Kinetics and Mechanism of The Photocycle of Photoactive Yellow Protein

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Abbreviations:

PYP  Photoactive Yellow Protein
WT  Wild Type
*Hr. Halophila* *Halorhodospira Halophila*
PAS  PER-ARNT-SIM, acronym formed from the names of the first three proteins of this family (period clock protein, aryl hydrocarbon receptor nuclear translocator, single minded protein)
NMR  Nuclear Magnetic Resonance
FTIR  Fourier Transform Infrared Spectroscopy
RR  Resonance Raman
CD  Circular Dichroism
SAXS  Small Angle X-ray Scattering
pCA  para-Coumaric Acid
PR  Photoreversal Signal
SVD  Singular Value Decomposition
BFTB  Blue Flash data acquisition Triggered on Blue flash
DFTB  Double Flash data acquisition Triggered on Blue flash
DFTV  Double Flash data acquisition Triggered on Violet flash
BFTV  Blue Flash data acquisition Triggered on Violet flash
VFTV  Violet Flash data acquisition Triggered on Violet flash
GFTV  Green Flash data acquisition Triggered on Green flash

List of amino acids:

A  Alanine (Ala)
C  Cysteine (Cys)
D  Aspartic acid (Asp)
E  Glutamic acid (Glu)
F  Phenylalanine (Phe)
G  Glycine (Gly)
H  Histidine (His)
I  Isoleucine (Ile)
K  Lysine (Lys)
L  Leucine (Leu)
M  Methionine (Met)
N  Asparagine (Asn)
P  Proline (Pro)
Q  Glutamine (Gln)
R  Arginine (Arg)
S  Serine (Ser)
T  Threonine (Thr)
V  Valine (Val)
W  Tryptophan (Trp)
Y  Tyrosine (Tyr)
Abstract

The kinetics and intermediates of the photocycle and photoreversal of photoactive yellow protein were investigated from pH 4.6 to 11 by time-resolved absorption spectroscopy. At pH 7, three intermediate states: I₁ (λ_max ~ 460 nm), I₂ (λ_max ~ 370 nm) and I₂' (λ_max ~ 350 nm) were observed. I₂' is the signaling state of this photoreceptor. The time courses and the spectra of these intermediates were constructed using the extrapolated difference method. From the rise of I₂' (~ 1.0 ms) to the end of the cycle, I₁, I₂ and I₂' are in equilibrium. The equilibrium between I₂ and I₂' is pH dependent. Upon increasing the pH from 4.6 to 8.4, the I₂ population decreases whereas I₂' increases with a pKₐ of ~6.4. This pKₐ might be assigned to the carboxylate side chain of E46. Due to the pH dependent I₂/I₂' equilibrium, the ground state recovery rate is also pH dependent with the same pKₐ.

At pH 10, three intermediate states: I₁, I₁' and I₂' were observed. The spectrum of I₁' (λ_max ~ 425nm) was determined. From the rise of I₂' (~ 1.0 ms) to the end of the cycle, I₁, I₁' and I₂' are in a pH dependent equilibrium with a pKₐ of ~ 9.9. Upon increasing the pH from 8 to 11, the I₁/I₁' population increases whereas I₂' decreases. This pKₐ is assigned to the deprotonation of the phenol of the exposed chromophore. I₂ contributes minimally in this pH range. The rate constant for the ground state recovery also has a pKₐ of ~ 10 suggesting that $k₃ ∼ [I₂']$. Thus, the pH controls the accumulation of the signaling state I₂'.

The kinetics of photoreversal from the I₁, I₂ and I₂' intermediates was investigated with double flash excitation. A first flash, at 430 nm, initiated the photocycle. After a variable time delay, the I₁ intermediate was photoreversed by a second flash, at 500 nm, or a mixture of I₂ and I₂' intermediates was photoreversed by a second flash, at 355 nm. The photoreversal times from the I₁, I₂ and I₂' intermediates are respectively <1 µs (unresolved), 57 µs and 380 µs at pH 6. The first step in photoreversal is unresolved cis-trans isomerization of the chromophore. The respective delay dependent photoreversal amplitudes corresponding to 57 µs and 380 µs confirmed the temporal variation of the I₂ and I₂' intermediates of the normal cycle, and I₂ and I₂' are in equilibrium after 5 ms. Measurement of the pH dependent photoreversal amplitudes with 355 nm as a second flash applied with a delay of 20 ms, and at pH range from 4.6 to 8.5 produced a pKₐ ~6.1. This confirms the normal cycle I₂/ I₂' equilibrium. Depending on the progression of the photocycle, reversal becomes slower with the time delay, thus mirroring the individual steps of the forward photocycle. Photoreversal experiments at pH 10 with the second flash at 355 nm confirm the presence of the I₁' and I₂' intermediates and absence of the I₂.
Chapter 1

Introduction

In this study, the mechanism of the photocycle kinetics of the blue light photoreceptor photoactive yellow protein (PYP) from *Halorhodospira Halophila* is investigated in detail. Transient absorption spectroscopy with single and double flash excitation is used to investigate the photocycle of wild type (WT) PYP. For an understanding of the mechanism of the photocycle it is necessary to determine the spectra of the intermediates and their temporal interconversions.

This thesis is organised as follows: basics about PYP related to the structure, function and dynamics are discussed in Chapter 1. In addition, the specific aims of this investigation are highlighted in this Chapter. Chapter 2 reports about the details of the materials and methods applied in this investigation. The experimental results of the photocycle kinetics are presented in Chapter 3 where the results are divided according to the pH ranges: acid/neutral and alkaline pH. These observations are supported by the experimental results with the double flash excitation as described in Chapter 4. Finally, a summary and outlook of this investigation are presented.

1.1 Photoactive Yellow Protein (PYP)

During the extraction of all coloured proteins from halophilic, photosynthetic purple bacteria *Ectothiorhodospira* (now *Halorhodospira*) *Halophila*, Terry E. Meyer discovered photoactive yellow protein (PYP) in the cytoplasm of this bacterium [Meyer, 1985]. This protein is highly soluble in water and has a brilliant yellow colour at neutral pH. PYP absorbs blue light with an absorbance maximum at 446 nm, Figure 1.1, with a high extinction coefficient ($\varepsilon=45000 \text{ M}^{-1}\text{cm}^{-1}$). This protein has a reversible photo bleaching (photocycle) upon blue light illumination [Meyer, 1987]. At neutral pH, the initial dark state is resumed within sub-s time range after the bleach [Meyer, 1987].
These bacteria, \textit{Hr. Halophila}\textsuperscript{1}, accumulate in photosynthetic green light \cite{Sprenger,1993} and the video analysis of freely swimming bacteria shows that they swim out of intense (but nondamaging) blue light. This physiological action is reversible upon reducing light intensity indicating that physiological action is inhibited by light. The action spectrum of this physiological reaction is similar to the absorption spectrum of PYP indicating that PYP might be mediating this action. \textit{Hr. Halophila} exhibits a repellent response to blue light, presumably to select the optimal light levels and living conditions \cite{Sprenger,1993}. Recently hybrid PYP/Phytochrome photoreceptors have been discovered with well-defined functions \cite{Kyndt,2004}.

![Figure 1.1: Absorption spectrum of an aqueous solution of photoactive yellow protein from \textit{Hr. halophila}.](image)

Photo-bleaching of PYP with blue light initiates the structural changes \cite{Meyer,1989} (for details, see below) of this protein (similar to partial unfolding). This structurally altered protein is supposed to bind to a response regulator (still unidentified) during signal transduction, a signaling process similar to that of other sensory proteins like rhodopsin, bacteriorhodopsin, phytochrome and phototropin \cite{Meyer,1989}. Therefore, PYP and its photocycle have been studied in considerable detail since its discovery, to elucidate this signal transduction mechanism up-to atomic level (detail later). In addition, the structural motif of PYP is found to be similar to that of PAS\textsuperscript{1} domain proteins (detail later) which are sensory modules found in signaling proteins of all kingdoms of life. Therefore, PYP is proposed as the structural prototype of this universal domain. A variety of biophysical techniques have been used to study the structure and dynamics of PYP. Foremost of them are: time resolved X-ray Laue crystallography, NMR\textsuperscript{1} in solution, FTIR\textsuperscript{1} in crystal and solution, RR\textsuperscript{1}, CD\textsuperscript{1}, SAXS\textsuperscript{1}, fluorescence and transient absorbance spectroscopy. Structure, function and dynamics of PYP were recently reviewed in \cite{Cusanovich,2003} and \cite{Hellingwerf,2003}.
1.1 Photoactive Yellow Protein (PYP)

Figure 1.2: A ribbon representation of the secondary structure of PYP from *Hr. halophila*. pCA¹ chromophore is shown in green and red sticks. Color code of the secondary structure: random coils-brown, helices-cyan (labeled with: α₁-α₆), central anti-parallel β sheet-pink (labeled with: β₁-β₅). Structure was acquired from [Ihee, 2005]. The crystal structure data are deposited in the protein data bank: [http://pdbbeta.rcsb.org/pdb/](http://pdbbeta.rcsb.org/pdb/) with PDB ID: 1TS6. Figure was produced using molecular visualisation system PyMOL: [http://pymol.org/](http://pymol.org/).

The secondary structure of PYP determined from X-ray crystallography at 1.6 Å spatial resolution [Ihee, 2005] shows that it has a mixed α/β fold with a five-stranded central anti-parallel β–sheet flanked by six α–helices, as shown in the Figure 1.2.

![Secondary structure of PYP](image)

Figure 1.3: Sequence and corresponding secondary structure of photoactive yellow protein [Ihee, 2005]. Corresponding secondary structures: random coils (black), α helices (red) and β strands (green) are indicated above this sequence and are represented by straight line, wave form and thick arrow respectively. Names of the corresponding secondary structure elements: alpha helices, α₁-α₆, and beta strands (β₁-β₅) are placed on the top.

The solution structure of PYP was also resolved with NMR resulting in an ensemble of 26 structures [Düx, 1998]. The solution and the crystal structures are essentially the same [Hellingwerf, 2003].

Blue light absorption of PYP is due to its 4-hydroxycinnamoyl chromophore also called para-coumaric acid (pCA¹) [Hoff, 1994], which is covalently attached to a unique
cysteine located at the position 69 in helix $\alpha_5$ via a thioester bond [Hoff, 1994], Figures 1.2 and 1.4.

![Chemical structure of PYP chromophore](image)

Figure 1.4: Chemical structure of PYP chromophore linked to Cys-69 of the apoprotein via a thiol ester bond.

The primary structure of this protein consists of a sequence of 125 amino acids as shown in Figure 1.3. The chemical structure of the PYP chromophore is shown in Figure 1.4. The molecular weight of this protein, determined by ionisation mass spectroscopy, is 14020 Daltons [Baca, 1994].

![Structure of the chromophore binding pocket of PYP](image)

Figure 1.5: Structure of the chromophore binding pocket of PYP in (A) dark and (B) $I_2$ intermediate. Hydrogen bonds are shown as yellow dotted lines. Carbon, oxygen, nitrogen and sulphur atoms are shown as sticks (thick: side chain, thin: main chain) with green, red, blue and orange colours respectively. Figure A is the detailed structure around the chromophore of Figure 1.2. The structural data for Figure B are acquired from PDB ID:1TS6.

The chromophore is buried in a hydrophobic pocket inside the protein surrounded by amino acids T50, E46, Y42, R52, M100, Y98, F96 (see abbreviations) as shown in Figure 1.5. The chromophore is anchored by three hydrogen bonds, two of them between the phenolate oxygen and the protonated side chains of Y42 and E46, Figure 1.5A. The third one is between the chromophore carbonyl and the backbone amide proton of C69.

In the dark the chromophore is in the anionic form [Baca, 1994], [Kim, 1995] and in the $trans$ configuration [Borgstahl, 1995], [Genick, 1997A]. The side chain oxygen of T50
forms a hydrogen bond with the hydroxyl group of Y42 and the main chain carbonyl oxygen of the E46 [Borgstahl, 1995]. In addition, the guanidinium group of R52 forms hydrogen bonds with the main chain oxygens of T50 and Y98. The chromophore is separated from the solvent only by the side chain of R52. The tight packing of the protein core strongly constrains the chromophore mobility and limits the trans to cis isomerization which occurs in fs only after light absorption [Genick, 1997A].

Free coumaric acid in water at neutral pH absorbs maximally at 284 nm [Kroon, 1996]. Native PYP denatured with 4 M GdnHCl absorbs maximally at 339 nm and 397 nm at pH 3 and pH 11 respectively with a pK$_a$ of 8.9 of phenolate oxygen. The red-shift of the absorbance maximum from 284 nm to 339 nm is due to binding of pCA covalently to Cys-69 of PYP. Deprotonation of the phenolate oxygen of bound pCA further red-shifts the absorbance maximum to 397 nm, which is at 446 nm for the buried pCA (deprotonated) inside the folded native protein. Thus the additional blue shift of ~49 nm is due to protein chromophore interaction [Kroon, 1996].

