single, docked citrate molecule, bound at the side chain position of F6 near amino acids H108, K106, K123, F28 and F121. The structural model is based on the 0.82 Å crystal structure (1nwz.pdb) of the pG state of PYP. Three of these side chains (H108, K106 and K123) interact with the negative charges of the citrate carboxylate groups (broken lines). The steric clash between citrate and the backbone of the N-terminal domain that would be present at position F6 in the non-truncated, full-length pG state is indicated by a circle.](image)

From our docking study with the simplified pB model we noted a possible role for residue F6, which seems to block citrate from a possible binding site in pG. In order to show the structural involvement of the aromatic ring of F6 to gain improved citrate binding, a model of a pB-citrate complex was constructed from the crystal structure of the pG state. The ring and backbone of F6 were manually rotated out of the pocket originally surrounded by side chains of H108, K106 and K123 and F28 (Fig. 4.16) to make place for a single citrate molecule. In the final energy-minimized model in water (see Fig. 4.16) a single citrate molecule (both the Citrate$^{3-}$ and HCitrate$^{2-}$ variants) fits nicely into this pocket without further major conformational rearrangements of other protein parts being necessary.
Discussion

An extensive amount of knowledge already exists about the bio-/photo-physics of PYP. As a result, various versions of its photocycle have appeared over the years to try and describe how PYP functions. We have found that it helps to interpret the photocycle based on just three key species (pG, pR, and pB). The key is that each of these species should be considered a large ensemble of similar but ultimately different structures. This is of course not a radically new idea; it is just the consequence of the dynamic nature of proteins. Each ensemble of species can subsequently be subdivided into sub-ensembles that may dominate under specific conditions, e.g., in a specific pH range.

For this study, we have generated an extensive dataset to study the recovery characteristics of PYP as a function of both pH and citrate concentration. It has already been established that citrate retards ground state recovery of PYP at low pH. By varying both pH and the concentration of citrate, we aimed to determine if the influence of citrate is general or linked to a specific species of citrate. Based on our experiments, we have learned that specifically hydrogen-citrate (HCitrate$^{2-}$) slows down PYP recovery between pH 4.5 and 7. Furthermore, it only influences a specific sub-set of pB (pB$_m$), which dominates between pH 6 and 10.

Additionally, at pH > 7.5 there appears to be a slight citrate induced acceleration of PYP recovery. This effect is rather small, however, and is likely linked to the Citrate$^{3-}$ species. In order not to overly complicate the photocycle model, we ignored the influence of Citrate$^{3-}$. Significantly though, solution-NMR measurements at pH 7.9 revealed that Citrate$^{3-}$ may bind to the same surface region of the β-sheet of PYP that is exposed in the signaling state (data not shown).

By treating the pB intermediate as an ensemble of sub-species, it was fairly straightforward to incorporate the influence of HCitrate$^{2-}$ on the photocycle. All that basically was required was to add an equilibrium reaction for the binding of HCitrate$^{2-}$ to the pB$_m$ sub-species. This equilibrium is suggested by the data through the observed influence of citrate, which appears to be linked to both the presence of HCitrate$^{2-}$ and pB$_m$ (see Fig. 4.7B). As a result, we were able to determine an equilibrium constant for the binding of HCitrate$^{2-}$ to pB$_m$. It is clear that this equilibrium constant is greater in the H108F mutant of PYP. In other words, HCitrate$^{2-}$ appears to bind less efficiently to H108F PYP compared to WT PYP. Note that when the equilibrium constant of WT PYP was used in the analysis of H108F PYP anyway, not only did the fit of kinetic data become significantly worse also the spectral analysis resulted in clearly incorrect spectra (data not shown). Unfortunately, we were not able to perform these measurements at citrate concentrations above 30 mM. Due to the nature of citrate, a higher citrate concentration would also require a higher ionic strength of the sample. A test using an ionic strength of 1 M, instead of 250 mM, revealed that the influence of the higher ionic strength overshadows the influence of citrate and no citrate effect was visible anymore (see Fig. 4.9). As such, we were not able to determine the citrate binding constant very accurately (see Table 4.1 and Fig. 4.11). Interestingly, the H108...
Binding of hydrogen-citrate to PYP is affected by the structural changes

residue also showed up as a possible citrate interaction partner in our Autodock
calculations, which could explain the lower binding affinity in H108F PYP.

Another aspect of the photocycle model we use here is the double transition at
high pH for pB, signifying first deprotonation of the chromophore, resulting in a red-
shift in the spectrum, followed by a transition signified by deprotonation of Tyrosine
residues. Data at high pH is difficult to measure and analyze, not only because of the
relatively extreme solvent conditions required to measure these transitions, but also due
to the overlap of the pG and pBm spectra. The values obtained for these high pH regions
should therefore be considered approximate. Even so, it is still possible to see the
typical absorption increase caused by tyrosine deprotonation, as also previously
observed for pG. Furthermore, recent NMR measurements have shown that Y76 and
Y98 have a pK of 10.2 in pG. It is likely these tyrosine residues are also involved in
the transition we see here for pB at pK ~10.6.

The pH dependence of the recovery rate in the Δ25 mutant has the same high
pH transitions that we see in WT PYP (and H108F PYP). However, an equivalent to the
transition around pH 6 that is observed in WT PYP could not be detected for the Δ25
mutant within the pH range we analyzed. Notably, the spectrum of the low-to-medium
pH range is similar to pBm in WT PYP. This is further proof that the transition from pB1
to pBm is caused by solvent exposure of the central β-sheet due to changes in the N-
terminus. As in the Δ25 mutant the central β-sheet is always solvent exposed, it is
reasonable to expect that the pB spectrum at low-to medium pH is similar to pBm in WT
PYP.