The absorbance maximum of native PYP at pH 2 is blue-shifted by ~96 nm to 350 nm (close to the absorbance maximum of the denatured form) and the chromophore is protonated indicating that the protein is partially denatured at low pH. The pK$_a$ of this chromophore protonation is 2.8 [Meyer, 1985]. PYP is indeed denatured with strong acid and gets hydrolysed above pH 11. In the active site of PYP the deprotonated form of the phenolate oxygen is stabilized, Figure 1.5A, indicating that the ionisation constants for both E46 and the chromophore are shifted strongly in opposite directions from their solution pK$_a$ values (4.5 and 9) [Baca, 1994]. For the bound chromophore the pK$_a$ is lowered to 2.8. Although the exact nature of the protein-chromophore interaction and of this unusual pK$_a$ is unclear, it is argued that the anion form of the chromophore is stabilized in the dark by forming unusually short hydrogen bonds with Y42 and E46 in this highly packed area [Xie, 2001], where hydrogen bonds acquire significant covalent character [Anderson, 2004].

The interaction of the chromophore with the protein dramatically changes the photochemical properties of this cofactor (see below). ApoPYP may be reconstituted with anhydrides of various chromophore analogues [Kroon, 1996]. Analogues lacking the 4-hydroxy substituent lack both chromophore deprotonation and chromophore protein interaction (hybrid PYPs have only the protonated form of the chromophore analogue) confirming the importance of this substituent in the spectral tuning of PYP. Hydroxy and methoxy substitutions in the 3- and/or 5- position, Figure 1.4, donot disrupt strong interactions with the protein (hybrid PYPs have both the protonated and anionic form of the
chromophore analogue) but increase their pK_a for protonation and fluorescence quantum yield [Kroon, 1996].

This protein is highly stable against heating as it can be heated at 90°C for 10 minutes without permanent damage [Meyer, 1987]. The protein denatures in 4 M urea, thus also shows high stability against denaturation.

PYP enters a photocycle upon blue light excitation. During the photocycle, thermally unstable and spectrally distinguishable intermediates are formed in the dark, which are in their electronic ground state. The photocycle events of PYP in solution at neutral pH and room temperature can be divided into four parts, as shown in Figure 1.6:

1. Photo-isomerization around the C_7-C_8 double bond: This is the early event just after reaching the Franck-Condon state with a short blue laser pulse. Using ultrafast infrared spectroscopy, it was shown that the electronic excited state, P*, decays to the early photointermediate I_0 (λ_max ~510 nm) in 3 ps [Heyne, 2005]. Structural changes on the femtosecond time scale are probably restricted to the closely packed chromophore within the hydrophobic binding pocket leading to breaking of the hydrogen bond between the carbonyl oxygen of the covalent thiol ester and the backbone amide hydrogen of the Cys 69, Figure 1.5A, along with a partial twist of the thioester bond [Heyne, 2005], [Genick, 1998]. This movement is associated with complete isomerization about the C_7=C_8 bond attached to the phenolic ring, Fig 1.4, without significant relocation of this phenolate moiety [Heyne, 2005]. This leads to the formation of the more relaxed planar chromophore [Heyne, 2005], [Genick, 1998]. Trans to cis isomerization process upon excitation is driven via charge translocation from the phenolic oxygen toward the ethylene chain, weakening of the isomerizabale C_7=C_8 bond and thus leading to the isomerization [Groot, 2003].

The fluorescence quantum yield from P* at room temperature is very low ~ 10^-3 [Meyer, 1991] suggesting that the photocycle processes occur with high efficiency [Groot, 2003]. I_0 decays with 1 ns life time to form the relatively stable intermediate I_1 [Groot, 2003].

I_1 absorbs maximally at 460nm [Meyer, 1987], [Yeremenko, 2006] and in this intermediate the carboxyl group of Glu 46 remains protonated [Brudler, 2001] and the hydrogen bond to the chromophore’s phenolate oxygen is further strengthened [Perman, 1998], [Genick, 1998], [Brudler, 2001], [Unno, 2000], [Ren, 2001], [Pan, 2004]. The chromophore configuration as a whole is altered significantly (photon energy is stored and the resulting chromophore is in a higher energy state).

2. Proton uptake and chromophore protonation: In 370 μs, I_1 decays to I_2, Figure 1.6, where the protein takes up one proton from solvent [Meyer, 1993], [Borucki, 2002]. The phenolate
oxygen of the chromophore, in *cis* form, is protonated in I$_2$ [Imamoto, 1997], [Unno, 2002], [Unno, 2003] and the local protein environment is altered [Brudler, 2001] [Xie, 2001], [Unno, 2003], [Pan, 2004] blue-shifting the absorption maximum to $\lambda_{\text{max}} = 370\text{nm}$ [Otto, 2005], [Yeremenko, 2006], [Shimizu, 2006]. Both hydrogen bonds with the phenolate oxygen are broken [Genick, 1997A], [Ihee, 2005], and the phenolate moiety of the chromophore is exposed to the solvent [Genick, 1997A], [Brudler, 2001] [Xie, 2001], [Pan, 2004], [Unno, 2003] allowing it to move towards Arg 52 forming an hydrogen bond with its side chain [Genick, 1997A], Figure 1.5B. The carbonyl part of the chromophore flips back towards the backbone reforming the hydrogen bond with the main chain of cysteine [Pan, 2004], [Ihee, 2005].

Figure 1.6: Photocycle of PYP in solution from *H. halophila* at neutral pH and room temperature. P is the ground state and P* the excited state. Intermediate states are shown along with the values of their absorption maxima and half-lifes. I$_0$ and I$_1$ are states with a *cis* form of the deprotonated chromophore and I$_2$ and I$_2'$ are *cis* states with protonated chromophore. The values of the absorption maximum and half-life of I$_0$ state were acquired from [Groot, 2003] and the remaining from [Joshi, 2005]. Key photocycle events associated with various transitions are shown in the interior part of the scheme.

There are a variety of explanations for the chromophore protonation mechanism. Whether the chromophore is protonated intramolecularly from the putative proton donor, E46 [Xie, 2001] or from the solvent upon outer exposure of the chromophore [Borucki, 2002] is still to be established.

The kinetics of proton uptake and formation of the I$_2$ intermediate was shown to be synchronised in flash photolysis experiments with a pH indicator dye, suggesting that protonation of the chromophore occurs by proton transfer from the solvent [Borucki, 2002].

According to a different explanation of the chromophore protonation mechanism, formation of I$_2$ involves intramolecular proton transfer from Glu-46 to the phenolate oxygen.
whereby the strong hydrogen-bonding interaction between the chromophore and E46 is lost [Imamoto, 1997], [Xie, 2001]. Therefore, I$_1$-to-I$_2$ transition is likely to be the stage where the stored photon energy is transferred from the distorted chromophore to the protein, producing a relaxed I$_2$ chromophore structure.

3. **Signaling state formation:** Recent time-resolved FTIR [Brudler, 2001], [Xie, 2001], UV/Vis transient absorption spectroscopy [Yeremenko, 2006] and dye binding [Borucki, 2002], [Hendriks, 2002] studies showed that about 3 ms after flash excitation I$_2$ decays to another blue-shifted intermediate with a protonated chromophore, I$_2'$ (decay time ~400 ms), Figure 1.6. This transition is associated with a major conformational change of the protein as indicated by structural and spectroscopic methods in solution such as NMR [Rubinstenn, 1998], FTIR ([Xie, 2001], [Hoff, 1999]), SAXS ([Sasaki, 2002], [Imamoto, 2002]), and CD ([Sasaki, 2002], [Lee, 2001], [Ohishi, 2001]). The global structural change from I$_2$ to I$_2'$ has also been described as a partial unfolding [Van Brederode, 1996] and the absorbance maximum is further blue shifted to ~350nm [Otto, 2005]. In I$_2'$, a hydrophobic surface area is exposed [Meyer, 1989], [Borucki, 2002] which is found to be near the chromophore binding site [Genick, 1997A], [Hendriks, 2002] as well as the N-terminal cap [Rubinstenn, 1998], Figure 1.7.

Long-lived I$_2$ (more precisely a photostationary mixture of I$_2$ and I$_2'$) intermediate shows considerable disorder in solution structure mainly in the N-terminal domain [Rubinstenn, 1998]. Movement of the N-terminal cap in the I$_2$ to I$_2'$ transition allows the exposure of a hydrophobic area which presumably involves the anti-parallel $\beta$-sheet of the PAS core.

A partial loss of ellipticity is observed in I$_2'$ [Lee, 2001], and assumed to be due to the swinging away of the N-terminus from the central $\beta$-sheet, and the associated structural change, Figure 1.7.

The conformational change detected by X-ray diffraction in the I$_2$ intermediate, was found to be limited to the chromophore and the binding pocket [Genick, 1997A], [Ihee, 2005]. In I$_2$ the chromophore reorients by about 60°, bringing its oxygen close to the protein surface [Genick, 1997A]. It is likely that in crystals packing constraints prevent the formation of I$_2'$ and that the crystal structure of ref [Genick, 1997A] and of Figure 1.5B both refer to I$_2$.

The structurally altered I$_2'$ state is supposed to be the signalling state where a response regulator (unidentified to date) binds via hydrophobic contact during the signal transduction process [Cusanovich, 2003].
The dyes bromocresol purple [Borucki, 2002] and Nile red [Hendriks, 2002] bind transiently to the surface of the structurally altered I₂' intermediate but not in I₂. Indicating that the long-living I₂' intermediate rather than I₂ is the activated or signaling state.

Direct comparison of the time-resolved FTIR spectroscopic data in solution and in crystals also showed that the structure of the putative signaling state is not developed in crystals [Xie, 2001].

PYP contains two hydrophobic pockets one on each side of the central β sheet [Borgstahl, 1995], [Düx, 1998]. The chromophore is buried inside the larger one of the pockets, Figure 1.2. Another hydrophobic pocket keeps the N-terminal cap attached to the central β sheet, Figure 1.7. The N-terminal domain is the region where the largest conformation change is observed during the formation of I₂' and presumably the binding site with the response regulator [Cusanovich, 2003].

Structural changes at the chromophore (yellow sticks, Figure 1.7) in the I₁ state (formed upon absorption of the photon energy) trigger the structural alteration surrounding the chromophore on one side of the β sheet (during I₂ transition), Figure 1.7 (β₅−β₆). This results in global structural changes in the subsequent transition to I₂' on the other side of the β sheet causing the transmission of the signal from the ligand-binding site to the interacting N-terminal part (pink). This signal transduction mechanism is still unclear and many hypotheses concerning such long range interactions have been proposed.

![Figure 1.7:](image)

One of them is the hydrophobic collapse model where the N-terminal cap of PYP is supposed be involved in signaling via N-terminal disorder induced by photo-activation of the chromophore followed by the breakage of the E46/chromophore hydrogen bond and by movement of the chromophore out of the hydrophobic cavity [Cusanovich, 2003]. This results in the collapse of this cavity which causes the distortion of the central β-sheet. This
distortion in turn causes the N-terminal cap to dissociate from the β-sheet [Cusanovich, 2003]. From the salt dependence of the $I_2$ to $I_2'$ transition it was recently discovered that a salt linkage between the conserved residues K110 and E12 needs to be broken in the formation of $I_2'$ [Borucki, 2005]. Breaking of this “ionic lock” allows the N-terminal cap to dissociate from the β-sheet.

Another molecular mechanism of PYP activation is the protein quake model [Xie, 2001], [Pan, 2004]. According to this model, the resulting conformational change following light absorption around the chromophore in the $I_1$ state triggers the chromophore rearrangement that results in the enhancement of proton affinity in the $I_1$ state which causes direct proton transfer from E46 to the chromophore during the formation of $I_2$ without changes in global protein conformation. The resulting negative charge on E46 is embedded in the highly hydrophobic cavity and thus energetically unstable, hence triggering a large amplitude protein quake with the epicentre located at the new buried charge, COO$^-$ of E46 [Xie, 2001].

4. Ground state recovery: The signalling state decays to the initial state in 400 ms, Figure 1.6. During recovery of the ground state, several processes have to occur. The chromophore has to re-isomerize to the trans form, the protonation state of chromophore has to change, and the protein conformation has to return to its ground state fold. Although all these events may occur in distinct steps, they appear to occur simultaneously. Re-isomerization of the chromophore could be the rate-limiting step [Devanathan, 1998]. Photoreversal, light induced reisomerization of the chromophore from cis-to-trans, during the photocycle leads to a 1000-fold increase in the recovery to P [Joshi, 2005]. In the M100A mutant, the light–activated recovery rate is 6 orders of magnitude faster than dark thermal recovery where $I_2^{trans}$ is formed probably in ps time scale [Devanathan, 1998]. $I_2^{trans}$ differs only in chromophore conformation. This shows that a large change of the protein fold and the state of protonation can be achieved quickly once the chromophore has been isomerised [Devanathan, 1998] and thus that chromophore isomerization could be the rate determining step during ground state recovery. However, the sequence of the events during recovery (e.g., reisomerization and deprotonation of chromophore, proton release to solvent and protein conformational reset) is still to be established.

The the solution photocycle bleach and recovery are strongly affected by alcohols and by the viscosity, indicating the protein conformational change that exposes hydrophobic region to solvent [Meyer, 1989]. A change in heat capacity inferred from curved Arrhenius plots for recovery of photobleached PYP as a function of temperature [Meyer, 1989] also indicates
that hydrophobic region (23% of the amide groups which are buried in P are exposed in $I_2'$) is exposed to solvent during the photocycle [Hoff, 1999].

There is a remarkable pH dependence for both bleach and recovery [Genick, 1997], [Demchuk, 2000]. The pH dependence of the recovery rate constant was observed to be bell-shaped with two $pK_a$'s of 6.4 and 9.4 and assigned to E46 and the chromophore respectively. Glu 46 and the chromophore have $pK_a$ values of 4.5 and 9 respectively in solution [Genick, 1997]. This is quite different from the chromophore $pK_a$ in the dark titration of 2.8.

Crystalline PYP was shown to undergo a different photocycle than the PYP in solution at pH 6.5 [Yeremenko, 2006]. The signaling state, $I_2'$ develops only minimally in the crystal. The majority of molecules return to P from $I_2$, whose lifetime is shortened by a factor of about $\sim 18$ (20 ms in the crystal, 360 ms in solution) compared to the recovery in solution (branching). This implies that the crystal kinetic model is not applicable to the solution kinetic model [Yeremenko, 2006]. The crystallographic structures are thus only relevant up to and including the $I_2$ intermediate.

From crystallography, it is found that the residues most likely to effect the properties of the chromophore are Y42, E46, T50, R52, Y98 and M100 (see abbreviations), Figure 1.5. Single site mutants Y42F, Y42A, E46Q, E46A, E46D, T50V, T50A, R52A, R52Q, M100A, M100L, M100K, M100E, Y98Q, Y98F have been constructed and partially characterized [Meyer, 2003], [Imamoto, 2001], [Borucki, 2002], [Borucki, 2003], [Borucki, 2005]. Some of the results that are relevant for this thesis are described below.

Major effects of the mutations at Y42 are on the spectral properties and less on the kinetics of the formation where formation of $I_2$ is faster by a factor 2 and recovery slowed by half in the Y42F mutant [Brudler, 2000]. In the absorption spectrum of the ground state of Y42F, an additional peak appears at 390nm, where the major absorption band has its $\lambda_{\text{max}}$ at 458 nm, i.e. red-shifted by 12 nm with respect to WT. Y42F shows a biphasic transition with $pK_a$'s of 4.4 and 6.4 during titration in the dark. This is likely due to the loss of the Y42 hydrogen bond [Brudler, 2000].

The major effect of the E46Q mutant ($\lambda_{\text{max}}$=460nm) is on the photocycle kinetics, in which both formation and decay of the $I_2$ intermediate are significantly accelerated with increasing pH in contrast to WT [Borucki, 2003], [Genick, 1997]. The pH dependence of the recovery rate of WT is bell-shaped while that of E46 mutants is sigmoidal ($pK_a=8.3$). The corresponding $pK_a$ is due to chromophore ionisation [Borucki, 2003]. The $pK_a$ of the chromophore in the dark folded protein is increased significantly in E46Q (from 2.7 to 4.8)
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and even further in E46A (to 7.9) [Borucki, 2003] and E46D (to 8.6), which approach that of the chromophore in solution (~9) [Devanathan, 1999A].

Mutants T50V and R52A show small red-shifts in the absorption maximum at 457 nm and 452 nm respectively and have minimal effects on the photocycle kinetics [Brudler, 2000], [Genick, 1997]. T50 hydrogen bonds with Y42 in WT but directly hydrogen bonds to the chromophore in Y42F [Brudler, 2000]. The double mutant Y42F/T50V has a greater proportion of 390 nm intermediate than the Y42F mutant. Thus, Y42 and T50 stabilize the chromophore in the 446 nm form. The minimal effect of the mutation at T50 indicates that it is less important than Y42 and other active site residues. Negative charge on the chromophore was supposed to be stabilized partially by the nearby positive charge on R52, but minimal effects of mutation suggest that this is not the case [Imamoto, 2001].

Mutations M100A, M100L and M100K have dramatic effects in slowing the recovery of the photocycle by at least 3 orders of magnitude such that room light is sufficient to produce steady-state bleach [Devanathan, 1998]. The more rapid photoreversal versus dark recovery suggests that cis-trans isomerization of the chromophore could be catalysed by the M100 sulphur [Devanathan, 1998].

![3D folding of the PYP molecule can be divided into four parts (Pellequer, 1998): 1) N-terminal cap, coloured in red, contains 1-28 residues (α1 and α2 helices). 2) PAS core, coloured in green, spanning 29-71 residues (first two beta strands and helices: α3 and α4 including α2-chromophore binding loop). 3) Helical connector, coloured in blue, spans 72 to 87 residues. 4) β-scaffold, coloured in yellow, spanning residues from 88 to 125 (contains last three antiparallel beta strands). Chromophore is shown by pink coloured sticks.](image)

PAS domains are the structural modules that can be found in proteins from all kingdoms of life [Taylor, 1999], [Pellequer, 1998]. These domains are sensory modules, sensing typically oxygen, redox potential, or light intensity, and have a common structural motif: PAS core, β-scaffold, helical connector. Although the amino acid sequences of the different PAS domains are different, their 3D structures are conserved [Pellequer, 1998]. Since all of the PAS domains resemble the structure of PYP, Figure 1.8, PYP is proposed as the PAS structural prototype [Pellequer, 1998] as it is the best studied up to now. Highly
diffracting crystals of the dark state as well as of many intermediates are available. Moreover, PYP is highly stable against heating, denaturant and light intensity. Understanding the mechanism of converting photon energy to conformational alteration by PYP may help to understand the signal transduction mechanism of PAS domain super family in general.

PYP’s have been identified in seven organisms up-to now: Halorhodospira halophila (Hr. halophila), Rhodothallasium sallexigens (Rt. sallexigens), Halochromatium sallexigens (Hc. Salexigens), Rhodobacter capsulatus (Rb. capsulatus), Rhodobacter sphaeroides (Rb. sphaeroides), Rhodocista centenaria (Rc. centenaria, Ppr) and Thermochromatium tepidum (Tc. tepidum, Ppd) [Kyndt, 2004], [Cusanovich, 2003]. All of the results presented in this thesis were obtained with the PYP from Hr. halophila.

In quantum–chemical studies, [Molina, 2001], applying second order perturbation theory to the experimentally determined available structure [Genick, 1998], it was shown that the $\pi-\pi^*$ transition to the lowest excited state is related to the typical blue light absorption observed at 446 nm. Differential electronic density for the vertical electronic transition between the ground state, $S_0$, and the lowest singlet excited state, $S_1$, of the dark ground-state of PYP is as shown in Figure 1.9, where, overall electronic charge shift takes place from the phenolate oxygen (blue, decrease) to the carbonyl group (green, increase) during this transition [Molina, 2001].

1.2 Specific Aims of the Investigation

It is apparent from the discussion in the previous section that upon light activation, PYP forms a signaling state during the photocycle, in which a large structural change takes place and the protein is partially unfolded. The mechanism leading to the formation and the decay of this signaling state at the atomic level is still unclear. For an understanding of the functional mechanism of PYP as a photoreceptor, a detailed characterization of its photocycle, in terms of the kinetic mechanism is essential. The kinetics of the photocycle mechanism consists of the detection of the number of intermediate states, via their characteristic absorption spectra or three dimensional structures, the connectivity among
them, and the rate constants by which these states interconvert. Therefore, a general question about PYP and the major aim of this thesis is:

- **What is the reaction mechanism of this blue light photoreceptor PYP?**

The answer will be discussed on the basis of the experimental results in Chapter 3. Detection of the temporal evolution of the three dimensional structures formed after laser excitation is one way to answer this question. These structures might be followed using time-resolved X-ray crystallography in crystals [Ihee, 2005]. In particular, signaling pathways have been determined via structural progression of intermediates using time-resolved X-ray crystallography from ns to seconds [Ihee, 2005]. However, the photocycle kinetics in crystals differs strongly from that in the solution [Xie, 2001], [Yeremenko, 2006]. The signaling state I₂⁻ is not well developed in the crystal, probably due to crystal packing constrains [Xie, 2001], [Yeremenko, 2006] of the PYP molecules in the crystal lattice. Since, PYP is in the aqueous state under physiological conditions, the relevance of the studies in crystals is questionable.

Alternatively, the sequential structures might be tracked by measuring some specific property, e.g. the change of the chromophore absorption or protein vibrational spectra using time resolved electronic [Meyer, 1987], [Hendriks, 2003] or vibrational (FTIR: [Brudler, 2001] [Xie, 2001], Resonance Raman: [Pan, 2004]) spectroscopy. Changes in secondary and tertiary structures have also been detected by CD and SAXS. Detection of the structural progression of the intermediates in the solution during dark relaxation after light activation is still in an early stage. The structural and the dynamic changes during the photocycle are also studied by NMR in solution [Rubinstenn, 1998], where the solution structure of the dark and the signaling state are resolved in the presence of background illumination. Time resolved absorption or vibrational spectroscopy is one way to track the intermediates under the physiological conditions to elucidate the activation mechanism of the photoreceptor.

In this investigation, time-resolved absorption spectroscopy with ns time resolution (section 2.2) is used to study the photocycle kinetics, where absorbance change is measured after laser excitation with a ns pulse at a wavelength near the absorption maxima, Figure 1.1. This absorbance change is due to the formation and decay of the various spectrally distinguishable intermediates during the photocycle. The purpose of this investigation is to characterize the intermediates spectrally from the measured data of the transient absorbance changes. Recently developed extrapolated difference method (section 2.4.1) and an alternative method scaled subtraction (section 2.4.2) will be used to determine the spectra of the intermediates. This is the key information to acquire the time courses of the intermediates.
that determine the interconversion of the intermediates (sections 3.1.1 and 3.2.1), leading to a plausible reaction mechanism.

This technique is comparable to the determination of signaling pathways via the transient structures in X-ray crystallography. Furthermore, this method has an advantage over X-ray crystallography. Transient absorption measurements are carried out in solution i.e. under the physiological conditions. Moreover, the photocycles in solution and crystal differ as described earlier.

During this investigation particular emphasis is given to the pH effect on the photocycle (sections 3.1.2 and 3.2.2). In earlier studies of the photocycle a bell-shaped recovery rate was observed with two pKa’s ~ 6.4 and ~ 9.4 [Genick, 1997]. In this study, these pKa’s will be explained in terms of titration curves of the intermediate populations, and assigned to particular functional groups. Thus, the extensive data set measured with ns time resolution from ns to s, in pH region 4.6 to 11, and the wavelength range from 320 nm to 510 nm will be explained in terms of the number of intermediates, their characteristic spectra and the interconnectivity. Detailed photocycle models will be proposed based on these observations.

Photoreceptors with their chromophores photoisomerizable around a specific bond have numerous features in common. One of these is that many photointermediates, in particular the signaling state, can be reversed by light directly back to the initial dark state. This is called photoreversal. Photoreceptors with this property are called photochromic: they can be switched back and forth by light between the inactive dark and active signaling states. Examples are rhodopsin, phytochrome, phototropin and PYP. Photoreversal is a common, almost universal, property of photoreceptors. Photoreversible reactions play a key role in signal transduction. In addition to its intrinsic biological significance, this photochromic molecular switching property is of potential technological interest in the development of optical storage and switching devices.

The mechanism of the photocycle kinetics is also supported by double flash excitation experiments. During a double flash experiment, the sample is excited by two flashes of the selected wavelengths applied with an appropriate time delay. Selection of these two parameters depends upon the absorption spectra and the kinetics of the photocycle intermediates. The first flash, usually a blue flash at 430 nm starts the cycle. The effect of the second flash applied during the photocycle is photoreisomerization of the chromophore followed by a rapid dark relaxation to the initial state through photoreversal process.
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The photoreversal signal ($PR^1$) may be resolved by triggering the data acquisition on the second flash. The second question considered during this investigation is:

- **What might be deduced from the effect of a second flash of the proper wavelength applied at a suitable time during the photocycle?**

The answer will be discussed on the basis of the experimental results in chapter 4. It will be shown that the answer to this question assists to verify the reaction mechanism of the normal photocycle described in chapter 3.

The choice of the wavelength of the second flash is important to be considered for this experiment. The largest effect is expected on the $I_2$ (370 nm) and $I_2'$ (350 nm) intermediates when a violet flash at 355 nm is applied during the photocycle, Figure 1.6. Similarly, the effect of a green flash at 500 nm might excite $I_1$ selectively, as other intermediates including the dark state absorb minimally around this wavelength. Moreover, the $I_1$ and $I_2/I_2'$ intermediates are formed respectively in $\sim \mu s$ and ms time scale during photocycle, Figure 1.6. A larger effect of a green flash on $I_1$ and a violet flash on $I_2/I_2'$ is expected, when the second flash is applied respectively with a $\sim \mu s$ and a ms time delay.

The effect of the second flash will be defined in terms of the photoreversal signal (section 4.1.1). This signal represents the photoback reaction kinetics. The kinetics may be time resolved if photoreversal is slower than the experimental time resolution. Spectral characterization of the photoreversal signal (section 4.1.3) describes of the photo-backreaction kinetics via the photoreversal intermediates involved. The delay dependence of the photoreversal amplitudes acquired from the fit of this signal (section 4.1.2) may follow the temporal variation of the normal cycle intermediate from which the photoreversal signal originates, when excited selectively. In this way, double flash excitation experiments facilitate the investigation of the normal photocycle, and contribute to an understanding of the reaction mechanism of the photoreceptor.