The experiments reported here were also initiated to obtain a better insight into
the elusive pG’ intermediate. We already postulated that two elements are important for
the catalytic properties of this intermediate, the protonation state of the chromophore
(deprotonated), and the folding state of the protein. The protonation state reveals
itself spectrally. The importance of the folding state has now revealed itself through
comparison of the WT, H108F, and Δ25 PYP results presented here. Previously, we
postulated that the recovery rate is linked to the amount of pG’ that is present. In other
words, the recovery rate is dependent on the equilibrium between pBm and pG’. This fits
nicely with what is observed in H108F PYP, where the recovery rate is slower than in
WT PYP and where we observe less pG’ as well. However, if this is the only factor, we
should not be able to observe pG’ for Δ25 PYP, which has a very slow recovery rate.
The fact that we do observe a small amount of pG’ in this mutant indicates that also the
catalytic efficiency of pG’ likely plays a role. As the N-terminus is removed in
Δ25 PYP it is reasonable to assume the N-terminus plays an important role in the
catalytic activity of pG’. Furthermore, as it is likely that the catalytic activity of pG’ is
largely absent in Δ25 PYP, the recovery rate of this mutant may be representative for
the non-catalyzed recovery rate of PYP. It may therefore be interesting to have another
look at mutants that severely influence the recovery rate characteristics such as the
M100 mutants\cite{146, 148} and the E46Q mutant\cite{108, 220}, and investigate the possible role of pG’ in their behavior.

Binding of H\text{Citrate}^{2-} to PYP is fairly specific. Based on the NMR experiments it is clear that it binds – if at all – with very low affinity to the pG state, but clearly perturbs spectra of the pB state (see Fig. 4.13). Based on this chemical shift perturbation, it is likely that citrate anions bind between the central \(\beta\)-sheet and the N-terminus, which is only possible when the N-terminus moves away from the central \(\beta\)-sheet while forming the pB intermediate. This fits nicely with the observation that binding of H\text{Citrate}^{2-} is specifically associated with the pB\text{m} sub-set of pB, for which large structural changes, likely involving the N-terminus, have been proposed.\cite{94, 98, 132}

Note that this does not mean citrate has no influence on pG. In fact, based on denaturation experiments, citrate seems to destabilize pG in WT PYP. Interestingly, where one can easily interpret the citrate induced slower recovery of WT PYP in pB as a stabilization of the pB structure by citrate, citrate appears to have no significant influence on the stability of pB. Therefore, the influence of citrate on the denaturation titrations seems mainly to be the result of competition for similar binding sites, and/or influencing the solvent accessible surface area of the protein. As such, the slower recovery rate that is observed in the presence of citrate in WT PYP is not so much a result of stabilization of a single pB state, but more the result of a change in the distribution of the different sub-species of pB. In fact, we would suggest that changes in recovery rate can often be related to a change in distribution of pB sub-species. In addition, a change in the distribution of the different sub-species of pB may also explain the NMR perturbation data where small shift-changes were observed in almost the whole pB structure.

A previously proposed binding site for anionic citrate near the chromophore pocket\cite{207} of the pG state of PYP does not agree with our observation of a citrate binding site between the central \(\beta\)-sheet and the N-terminus. Therefore, we used the program Autodock\cite{212} to obtain a more detailed impression of the site(s) where H\text{Citrate}^{2-} may bind. The major problem here is the dynamic nature of pB, which makes it essentially impossible to assign a single structure to this intermediate. Therefore we used two widely different structures for pB, each with their own pros and cons (see above). The analysis revealed several possible binding sites, none of which include the previously proposed binding site.\cite{207} The most favored binding sites are indeed between the central \(\beta\)-sheet and the N-terminus, as we predicted based on our measurements. Furthermore, for both pB structures a binding site was found where H108 was involved. The involvement of H108 is in line with the observation that the influence of citrate in H108F PYP is either diminished or eliminated.
Conclusion

We have used the pH dependence of PYP as a tool to further refine the interpretation of the photocycle of this protein. To do this successfully we deem it essential to interpret the ‘classic’ photocycle intermediates/species of this protein as ensembles of sub-species. Here the distribution of these sub-species is dependent on characteristics such as pH. As a result we were able to show that addition of citrate can influence photocycle recovery by influencing the distribution of pB sub-species. More specifically, we were able to show that HCitrate$^{2-}$ can interact with the pB$_m$ sub-species to retard photocycle recovery. Furthermore, by doing the same for several mutants of PYP (H108F and Δ25) we could deduce that the relevant interaction of HCitrate$^{2-}$ with the pB$_m$ sub-species was most likely situated between the central β-sheet and the N-terminus. This was then confirmed via NMR measurements and Autodoc calculations.

The principles behind how citrate influences the PYP photocycle may translate very well to how light signals received by PYP may be transduced to the cell, or how a transduction interaction partner for PYP may influence the photocycle of PYP in vivo; an aspect that has been very poorly characterized for PYP up to this point. Especially since, a transduction interaction partner for PYP may well interact at a similar place to where HCitrate$^{2-}$ interacts with pB$_m$, i.e., between the central β-sheet and the N-terminal cap.

We feel that the idea, that changes in the distribution of photocycle intermediate sub-species influences photocycle kinetics and even the route taken in the photocycle reaction scheme, is a powerful one that can and should be used in the (re)interpretation of PYP data.

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