The initial evidence for photoreversals in PYP are from single flash kinetic experiments with a photostationary mixture of $I_2$ and the ground state in wild type [Miller, 1993], [Hendriks, 1999] and in the mutant M100A [Devanathan, 1998]. Transient absorption measurements with excitation at 355 nm and nanosecond time resolution showed that rapid unresolved photoisomerization occurred, converting $I_2$ (which is the *cis* isomer) to an $I_2^{\text{trans}}$ form. $I_2^{\text{trans}}$ then decayed monoexponentially to the initial dark state (P) with an exponential time constant of 147 $\mu$s at room temperature [Hendriks, 1999]. Compared to the thermal decay, the light-induced decay of $I_2$ in wild-type PYP is approximately $10^3$ times faster. In the mutant M100A the return to P via the photo-backreaction from $I_2$ was also
monoeponential with a time constant of 230 µs [Devanathan, 1998]. At that time the existence of two I₂- like intermediates, I₂ and I₂′ was not recognised. So in these early experiments an undefined photostationary equilibrium of I₂ and I₂′ was photoreversed.

The double flash experiments presented in this investigation are superior to the earlier reported photoreversal experiments ([Miller, 1993], [Hendriks, 1999], [Devanathan, 1998]) where a photostationary mixture of intermediates was subjected to a single UV flash. Moreover these experiments were limited to the conditions where the cycle is slow enough to accumulate the intermediates in sufficient amounts.

The next three chapters report the details of the experimental and data analysis methods (Chapter 2), and the results of the single (Chapter 3) and double flash excitation (Chapter 4) experiments.
Chapter 2

Materials and Methods

The mechanism of the photocycle kinetics of PYP was studied in detail using time resolved absorption spectroscopy, also known as flash photolysis. In this technique, the sample is excited with a short laser pulse and dark relaxation of the protein is monitored via time resolved absorbance changes. In this chapter, the principles of the transient absorbance changes will be explained (section 2.2.1) and the experimental setup used to measure such changes in single or double flash excitation experiments will be described (section 2.2.2). The aim of the data analysis is to determine the spectra and time courses of the intermediates as described in section 2.4. Spectra of the intermediates formed during the photocycle and the intermediate time courses can be calculated from the measured time resolved absorbance changes using the extrapolated difference method (section 2.4.1) or the scaled subtraction method (section 2.4.2). A photocycle model can be proposed from the fit of the time courses with a sum of exponentials. As some of the photocycle intermediates are in equilibrium during the photocycle, the time courses were also measured for various values of a certain parameter such as pH, to identify the nature of the equilibria. Moreover, pH dependent equilibria and associated decay rates are related in section 2.5.2, which explains the underlying cause of the pKₐ of the pH dependent decay rates.

2.1 Sample Preparation

Wild type PYP was investigated in this study and acquired from Terry E. Meyer/ Michael A. Cusanovich, University of Arizona, USA. Native PYP from *H. halophila* was prepared as described [Meyer, 1989]. *H. halophila* holo-PYP was produced also by the use of the biosynthetic enzymes TAL and pCL and subsequently purified from *Escherichia coli* BL21(DE3) as described in [Kyndt, 2003].


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2.2 Flash Photolysis

2.2.1 Transient Absorbance Changes

When monochromatic light is passed through an absorbing material, the transmitted intensity is proportional to the incident intensity \( I \), the concentration \( c \) of the absorbing material, the infinitesimal path length \( dx \) and the characteristic extinction coefficient \( \varepsilon(\lambda) \):

\[
dI = -\varepsilon(\lambda) c(x) I(x) dx
\]

(2.1)

If the concentration \( c \) is constant over the path \( d \), integration of this relation leads to the Lambert-Beer law:

\[
I = I_0 10^{-\varepsilon(\lambda) cd} \Rightarrow A(\lambda) = \varepsilon(\lambda) cd = \log \left( \frac{I_0}{I} \right)
\]

(2.2)

The wavelength dependence of the extinction coefficient \( \varepsilon(\lambda) \) (unit: \( M^{-1}cm^{-1} \)) determines the absorption spectrum of the species. The dimensionless exponent \( \varepsilon(\lambda)cd \) is called absorption and also measured in the unit of OD (optical density). To measure the absorption spectrum, the light intensity of monochromatic light produced by a monochromator has to be measured before (\( I_0 \)) and after (\( I \)) the sample for every wavelength of the measuring light. In a two beam spectrophotometer, instead of measuring the light intensity \( I \), the transmitted intensity through a reference sample (usually water), placed in one of the beams of the spectrophotometer, is measured to calculate the absorption. A typical absorption spectrum of PYP is as shown in Figure 1.1.

In general, during the photocycle after laser excitation, a spectrally distinguishable mixture of species \( i \), each with a distinct spectrum \( \varepsilon_i(\lambda) \), is formed transiently. In that case, the absorption is a function of both time and wavelength:

\[
A(\lambda, t) = \sum_{i=1}^{N} A_i(\lambda, t) = \sum_{i=1}^{N} \varepsilon_i(\lambda) c_i(t) d
\]

(2.3)

In time resolved absorption spectroscopy, the absorbance change before and after laser excitation is measured rather than the absolute absorption. In these measurements, the transmitted intensity of the measuring light at a selected wavelength after laser excitation is measured, and transient recording of the intensity \( I(\lambda,t) \) is started after the laser flash. In this case, the absorbance change during the photocycle is calculated using the relation:

\[
\Delta A(\lambda,t) = A(\lambda,t) - A_p(\lambda) = \log \left( \frac{I(\lambda)}{I(\lambda,t)} \right)
\]

(2.4)

where,

\[
A_p(\lambda) = \varepsilon_p(\lambda) c_T d
\]

(2.5)
is the ground state (dark state, P) absorption and \( I(\lambda) \) is the transmitted intensity from the sample before laser excitation. \( c_T \) is the initial PYP concentration (total). In this case, measurement of the intensity \( I_0 \) before the sample is not necessary. Substituting the values of \( A(\lambda,t) \) and \( A_p(\lambda) \) in eq 2.4 from 2.3 and 2.5 respectively:

\[
\Delta A(\lambda,t) = \sum_{i=1}^{n} \epsilon_i(\lambda) c_i(t) d - \epsilon_p(\lambda) c_T d \tag{2.6}
\]

where, the absorbance change is a function of the transient concentration changes of the intermediates. Since,

\[
c_T = \sum_{i=1}^{n} c_i(t) \tag{2.7}
\]

Eq 2.6 becomes:

\[
\Delta A(\lambda,t) = \sum_{i=1}^{n} \left[ \epsilon_i(\lambda) - \epsilon_p(\lambda) \right] c_i(t) d \tag{2.8}
\]

and further,

\[
\Delta A(\lambda,t) = \sum_{i=1}^{n} \left[ \epsilon_i(\lambda) - \epsilon_p(\lambda) \right] \left( \frac{1}{c_T} \right) c_i(t) d * c_T \tag{2.9}
\]

with,

\[
n_i(t) = \frac{c_i(t)}{\sum_{i=1}^{n} c_i(t)} \tag{2.10}
\]

as the relative concentration, eq 2.9 becomes:

\[
\Delta A(\lambda,t) = \sum_{i=1}^{n} \left[ A_i(\lambda) - A_p(\lambda) \right] n_i(t) \tag{2.11}
\]

Thus, the absorbance change from the time resolved absorption measurements at any wavelength \( \lambda \) is the sum of the difference spectra multiplied by the associated relative concentrations of the intermediates. When the coupled system is described with first order kinetics, the rate of change of the concentration depends linearly on the concentration itself:

\[
\frac{d}{dt} n_i = \sum_{j=1}^{m} K_{ij} n_j \tag{2.12}
\]

with \( K_{ii} = -\sum_{j=1,j\neq i}^{m} K_{ij} \); \( i = 1, \ldots, m \)

where \( K_{ij} \) is the decay rate from the j to i intermediate. The general solution of this system of coupled linear differential equations can be written as the sum of r exponentials:
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\[ n_i(t) = \sum_{j=1}^{r} C_{ij} e^{-k_j t} \]  

(2.13)

with decay rates \( k_j \). Substituting the solutions for \( n_i(t) \) from eq 2.13 in eq 2.11 we obtain:

\[ \Delta A(\lambda,t) = \sum_{j=1}^{r} \sum_{i=1}^{n} C_{ij} [A_i(\lambda) - A_p(\lambda)] e^{-k_j t} = \sum_{j=1}^{r} B_j(\lambda) e^{-k_j t} \]  

(2.14)

where,

\[ B_j(\lambda) = \sum_{i=1}^{n} [A_i(\lambda) - A_p(\lambda)] C_{ij} \]  

(2.15)

is the amplitude spectrum corresponding to the \( j^{th} \) transition which depends upon the kinetic parameters \( C_{ij} \) and the difference spectra.

In matrix notation, relations (2.11) and (2.15) are:

\[ \Delta A = (A - A_p) n \]  

(2.16)

\[ B = (A - A_p) C \]  

(2.17)

The row index of \( \Delta A \), \( B \), \( A \) and \( A_p \) corresponds to the wavelength \( \lambda \), the column index of \( \Delta A \) and \( n \) corresponds to the time \( t \), the column index of \( B \) and \( C \) to the \( j^{th} \) component and the column index of \( A \), \( A_p \) and row index of \( C \) and \( n \) to intermediate \( i \). Matrix \( A_p \) consists of identical columns of the ground state spectrum.

The purpose of the analysis of the photocycle kinetics is to extract the spectra of the intermediates, \( \varepsilon_i(\lambda) \), their time courses \( n_i(t) \) and, under favourable circumstances, the reaction scheme from the exponential fit of \( n_i(t) \). The extrapolated difference method (section 2.4.1) and the subtraction method (section 2.4.2) are the methods used to obtain the spectra of the intermediates, i.e. matrix \( A - A_p \). Corresponding time courses of the intermediates can be obtained from the measured absorbance changes (time traces) using the matrix inversion of the eq 2.16:

\[ n = (A - A_p)^{-1} \Delta A \]  

(2.18)

2.2.2 Experimental Set Up for the Measurement of Transient Absorbance Changes with Single and Double Flash Excitation

The experimental set up for the measurement of transient absorbance changes with single or double flash excitation is shown in Figure 2.1. The whole experiment was controlled through a PC (486, 133 MHz, 8 MB RAM) via a homemade software, an algorithm developed in C++ [Dickopf, 1997]. For the efficient excitation of the ground state to start the photocycle, a short laser pulse at the wavelength near the absorption maximum of 446 nm is required. This is produced through an excimer pumped dye laser. When the measurement is
started, an I/O serial port (Fa. Kolter) transmits a TTL pulse to a home made delay generator (DG), Figure 2.1, via an interface (home made).

The excimer laser (Radiant Dyes, RD-EXC-100), EL, with XeCl as laser medium can be started using two pulses P1 and P2 at a delay of 33 ms produced by the delay generator. An output laser pulse at 308 nm of about 100 mJ pulse energy and 10 ns pulse length is used to excite the dye (Stilbene 3 dissolved in methanol, Radiant Dyes) DC, which produces a laser pulse at 430 nm (blue flash), energy of which is increased further to 4-6 mJ by using a mirror (m1) on one side of the beam, Figure 2.1.


Mirror m2 was used to align this beam to excite the sample placed in the cuvette, C (5X5 mm, Quartz glass, Helma, QS 1.000). The beam diameter was adjusted with two lenses (6 cm diverging and 16 cm converging lens, not shown) which was about 1 cm at the sample cuvette. Beam profile and intensity adjustment before the sample cuvette and beam alignment
Chapter 2 Materials and Methods

were made by optimising the output signal of the photomultiplier (see below) measured by an oscilloscope with the sample in the cuvette.

The volume of the protein solution in the cuvette was 500-700 µl. The temperature of the sample was controlled by a thermostat (Julabo Labortechnik GmbH) with the circulation around the cuvette holder.

A 100 W tungsten-halogen lamp (Spindler and Hoyer) was used as a source of measuring light (LS). This is highly stable and durable with sufficient light intensity in the spectral range of 320-800 nm which is one of the requirements of the flash photolysis experiment. The light beam passed through an infrared filter, from water lens (WL), that suppresses heat radiation, was incident on the monochromator M₁ (Jobin-Yvon, “division d’instruments” SA, H10 VIS, spectral bandwidth (FWHM) of 6 nm with 1 mm slits).

The beam is further collimated by a lens L₁, limited by an aperture A₁ (5-7 mm diameter) and passed through the sample cuvette (path length 5 mm) crossing through almost the same volume and at right angles to the excitation beam. The transmitted beam is further limited with a bit larger aperture A₂ (10-12 mm diameter) and focussed on the double monochromator, M₂ (Jobin-Yvon, “division d’instruments” SA, H10 VIS, spectral resolution of 6 nm with 2 mm width of all three slits) by a lens L₂. The photomultiplier, PM (Hamamatsu S4710 1 GHz bandwidth) was placed at the exit of this monochromator. The double monochromator, M₂ was used to suppress the light of the laser flash scattered in the direction of the photomultiplier.

The light intensity of the measuring beam was 100 µW/cm² at 450 nm at the sample after the first monochromator. Attenuation of the intensity of the measuring beam had no effect on the observed kinetics (see later), suggesting that the intensity was small enough to avoid the build up of photostationary states. The spectral bandwidth of the monochromators in the measuring beam was ± 6 nm which is the spectral resolution of the experiment.

As the monochromator M₂ doesn’t suppress fully the scattered light, the photomultiplier detects the saturation intensity as the flash effect in the time range of < 100µs at the wavelength of the laser pulse (± 6 nm). To reduce further this artefact, the light intensity of the measuring light has to be increased so that the scattered light intensity is negligible compared to the measuring light intensity. This can also be done by using slits of larger widths in the monochromator M₁ and M₂. However, with measuring light of higher intensity, a photostationary mixture of the ground state and the photointermediates was observed at pH values where the photocycle is slow (see below). In that case, the measuring
light intensity has to be further reduced. To overcome the problem with lower signal to noise ratio in this case, the number of measuring scans were increased (see below).

The photomultiplier voltage, $V(t)$ is proportional to the intensity $I(t)$ transmitted through the sample. In this experiment, the absorbance change is measured rather than the absolute absorbance which depends upon the measured voltage, $V(t)$ with respect to the reference voltage, $V_r$. In this case, a voltage of opposite sign $V_o$ is applied to the photomultiplier so that signal is combined with the $V_o$, and $V_r$ is compensated ($V_o \approx V_r$). Then, the time course of the measured voltage, $V^M(t)$, after laser excitation is:

$$V^M(t) = V(t) + V_o$$

Absorbance change of eq 2.4 is obtained using the following relation:

$$\Delta A(\lambda, t) = -\log \left( \frac{I(\lambda, t)}{I(\lambda)} \right) = -\log \left( \frac{V_r + V^M(t) - V_r^M}{V_r} \right)$$

(2.20)

To measure $V_r$, the voltage of opposite sign $V_o$ has to be switched off for a defined time interval and $V_r^M$ is the voltage just before the trigger (pretrigger value). Before the start of the measurement, the photomultiplier voltage is increased so that the offset is 200 mV. During measurement a photomultiplier voltage of the typical range of 200-500 volts was necessary with this additional offset voltage, $V_r$ of ~ 200 mV. Photomultiplier voltage depends upon the intensity of the transmitted light which in turn depends on the type of the sample and the other experimental conditions (e.g. slit width, measuring light intensity, geometry of optical set up etc.). Once the photomultiplier is sensitive to the transmitted light, output signal from the photomultiplier is produced. As the absorption of the sample changes during the photocycle after laser excitation, the output of the photomultiplier changes accordingly.

The signal from the photomultiplier is amplified (amplifier, AD9610, Analog Devices) by a factor of 40, passed through a low pass filter and divided in two channels (Ch1, Ch2, Figure 2.1) for digitalization via two overlapping channels. The corresponding ranges of the sampling rate of the fast and slow channels are $10^8-10^7 \text{ s}^{-1}$ and $10^5-10^4 \text{ s}^{-1}$ respectively. Data acquisition, signal processing, and digitalization were performed as described [Dickopf, 1997], [Borucki, 1999].

Output (data acquisition) from the two channels is converted into signals of different time base using a Analog-Digital converter. One of the channels is connected to the fast oscilloscope (Lecroy 9350A, DO: Figure 2.1), which has an analog band width of 500 MHz and sampling rate of 1 GHz, 8-bit amplitude resolution and equipped with memory capacity of 100kbyte. The saved data were read via IEEE-interface (488-PC2, Meilhaus) to the PC for
further processing. Data acquisition for the slower channel is done with data acquisition board (T512, IMTEC). This has 12-bit resolution, 4 Mega samples storage capacity and 200 ns time resolution.

Both data acquisition units, the oscilloscope and the A/D card of the PC, can be triggered simultaneously by the output signal from photodiode T₁ which is triggered by the scattered light of the laser flash. This is the zero time of the measurement. The measured time range in this experiment is 1 ns to 50 s with a sufficient overlap of both channels. The effective time resolution during the laser flash is limited to the range of 20-50 ns due to the rise time of the photomultiplier and the amplifier.

2x10⁵ data points per flash are collected at the temporary memory of the PC after triggering of the data acquisition units which is the input for further data processing.

Each measurement produces a large amount of data (above 8 MByte). To describe the exponential relaxation with time after laser excitation, fewer time points per time interval are sufficient (logarithmically equidistant 100 data points per decade are sufficient to produce a adequately smooth time trace, see below). Therefore, logarithmic data reduction of measured data points is valuable. Data reduction is also useful to enhance the efficiency of the PC. To reduce the data points measured on the linear time scale to logarithmically equidistant time patterns with 100 data points per decade the following relation can be used:

\[ t_i = 10^{i/100} \text{ ns}, \text{ i=0, \ldots, 1000} \quad (2.21) \]

In this way, measured linear time points are converted to logarithmic time patterns. The total number of data points is about 700 after data reduction of 2x10⁵ measured data points. Then, the absorbance change is calculated from \( V_r \), \( V_r^M \) and the temporal variation voltage \( V^M \) using equation 2.20. Due to slightly different amplification factors of the two channels, the two signals deviate slightly in the overlap region and an offset is used to correct the mismatch. Signals from the 2 channels are merged into a single curve. To increase further the signal to noise ratio, measurements are repeated many times, generally 10 or 20 scans, and the averages of all the scans are saved.

Due to this averaging process, the scattered data points produce more or less continuous time traces. As more linear data points contribute to a measured logarithmic time point at later times than at early times, the signal to noise ratio of the later time point is much better than that of the early time point, (Figure 3.3). For this reason a slower process will have a higher accuracy than a faster one. This effect was taken into account in the data analysis by using appropriate weighting factors which increase the weight of the later data points (see below).
To investigate the effect of a second flash applied during the photocycle, double flash experiments were performed. In this experiment, the sample is excited with a second flash at a selected wavelength after a suitable time delay. Selection of these two parameters depends upon the absorption spectra and the kinetics of the photocycle intermediates. In general, the effect of the second flash applied during the photocycle is a fast isomerization followed by return to the initial state which is faster than the thermal relaxation in the normal cycle. This is called photoreversal. In PYP, intermediates like $I_1$ ($\lambda_{\text{max}}=460\text{nm}$), $I_2$ ($\lambda_{\text{max}}=370\text{nm}$) and $I_2'$ ($\lambda_{\text{max}}=350\text{nm}$) are formed during the photocycle on the µs and ms time scales respectively. The largest effect is expected on the $I_2$ and $I_2'$ intermediates when a violet flash at 355 nm is applied between 200 µs and 20 ms, Figure 4.1, after initiation of the photocycle (as other intermediates as well as the ground state have smaller extinction coefficients at this wavelength). A green flash around 500 nm wavelength applied at a µs time delay might excite selectively the $I_1$ intermediate (as $I_2$ and ground state have practically zero absorbance at this wavelength).

For photoreversal experiments on $I_2$ and $I_2'$, the sample was excited with a second laser pulse at 355 nm (Q-switched frequency tripler of Nd:YAG Laser, Spectra-Physics Inc., GCR-16, pulse energy 10 mJ/pulse, pulse width 5-6 ns), Figure 2.1. The delay generator (DG) produced two pulses- 2 and 3 from the start pulse with defined delay which were used to start the lasers. First pulse- 2 starts the excimer pumped dye laser (430 nm) which initiates the photocycle of PYP. Second pulse- 3 starts the Nd:YAG Laser laser at a defined time delay during the photocycle.

The time delay between the two pulses was checked by an universal counter (Philips PM6666) UC, using the output pulse from the photodiode for the excimer laser and the electronic pulse synchronized to the Q-switch for the violet flash. Since the fluctuation of time delay between the triggers is around ±200 ns, time delays larger than 1 µs are accurate. A delay generator (DG) allowed delays to be set between the two laser pulses from 1 µs to 50 s with an accuracy of 100 ns.

To resolve the kinetics of photoreversal after the second flash, data acquisition was also triggered on the second flash. This was the Q-switch synchronized pulse for the violet flash.

For the photoreversal of $I_1$, the sample was excited with a second laser pulse at 500 nm from an excimer pumped dye laser (Lambda Physik, EMG 50E, pulse width 10 ns, average energy 100 mJ/pulse). Coumarine 307 dissolved in methanol was used as the laser
dye. The laser energy in front of the dye cuvette was 13 mJ/pulse. Photodiode T₂ was used to monitor the delay and used for triggering of the data acquisition.

The dark spectrum (A₀) was measured by using a double beam UV-Vis spectrophotometer (Shimadzu Corporation, Japan, UV-260). The pH was controlled with a glass electrode of a digital pH-Meter (Knick). Titrations were carried out by the addition of the small amounts of HCl or KOH.

### 2.3 Amplitude Spectra of the Transitions

The amplitude spectra Bᵢ(λ) of eq 2.15 can be obtained from the measured transient absorbance changes ΔA(λ, t) with the following two methods: global fit analysis and singular value decomposition (SVD¹). Amplitude spectra of the transitions are further used in the extrapolated difference method (see section 2.4.1) or scaled subtraction method (see section 2.4.2) to estimate the spectra of the intermediates of eq 2.16 and to calculate their time courses from eq 2.18.

#### 2.3.1 Global Fit Analysis

Absorbance changes ΔA(λ, t), in general, can be represented with the sums of r exponentials with apparent rate constants kᵢ and amplitudes aᵢ.

\[
ΔA(λ,t) = \sum_{i=1}^{r} a_i(λ) e^{-k_i t} + a_0(λ) \tag{2.22}
\]

In global fit analysis, the weighted sum of squared differences f between the fit with r rate constants kᵢ and the data points at nₑ measured wavelengths λᵢ and N times tⱼ is minimized [Hebling, 1993]:

\[
f = \sum_{i=1}^{nₑ} \sum_{j=1}^{N} [W_i(t_j)]^2 \left[ ΔA_{measured}(λ_i, t_j) - \sum_{i=1}^{r} a_i(λ_i) e^{-k_i t_j} - a_0(λ_i) \right]^2 \tag{2.23}
\]

The assumption in this procedure is that the rate constants are the same for all spectral elements. The number of rate constants r is chosen, along with the optimal weight, with the criterium that the residual plot has a random distribution with r rate constants, however this plot results a systematic deviation with r-1 rate constants. The weight is chosen with respect to the estimated noise using the relation:

\[
W_i(t_j) = \frac{(w_i)^j}{\sum_{j=1}^{N}(w_i)^j} \times N \quad ; \quad j=1,2,\ldots,N \tag{2.24}
\]
with associated error, $E(t_j) = \frac{1}{W(t_j)}$. Here $N$ is the total number of measured time points, and the value of $w_i$ of eq 2.24 is chosen from the range of $1<w_i<1.05$ which is the same for all wavelengths $\lambda_i$ and differs for different time points $t_j$. This is because in the measured absorbance changes, the signal to noise ratio increased with increasing time which is due to the averaging procedure during data acquisition (explained in section 2.2.2). A proper choice of weights produces the best fit across the whole time range. Optimisation is done using software Microcal origin 7.5. The amplitudes from the fit according to eq 2.22 represent the amplitude spectra:

$$B_i(\lambda) = a_i(\lambda)$$

(2.25)

which are equal to the amplitude spectra described in eq 2.15 and 2.27.

In general, a global fit analysis was used to obtain the amplitude spectra from the single and double flash excitation measurements of PYP.

### 2.3.2 Singular Value Decomposition

Another method to obtain the amplitude spectra of transitions is singular value decomposition [Henry, 1992]. Transient absorbance changes measured at various wavelengths covering the whole spectral range of the photocycle are arranged in a matrix $\Delta A$. This matrix has dimensions $m \times n$ where $m$ time traces ($m$ is the number of wavelengths) are arranged in $n$ columns for each time point. A column represents the transient absorbance change at a particular time point for the measured spectral range. Singular value decomposition of the matrix $\Delta A$ is the decomposition of this matrix into the product of three matrices $U, S$ and $V$ in such a way that [Henry, 1992]:

$$\Delta A = U S V^T$$

where, $U$ and $V$ are unitary matrices having the property $U^{T*}U = V^{T*}V = I_n$ where $I_n$ is identity matrix. The columns of $U$ (dimension $m \times n$) and $V$ (dimension $n \times n$) represent the orthonormal basis vectors corresponding to the amplitude spectra (time independent) and the time traces respectively. The third matrix $S$ (dimension $n \times n$) is a diagonal matrix with the diagonal elements arranged in decreasing order called singular values. The smallest value of the magnitude of the difference $\|\Delta A - U_k S_k V_k^T\|$ is feasible with a suitable choice of the number of singular values $k$ (also called significant singular values) of the diagonal matrix $S$. An important property of SVD is that for all $k \leq n$, the first $k$ columns of $U$, along with the corresponding columns of $V$ and the rows and columns of $S$, provide the best least square
approximation to the matrix $\Delta A$. The number of singular values $k$ is chosen, with the criterium that the difference $\|\Delta A - U_k S_k V_k^T\|$ has a random distribution with $k$ columns of $U$, $S$ and $V$ matrices, however this difference results a systematic deviation with $k-1$ columns. The remaining data matrix contains mainly the noise (up to the point at which a particular subset of significant singular values and the vectors is chosen). Thus, singular value decomposition acts as a mechanism-independent filter of noise. Time traces of the basis vectors $V_l(t)^T$, with $l=1, 2, 3, \ldots, k$ ($V_k^T$ has a dimension of $k \times n$), weighted by the corresponding singular values $S_l$ can be fitted simultaneously by a sum of $r$ exponentials:

$$S_l V_l(t)^T = G_{l0} + \sum_{j=1}^{r} G_{lj} e^{k_j t}$$

where, $k_j$ represents the rate constant of the $j^{th}$ transition. From the amplitudes $G_{l0}$ and $G_{lj}$ of this fit and the basis spectra $U_l(\lambda)$ ($U_k$ has dimension of $m \times k$), the amplitude spectra $B_j(\lambda)$ can be calculated [Borucki, 2003A] from:

$$B_j(\lambda) = \sum_{l=1}^{k} [U_l(\lambda)] \ast G_{lj}$$

which, in matrix notation is:

$$B = U_k G$$

Here, $B_j(\lambda)$ ($B$ has a dimension of $m \times r$) represents the amplitude spectrum corresponding to the $j^{th}$ transition in total of $r$ transitions. In general for PYP, the amplitude spectrum corresponding to the infinite time, $G_{l0}$ is negligible compared to the amplitude spectra of the other transitions as the absorbance change vanishes after completion of the photocycle. Another advantage of SVD is that the number of data points to be analysed is greatly reduced ($m \times n$ data points before SVD vs $m \times k$ after SVD). This procedure provides information about the number of significant spectral components ($k$) and the corresponding transitions ($r$) during the photocycle.

Matrix calculations were performed with Matlab version R12.1. Fitting the weighted basis vectors of time traces with sums of exponentials was carried out with Microcal Origin 7.5.

### 2.4 Spectra and Time Courses of the Intermediates

Two independent methods, the extrapolated difference and the scaled subtraction methods, use the amplitude spectra $B_j(\lambda)$ of eq 2.25 or eq 2.27 to obtain the matrix containing
the difference spectra of intermediates $A - A_p$ as described below. The matrix of the corresponding time courses $n$, can be obtained using eq. 2.18.

### 2.4.1 Extrapolated Difference Method

SVD analysis of the measured transient absorbance changes shows that there are three significant singular values and thus three spectrally distinguishable intermediates contributing in the photocycle of PYP, with three transitions (see sections 3.1.1 and 3.2.1), mainly $I_1$, $I_2$ and $I_2'$ in the acid/neutral pH range (pH 4.6 to 8.4, section 3.1.2) and $I_1$, $I_1'$ and $I_2'$ in the alkaline pH range (pH 8 to 11, section 3.2.2). $I_1$ decays partially to $I_2$ (at acid pH) or to $I_1'$ (at alkaline pH) in the first transition. The mixture of $I_1/I_2$ or $I_1/I_1'$ decays together to $I_2'$ in the second transition in acid or alkaline pH respectively. All intermediates in the respective pH range are in equilibrium after about ~20 ms and in turn, decay to P in the third transition.

The amplitude spectra $B_j(\lambda)$ of eq 2.25 or 2.28 are arranged in columns ordered from low to high apparent time constants, to form the amplitude matrix $B$ of eq 2.17. From the columns of $B$ and $C$ new matrices, $\tilde{B}$ and $\tilde{C}$ are formed by linear combinations of sum of columns starting with index $k$ to index $r$ in the following way:

$$ (\tilde{B})_k = \sum_{j=k}^{r} (B)_j; \quad (\tilde{C})_k = \sum_{j=k}^{r} (C)_j; \quad k = 1,2,\ldots, r \tag{2.29} $$

The columns of $\tilde{B}$ represent the extrapolated absorption difference spectra, [Borucki, 1999] and the columns of $\tilde{C}$ of eq (2.30), contain the relative contributions of the intermediates in these difference spectra.

$$ \tilde{C} = \begin{pmatrix} x_1 & x_2 & x_3 \\ y_1 & y_2 & y_3 \\ z_1 & z_2 & z_3 \end{pmatrix} \tag{2.30} $$

$x_i, y_i, z_i$ represent the relative contributions of the respective spectral components $x$, $y$ and $z$ in the $i^{th}$ transition. The matrix equation for $\tilde{B}$ follows from eq 2.15 and 2.17:

$$ \tilde{B} = (A - A_p)\tilde{C} \tag{2.31} $$

and can be solved for $A$:

$$ A = \tilde{B}\tilde{C}^{-1} + A_p \tag{2.32} $$

Once the $\tilde{C}^{-1}$ matrix, the inverse of the matrix $\tilde{C}$, defined in eq 2.33, is known, the spectra of the intermediates can be calculated.
\[
\mathbf{C}^{-1} = \begin{pmatrix}
(C^{-1})_{11} & (C^{-1})_{12} & (C^{-1})_{13} \\
(C^{-1})_{21} & (C^{-1})_{22} & (C^{-1})_{23} \\
(C^{-1})_{31} & (C^{-1})_{32} & (C^{-1})_{33}
\end{pmatrix}
\]

(2.33)

For each column \((C^{-1})_i\), the related spectrum \(A_i\) can be calculated from column \(i\) of the matrix eq 2.32:

\[
(A)_i = \mathbf{B}(C^{-1})_i + (A_p)_i
\]

(2.34)

In general, it is not possible to find a unique solution for the unknown elements of \(\mathbf{C}\) and \(\mathbf{A}\) from these equations, since the columns of \(\mathbf{B}\) are not linearly independent. However, spectra of intermediates, i.e. columns of \(\mathbf{A}\), can be calculated by introducing the following constraints on the intermediate spectra \(\mathbf{A}\) and the \(\mathbf{C}^{-1}\) matrix:

1. The sum of the contributions of the intermediates in the extrapolated difference spectra (columns of \(\mathbf{B}\)) is constant and equals the fraction of molecules cycling, \(\eta\), provided that the time constants of the relaxations are sufficiently separated. This means that the sum of \(x\), \(y\) and \(z\) for each extrapolated difference spectrum equals \(\eta\), or that the sum of the matrix elements of each column of \(\mathbf{C}\) equals \(\eta\):

\[
\sum_{j=1}^{3} C_{ij} = \eta \Leftrightarrow \sum_{j=1}^{3} C^{-1}_{ij} = 1/\eta \quad j = 1, 2, 3
\]

(2.35)

Here, \(\eta\) is the fraction of cycling molecules which is assumed to be conserved during these transitions.

2. The absorption of \(I_2'\) is identical to zero for wavelengths larger than or equal to 410 nm: \(A_{I2'}(\lambda \geq 410\text{nm}) = 0\). This is because \(I_2'\) has its \(S_0\) to \(S_1\) transition around 355 nm. No higher wavelength transition thus occur. Assuming that the spectra of all intermediates have similar bandwidths, one can then estimate from the spectrum of \(P\), which does not absorb beyond 500 nm, that \(I_2'\) does not absorb beyond 410 nm.

Taking into account the second constraint, we consider eqs 2.31, 2.32 and 2.34 only in the wavelength region \(\lambda \geq 410\text{nm}\), i.e. we drop the rows which are related to smaller wavelengths. Hence the third column of the reduced matrix \(\mathbf{A}\) corresponding to \(I_2'\) intermediate is the null vector \((A)_3 = 0\). Since \((A)_3\) is zero, eq 2.34 allows to determine \((\mathbf{C}^{-1})_3\), the third column of \(\mathbf{C}^{-1}\). Provided that the number of wavelength points is larger than 3, eq 2.34 is an over determined system of linear equations, for which the least squares solution
may be found by multiplication with the pseudoinverse matrix $\tilde{B}^{-1}$ from the left and rearranging:

$$(\tilde{C}^{-1})_3 = (\tilde{B})^{-1}(A_p)_3 \quad (2.36)$$

Considering eq 2.34 not only for $\lambda \geq 410\text{nm}$ but in the full spectral range, $(\tilde{C}^{-1})_3$ allows to determine the spectrum of the $I_2'$ intermediate. Using the conservation constraint of eq 2.35, $(\tilde{C}^{-1})_3$ also provides $\eta$, the fraction of cycling molecules or the excitation efficiency of the laser flash.

By making the very reasonable assumption that only $I_1$ contributes in the first extrapolated state $\tilde{B}_1$ (which is also verified with an independent method, see section 2.4.2), the problem is simplified with $y_1=0= z_1$ in eq 2.30. The elements of $(\tilde{C}^{-1})_1$, of eq 2.33, can be expressed in terms of $(\tilde{C}^{-1})_2$, $(\tilde{C}^{-1})_3$, and the elements of the first column of $\tilde{C}$ using the matrix relation $\tilde{C}^{-1} \tilde{C} = I$, where we get:

$$(\tilde{C}^{-1})_{1i} = (1 - ((\tilde{C}^{-1})_{i2} * y_1 - (\tilde{C}^{-1})_{i3} * z_1)) / x_1$$

$$(\tilde{C}^{-1})_{2i} = -( (\tilde{C}^{-1})_{i2} * y_1 + (\tilde{C}^{-1})_{i3} * z_1)) / x_1$$

$$(\tilde{C}^{-1})_{3i} = -( (\tilde{C}^{-1})_{i2} * y_1 + (\tilde{C}^{-1})_{i3} * z_1)) / x_1 \quad (2.37)$$

and thus,

$$(\tilde{C}^{-1})_1 = \begin{pmatrix} 1 / x_1 \\ 0 \\ 0 \end{pmatrix} \quad (2.38)$$

Here, the conservation constraint of eq 2.35 is applied, from which it follows that $x_1$ is fraction of cycling molecules $\eta$. Hence, from eq 2.34 (where $i=1$), with known $(\tilde{C}^{-1})_1$, the $I_1$ spectrum is determined.

It is an experimental observation that the second column of the $\tilde{B}$ matrix, $\tilde{B}_2$ has no contribution from $I_2'$ (detail in the section 3.1.1), implying $z_2=0$ in $\tilde{C}$ matrix of eq 2.30. As we already know the $(\tilde{C}^{-1})_1$ and $(\tilde{C}^{-1})_3$, elements of $(\tilde{C}^{-1})_2$ can be expressed in terms of $(\tilde{C}^{-1})_1$, $(\tilde{C}^{-1})_3$, and elements of the second column of $\tilde{C}$ using the matrix relation $\tilde{C}^{-1} \tilde{C} = I$, where we get:

$$(\tilde{C}^{-1})_{12} = -( (\tilde{C}^{-1})_{12} * x_2 + (\tilde{C}^{-1})_{13} * z_2) / y_2$$

$$(\tilde{C}^{-1})_{22} = (1 - (\tilde{C}^{-1})_{21} * x_2 - (\tilde{C}^{-1})_{23} * z_2) / y_2$$

$$(\tilde{C}^{-1})_{32} = -( (\tilde{C}^{-1})_{32} * x_2 + (\tilde{C}^{-1})_{33} * z_2) / y_2 \quad (2.39)$$
Again, the conservation constraint of eq 2.35 implies: \( x_2 + y_2 + z_2 = \eta \). Thus \((\tilde{C}^{-1})_2\) can be expressed as a function of \( y_2 \):

\[
(\tilde{C}^{-1})_2 = \begin{pmatrix}
\frac{(\eta - y_2)}{\eta^* y_2} \\
\frac{1}{y_2} \\
0
\end{pmatrix}
\]  \hspace{1cm} (2.40)

Since every element of the \( \tilde{C} \) matrix can take only positive values and the conservation constraint has to be obeyed, \( y_2 \) can have values between 0 and \( \eta \). Now, from eq 2.34 (in this case \( i = 2 \)), the \( I_2 \) (at acid/neutral pH) or \( I'_2 \) (at alkaline pH) spectrum is calculated for various values of \( y_2 \). Physically meaningful absorption spectra are those which have positive absorbance. Of the many possible spectra one is selected which has a reasonable spectral bandwidth (see section 3.1.1 and 3.2.1).

The completely determined spectra of intermediates in the matrix of difference \( A - A_P \) provides information about the ratio of concentrations of the intermediates in the extrapolated states and can be used to calculate their time courses, \( n \), using eq 2.18.

The extrapolated difference method was developed by Dr. B. Borucki [Borucki, 1999] and successfully applied to the photocycle of bacteriorhodopsin [Borucki, 1999]. See [Borucki, 1999] for further details.

### 2.4.2 Scaled Subtraction Method

A different method to obtain the intermediate spectra from the amplitude spectra of eq. 2.25 or 2.28 is the scaled subtraction method. This method is used for the data set at pH 10 (contributing \( I_1 \), \( I'_1 \) and \( I'_2 \) spectra, section 3.2.1.2). Details of this procedure are explained in the section 3.2.1.2.

To start with this method, a correct \( I_1 \) spectrum is essential. It is an experimental observation that the initial bleach \( \Delta A(\lambda, \sim 1 \mu s) \), which is also the \( I_1 - P \) difference spectrum, is the same for acid/neutral pH as well as alkaline pH, showing that \( I_1 \) is the same for the whole pH range (see \( \tilde{B}_I \) of Figure 3.3 and 3.10). To obtain the \( I_1 \) spectrum, the proper amount of dark spectrum has to be added to this bleach (detail in section 4.1.1, Figure 4.1C). This amount depends upon the excitation efficiency and can be approximated by scaling the dark spectrum to the bleached signal \( \Delta A(\lambda \sim 430 \text{nm}, \sim 10 \text{ ms}) \) at pH 6 assuming that mainly \( I_2/I'_2 \)
contribute in this time range, which do not absorb beyond λ≥430nm, as well as I\textsubscript{1} contribute minimally in this time range.

When the I\textsubscript{1} contribution (Figure 3.9A, ■) is subtracted from the amplitude spectrum of the first transition (B\textsubscript{1}) [●, Figure 3.9A], the pure I\textsubscript{1}\textsuperscript{'} spectrum (Figure 3.9A, ○) can be obtained. This subtraction is done by matching the scaled I\textsubscript{1} spectrum and the (B\textsubscript{1}) amplitude spectrum in the spectral range λ≥485 nm as shown in Figure 3.9A. In successive subtraction of the I\textsubscript{1} contribution from the other two amplitude spectra (B\textsubscript{2}) [□, Figure 3.9A], and (B\textsubscript{3}) [○, Figure 3.9B], the mixtures of I\textsubscript{1}′/I\textsubscript{2}′ (Figure 3.9C, □) and I\textsubscript{1}′/I\textsubscript{2}′/P (Figure 3.9B, ●) amplitude spectra are obtained respectively.

Using the pure I\textsubscript{1}\textsuperscript{'} spectrum (Figure 3.9A, ○), the pure I\textsubscript{2}\textsuperscript{'} spectrum (Figure 3.9C, ■) can be calculated upon subtraction of the I\textsubscript{1}\textsuperscript{'} contribution (Figure 3.9C, □) from I\textsubscript{1}/I\textsubscript{2}′ mixture (Figure 3.9C, □) by matching in the spectral range λ≥430 nm as shown in Figure 3.9C.

The I\textsubscript{2}\textsuperscript{'} spectrum can also be calculated from the I\textsubscript{1}/I\textsubscript{2}/P amplitude spectrum (Figure 3.9B, ●), see above. In this case, the dark state contribution (P, *: Figure 3.9B) has to be subtracted at first from the I\textsubscript{1}/I\textsubscript{2}/P mixture (Figure 3.9B, ●), where this I\textsubscript{1}/I\textsubscript{2}/P amplitude spectrum and P match in λ≥465 nm range as shown in Figure 3.9B. This subtraction results in the I\textsubscript{1}/I\textsubscript{2}′ amplitude spectrum (Figure 3.9C, ○). Finally, the I\textsubscript{2}\textsuperscript{'} spectrum can be calculated from this I\textsubscript{1}/I\textsubscript{2}′ amplitude spectrum upon subtraction of the I\textsubscript{1}\textsuperscript{'} contribution (Figure 3.9C, ●) by matching in the spectral range λ≥430 nm as shown in Figure 3.9C.

The relative contributions of the corresponding factors obtained during the subtraction procedure are the elements of the matrix C of eq. 2.17. Thus, n can be obtained using eq 2.18, once the matrix A\textsubscript{A-P} is known from the subtraction procedure.

### 2.5 pH Dependent Time Courses of the Intermediates

As we will describe in detail in section 3.1.1 and 3.2.1, during the photocycle the I\textsubscript{1}, I\textsubscript{2} and I\textsubscript{2}\textsuperscript{'} intermediates are in equilibrium at pH 6 and I\textsubscript{1}, I\textsubscript{1}\textsuperscript{'} and I\textsubscript{2}\textsuperscript{'} at pH 10 after about ~20 ms. It is further investigated whether this equilibrium is pH dependent. For that purpose, transient absorbance changes ΔA(λ,t) are measured for various pH values and the pH dependent time courses of the intermediates I\textsubscript{1}, I\textsubscript{2} or I\textsubscript{1}\textsuperscript{'} and I\textsubscript{2}\textsuperscript{'} were obtained using combined singular value decomposition (section 2.5.1). The equilibrium of the I\textsubscript{2}/I\textsubscript{2}\textsuperscript{'} states is pH dependent at acid/neutral pH (see section 3.1.2). Likewise the I\textsubscript{1}/I\textsubscript{1}\textsuperscript{'}/I\textsubscript{2}\textsuperscript{'} states are in equilibrium in the alkaline pH (see section 3.2.2). In addition, the decay rates of these equilibria to P are also strongly pH dependent. Both observations might be related with the photocycle scheme as described below (section 2.5.2).
2.5.1 Combined Singular Value Decomposition

It is possible to perform the singular value decomposition of the combined data matrix constructed from the large data set measured at the various pH values. This is particularly useful if the same number of intermediates are involved in the whole pH range [Borucki, 2002]. The large matrix, $\Delta A$, can be constructed by joining successively the matrices $\Delta A_1(\lambda, t_1)$, $\Delta A_2(\lambda, t_2)$, $\ldots$, $\Delta A_n(\lambda, t_m)$ from the measured data sets of the n individual pH values. Under the condition that the same spectral components contribute throughout the whole pH range, it is necessary to have the time traces in exactly the same spectral range for every pH. In this case, kinetics might change with the pH, i.e., the number of measured time points might differ. The number of the rows in this large matrix is the number of wavelengths and the number of columns is the sum of the time points from the whole pH range. Singular value decomposition of such a combined matrix $\Delta A$ is:

$$
\Delta A = [\Delta A_1(\lambda, t_1) \Delta A_2(\lambda, t_2) \ldots \Delta A_n(\lambda, t_m)] = U_k S_k V_k^T
$$

(2.41)

where,

$$
V_k^T = [V_{k1}(t_1) \ V_{k2}(t_2) \ldots \ V_{kn}(t_m)]^T
$$

(2.42)

with $U_k$ and $V_k$ the unitary matrices. The best fit to the data matrix for the individual pH data set $\Delta A_i(\lambda, t_j)$ can be obtained from the matrix product $U_k S_k V_k^T x_j(t_j)$ with the significant singular values $k$. The advantage of the combined singular value decomposition is that the error is distributed over the whole data set during the matrix decomposition. The time course of the intermediates, $n_i(t_j)$, can be calculated from the following relation:

$$
\Delta A = U_k X^{-1} X S_k V_k^T
$$

(2.43)

where,

$$
U_k X^{-1} = A - A_P
$$

(2.44)

and,

$$
[n_1(t_1) \ n_2(t_2) \ldots \ n_n(t_m)]^T = X S_k V_k^T
$$

(2.45)

if the transformation matrix, $X$ is known. Here, the matrix $X$ is required because this matrix transforms the basis vectors of the spectral part ($U_k$) to the spectra of intermediates (eq 2.44), and the basis vectors of the temporal part ($V_k^T$) to the time courses of the intermediates (eq 2.45). The advantage with this matrix $X$ is that this is a single transformation matrix for the whole measured data set subjected to the combined SVD. This matrix $X$ might be determined from the basis spectra, $U_k$ and known difference spectra $A - A_P$ using eq 2.44. The difference spectra $A - A_P$ in turn, can be obtained from the analysis of the data set at a particular pH using the extrapolated difference spectra method or scaled subtraction method (section 2.4).

Combined SVD of the data set from pH 4.6 to 8.4 shows that three spectral components $I_1$, $I_2$ and $I_2'$ contribute during the photocycle where $I_2$ and $I_2'$ are in pH dependent
equilibrium (after ~20 ms) with increasing $I_2'$ at higher pH (section 3.1.2). Data sets from pH 8 to 11 show that three spectral components $I_1$, $I_1'$ and $I_2'$ contribute in this pH range where $I_1'$ and $I_2'$ are in pH dependent equilibrim with increasing $I_1$ at higher pH (section 3.2.2.). In the overlapping pH range 8 to 8.5, all four intermediates $I_1$, $I_1'$, $I_2$ and $I_2'$ might contribute. However, the major contribution is due to $I_2'$.

### 2.5.2 pH Dependence of Equilibrium and Decay Rate

During the photocycle of PYP, $I_1'$ (deprotonated form of chromophore) and $I_2'$ (protonated form of chromophore) are in pH dependent equilibrium (section 3.2.2). In addition, the decay rate of this equilibrium to the initial state P is also pH dependent with similar pK_a. The pH dependence of this rate constant can be related to the relative population of the species with the following analysis using the reaction scheme of Figure 2.2, [Kenneth, 1990].

$I_1'$ and $I_2'$ are in pH dependent equilibrium:

$$[I_1'] + [H^+] \Leftrightarrow [I_2']$$  \hspace{1cm} (2.46)

The acid dissociation constant is:

$$K_a = \frac{[H^+] [I_1']}{[I_2']} = 10^{pK_a}.$$  \hspace{1cm} (2.47)

The normalized fractions of $I_1'$ and $I_2'$ are respectively:

$$F_{I_1'} = \frac{[I_1']}{[I_1'] + [I_2']} = \frac{K_a}{[H^+] + K_a} = \frac{1}{1 + 10^{-n(pH-pK_a)}}$$  \hspace{1cm} (2.48)

$$F_{I_2'} = \frac{[I_2']}{[I_1'] + [I_2']} = \frac{[H^+]}{[H^+] + K_a} = \frac{1}{1 + 10^{n(pH-pK_a)}}$$  \hspace{1cm} (2.49)

where, $[H^+] = 10^{pH}$ and $n$ is the Hill coefficient. Thus, $F_{I_1'}$ and $F_{I_2'}$ are sigmoidal functions of the pH.

![Figure 2.2](image)

**Figure 2.2:** A reaction scheme showing pH dependent equilibrium between $I_1'$ and $I_2'$ and transition to P with decay rates $k'$ and $k''$ respectively.

Let us suppose that $I_1'$ and $I_2'$ decay to P with decay rates of $k'$ and $k''$ respectively and that the $I_1'/I_2'$ equilibrium formation is very fast compared to these two rates. Under these conditions, the decay rate of the sum of the states $I_1'$ and $I_2'$ can be expressed as their sum weighted by respective rate constants:
\[- \frac{d([I_1^\prime] + [I_2^\prime])}{dt} = k^\prime [I_1^\prime] + k^{\prime\prime} [I_2^\prime] \]  
(2.50)

Replacing the values for \([I_1^\prime]\) and \([I_2^\prime]\) using eq 2.48 and 2.49:
\[- \frac{d([I_1^\prime] + [I_2^\prime])}{dt} = \left\{ k^\prime \left( \frac{1}{1 + 10^{-n(pH-pK_a)}} \right) + k^{\prime\prime} \left( \frac{1}{1 + 10^n(pH-pK_a)} \right) \right\} ([I_1^\prime] + [I_2^\prime]) \]  
(2.51)

Thus, the apparent rate constant for the ground state (P) recovery is:
\[ k = \left\{ k^\prime \left( \frac{1}{1 + 10^{-n(pH-pK_a)}} \right) + k^{\prime\prime} \left( \frac{1}{1 + 10^n(pH-pK_a)} \right) \right\} \]  
(2.52)

Under the condition that \(k^{\prime\prime} \gg k^\prime\), the apparent rate constant \(k\) is a sigmoidal function of pH, the same as that of the \(I_2^\prime\) population in the \(I_1^\prime/I_2^\prime\) equilibrium. Thus,
\[ k = k^{\prime\prime} \left( \frac{1}{1 + 10^n(pH-pK_a)} \right) \]  
(2.53)

So, the pK\(_a\) of the \(I_1^\prime/I_2^\prime\) equilibrium and of the decay rate of this \(I_1^\prime/I_2^\prime\) equilibrium to P are identical under these conditions.
Chapter 3

Photocycle Kinetics Mechanism of Photoactive Yellow Protein

The experimental results of the measurements on the pH dependence of the photocycle kinetics of photoactive yellow protein are presented in this Chapter. They are divided into two parts covering the acid/neutral (pH 4.6 to 8.4) and the alkaline (pH 8 to 11) pH ranges. In the whole pH range and the measured time window of 50 ns to 50 s, three transitions are observed. The intermediates, I₁ and I₂′ contribute over the whole pH range. However, I₂ and I₁′ contribute only in the acid and alkaline pH range respectively.

Spectra and time courses at pH 7 (section 3.1.1) and at pH 10 (section 3.2.1.1) are determined using the extrapolated difference method. Moreover, the spectra at pH 10 are confirmed with an independent method, scaled subtraction (section 3.2.1.2). The pH dependent time courses in the acid/neutral pH range (section 3.1.2) were determined using the I₁, I₂ and I₂′ intermediate spectra acquired at pH 7. The corresponding pH dependent time courses in the alkaline pH range (section 3.2.2) were calculated using the I₁, I₁′ and I₂′ spectra obtained at pH 10. Finally, the intermediate populations measured at about ~20 ms during the cycle confirmed the pH dependent equilibrium between the I₂ and I₂′ intermediates in the acid/neutral pH range and between the I₁′ and I₂′ intermediates in the alkaline pH range respectively. From these observations and the measured pH dependent decay rates photocycle models are proposed for both the acid/neutral (section 3.1.3) and alkaline pH (section 3.2.3) range. A different photocycle model is proposed for each pH range, since the intermediates contributing are different (I₂′ and I₁′ contributing only in the acid and alkaline pH ranges respectively).
3.1 Photocycle Kinetics at Acid/Neutral pH

The intermediate spectra are determined at pH 7. I₁, I₂ and I₂’ contribute to the photocycle at this pH. Using these spectra, the corresponding pH dependent time courses of the photocycle intermediates in the pH range 4.6 to 8.4 are calculated. We find that the pH dependent intermediate populations are related to the transition rates. A photocycle model is proposed based on these observations.

3.1.1 Spectra and Time Courses of Intermediates at pH 7

Transient absorbance changes were measured at pH 7 at 19 wavelengths, ranging from 330 to 510 nm, in the time range from 50 ns to 5 s, Figure 3.1A. For clarity, only eight of the time traces are shown.

![Figure 3.1: (A) Transient absorption changes after excitation at 430 nm at 19 wavelengths varying from 330 to 510 nm. For clarity, only the traces at the indicated wavelengths are shown. The vertical dashed lines indicate the time constants for a global fit to the weighted SVD time traces with a sum of three exponentials. \( \tau_1 = 270 \, \mu s \) is the rise time of I₂, \( \tau_2 = 2.0 \, ms \) is the rise time of I₂’, and \( \tau_3 = 260 \, ms \) is the return to P. The dotted lines, only distinguishable from the data in the \( \mu s \)-time range, are the fits. Conditions: pH 7, 20 °C, 50 mM KCl and 50 mM Tris. PYP concentration: 35 \( \mu M \). (B) Amplitude spectra B(\( \lambda \)) calculated from the amplitudes of the exponential fits to the SVD time traces and the corresponding basis spectra of the data in (A). The three amplitude spectra correspond to the following time constants: \( \tau_1 = 270 \, \mu s \) (●), \( \tau_2 = 2.0 \, ms \) (□), \( \tau_3 = 260 \, ms \) (○). The solid curve is a scaled and inverted ground state spectrum. (C) Extrapolated difference spectra obtained from the amplitude spectra of (B) as described in the text: \( \tilde{B}_1 \) (▲), \( \tilde{B}_2 \) (○), \( \tilde{B}_3 \) (○).]

The complete data set in the time range 10 \( \mu s \) to 5 s was subjected to SVD analysis as described in section 2.3.2. The first six singular values were: 11.2, 1.8, 0.14, 0.05, 0.03, and 0.02. We consider the first three to be significant, suggesting the presence of only three spectrally distinguishable intermediates. The additional components, corresponding to the
singular values \(s_4, s_5\) and \(s_6\) show very noisy time traces and were thus neglected. The three weighted time traces from SVD were fitted simultaneously, as described in eq 2.26, starting at 10 \(\mu\)s with a sum of three exponentials with time constants \(\tau_1 = 270 \ \mu\)s, \(\tau_2 = 2.0 \ \text{ms}\) and \(\tau_3 = 260 \ \text{ms}\). These times are marked by vertical dashed lines in Figure 3.1A. The dotted lines in Figure 3.1A, which can barely be distinguished from the data, represent these fit curves for the individual time traces. From the fit to the SVD time traces with the sum of three exponentials and the corresponding basis spectra, the amplitude spectra \(B_i(\lambda)\) were calculated using eq. 2.27. These are presented in Figure 3.1B. The amplitude spectra provide considerable insight into the spectra of the intermediates. \(B_1(\lambda)\) clearly describes the transition from \(I_1(\lambda_{\text{max}} \sim 460 \ \text{nm})\) to \(I_2\) with a \(\lambda_{\text{max}}\) value above 360 nm. \(B_2(\lambda)\) is apparently a transition from an equilibrium of \(I_1\) and \(I_2\) to \(I_2'\) with \(I_2'\) blue-shifted with respect to \(I_2\). \(B_3(\lambda)\) represents the ground state recovery and suggests that \(I_2'\) has its \(\lambda_{\text{max}}\) value near 350 nm. Comparison of the negative minimum of \(B_1\) with the positive maximum of \(B_3\), suggests that the transition from \(I_2\) to \(I_2'\) is associated with a blue-shift of the order of \(\sim 20 \ \text{nm}\).

![Figure 3.2](image-url)

Figure 3.2: (A) Intermediate spectra \(I_1 (\square), I_2 (\bigcirc)\) and \(I_2' (\blacksquare)\) calculated from the extrapolated difference spectra of Figure 3.1C. The solid curve represents the spectrum of dark state \(P\) for comparison. Vertical dashed lines indicate the wavelengths of the blue (430 nm) and violet (355 nm) excitation flashes used. (B) \(I_2\) and \(I_2'\) spectra for various allowed values of \(y_2\) as described in the text. \(y_2 = 0.12 (\bullet), y_2 = 0.15 (\blacksquare), y_2 = 0.18 (\blacktriangle), y_2 = 0.22 (\bigcirc), y_2 = 0.27 (\square)\) and \(y_2 = 0.37 (\Delta)\). (C) Time-courses of the relative concentrations of \(I_1 (\longrightarrow), I_2 (\dashlongrightarrow), \text{and } I_2' (\cdashlongrightarrow)\) calculated according to eq 2.18. The time-courses of the sum of the relative concentrations of \(I_1, I_2\) and \(I_2'\) is indicated by \(\longrightarrow\). The vertical dashed lines indicate the time constants from the global SVD fit of Figure 3.1A.

The three amplitude spectra \(B_1, B_2\) and \(B_3\) were used to construct the \(\tilde{B}\) matrix according to eq 2.29. \(\tilde{B}_1\) is the sum of amplitude spectra \(B_1, B_2\) and \(B_3\) (arranged in
columns). $\mathbf{B}_2$ is the sum of amplitude spectra $\mathbf{B}_2$ and $\mathbf{B}_3$, and $\mathbf{B}_3$ is the same as $\mathbf{B}_3$. The three columns, $\mathbf{B}_1$, $\mathbf{B}_2$ and $\mathbf{B}_3$, representing the extrapolated difference spectra, are presented in Figure 3.1C. $\mathbf{B}_1$ equals the initial absorbance change right after the flash, and suggests that the initial bleach led to the formation of the I₁ intermediate (positive absorbance change near 480 nm).

Using the second constraint as described in Materials and Methods (section 2.4.1), that $I_2'$ does not absorb beyond 410 nm, eq 2.34 is considered only in the range $\lambda > 410$ nm, i.e. the rows for the shorter wavelengths are dropped. Then the third column of the reduced matrix $\mathbf{A}$, corresponding to the spectrum of $I_2'$, is the null vector: $(\mathbf{A})_3 = \mathbf{0}$. Since $(\mathbf{A})_3$ is zero, we can solve eq 2.34 for $(\mathbf{C}^{-1})_3$ resulting in eq 2.36. In this way, the third column of $\mathbf{C}^{-1}$ is determined. Using the first constraint described in the section 2.4.1, the sum of these matrix elements equals $\eta^{-1}$. In this way, the fraction of cycling molecules, $\eta = 0.371$, is determined.

Finally, using $\mathbf{B}$ and $\mathbf{A}_p$ for the whole spectral range allows us to calculate the spectrum of $I_2'$ from $(\mathbf{C}^{-1})_3$ using eq 2.34. The result is shown in Figure 3.2A ($\square$). The $\lambda_{\text{max}}$ value of the spectrum is at about 350 ± 5 nm.

Since only $I_1$ contributes to $\mathbf{B}_1$ (see Figure 3.1C), the elements $\mathbf{C}_{21} = y_1$ and $\mathbf{C}_{31} = z_1$ of $\mathbf{C}$ of eq 2.30 are given by $y_1 = z_1 = 0$. This allows us to calculate the elements of the first column of $\mathbf{C}^{-1}$ of eq 2.33. The result is $\mathbf{C}^{-1}_{11} = 1/x_1$, $\mathbf{C}^{-1}_{21} = 0$, $\mathbf{C}^{-1}_{31} = 0$. Since the sum of these elements equals $1/\eta$ (eq 2.35, conservation constraint), we have $x_1 = \eta = 0.371$. With $(\mathbf{C}^{-1})_1$ now completely known, we can calculate the spectrum of $I_1$ from $(\mathbf{C}^{-1})_1$, $\mathbf{B}$ and $\mathbf{A}_p$ using eq 2.34. The result is shown in Figure 3.2A ($\square$). This spectrum of $I_1$ is in good agreement with that obtained at pH 10 (Figure 3.8A ($\square$), below).

To calculate the spectrum of the third spectral species, $I_2$, the procedure is as follows. From Figure 3.1B, we note that $\mathbf{B}_1$ reflects a transition between two intermediates with $\lambda_{\text{max}}$ values of about 460 nm (decay of $I_1$) and 370 nm (rise of $I_2$). Since there is apparently no contribution from the more blue-shifted species $I_2'$ ($\lambda_{\text{max}}$ about 350 nm) in $\mathbf{B}_1$, which is well known to be formed from $I_2$ in the next transition [Xie, 2001], $I_2'$ does not contribute to $\mathbf{B}_2$ either. Moreover, dye binding experiments showed that the formation of the signaling state $I_2'$ is delayed with respect the formation of $I_2$ [Borucki, 2002]. Therefore it is concluded that $I_2'$ is not involved in the first transition and thus $z_2 = 0$ in eq 2.30. The elements $x_2$, $y_2$, $z_2$ of the second column of $\mathbf{C}$ of eq 2.30 can now be expressed in terms of $x_2$, $y_2$, and $\eta$ with the
help of $\tilde{C}^{-1}\tilde{C} = \mathbf{I}$ as described in eq 2.39. Using the conservation constraint, $x_2 + y_2 = \eta$ of eq 2.35, we finally obtain for the elements of the second column of $\tilde{C}^{-1}$ (eq 2.40): $\tilde{C}^{-1}_{12} = - (\eta - y_2)/\eta y_2$, $\tilde{C}^{-1}_{22} = 1/y_2$, $\tilde{C}^{-1}_{32} = 0$. So we have now determined all elements of the second column of $\tilde{C}^{-1}$, the only free parameter remaining is $y_2$ (eq 2.40). Since $x_i$, $y_i$ and $z_i$ can only assume positive values and $x_2 + y_2 = \eta$, $y_2$ is restricted to values between 0 and $\eta$. The spectrum of $I_2$ can now be calculated from $(\tilde{C}^{-1})_2$, $\tilde{B}$ and $A_p$ using eq 2.34. The results are shown in Figure 3.2B for six values of $y_2$ from 0.12 to 0.37 ($-\eta$). Since the extinction coefficient has to be positive, physically meaningful absorption spectra are only obtained for $y_2 \geq 0.22$. Of the spectra remaining in Figure 3.2B, we pick the one associated with $y_2 = 0.22$, since it has the smallest spectral bandwidth.

For $y_2$ considerably larger than 0.22, the spectral bandwidth becomes much larger than for $P$ and $I_1$ and a secondary absorption maximum develops near 460 nm. This contradicts the original assumption that the UV transitions of $I_2$ and $I_2'$ are the longest wavelength transitions of these intermediates, which precludes transitions at higher wavelengths. The spectrum of $I_2$ for $y_2 = 0.22$ is redrawn in Figure 3.2A ($\mathcal{O}$). Its $\lambda_{\text{max}}$ value is at about $370 \pm 5$ nm. We note that the value of $\lambda_{\text{max}}$ is independent of the choice of $y_2$.

Using the spectra of $I_1$, $I_2$ and $I_2'$, the time courses of the intermediates were calculated from the experimental $\Delta A(\lambda,t)$ data by using eq 2.18. The time-dependence of the relative concentrations of the $I_1$, $I_2$ and $I_2'$ intermediates at pH 7 are shown in Figure 3.2C.

$I_1$ partially decays to $I_2$ in 270 µs. $I_1$ and $I_2$ then further decay around 2 ms to an $I_1/I_2/I_2'$ equilibrium. This equilibrium finally decays to $P$ in 260 ms. Also shown is the sum of the relative concentrations of these intermediates (dash-dot line). To a good approximation, this sum is constant over the entire time range before the decay to $P$, validating the data analysis. Its value is very close to $\eta = 0.371$, the fraction cycling, showing the internal consistency of the analysis.

The matrix containing the relative contributions of the intermediates, $\tilde{C}$ of eq. 2.30, it’s inverse, $\tilde{C}^{-1}$ of eq. 2.33, and the coefficient matrix, $C$ of eq. 2.17 calculated in this analysis are:

$$\tilde{C}^{-1} = \begin{pmatrix} 0.371 & 0.151 & 0.032 \\ 0 & 0.22 & 0.13 \\ 0 & 0 & 0.209 \end{pmatrix}.$$
Chapter 3 Photocycle Kinetics Mechanism of PYP

\[ \mathbf{C}^{-1} = \begin{pmatrix} 2.7 & -1.85 & 0.742 \\ 0 & 4.55 & -2.83 \\ 0 & 0 & 4.78 \end{pmatrix} \text{ and } \]

\[ \mathbf{C} = \begin{pmatrix} 0.22 & 0.119 & 0.032 \\ -0.22 & 0.09 & 0.13 \\ 0 & -0.209 & 0.209 \end{pmatrix} \]

respectively.

3.1.2 pH Dependence

To answer the question whether the equilibrium between \( I_1, I_2 \) and \( I_2' \) observed at 20 ms during photocycle (Figure 3.2C) is pH dependent, and to learn more about the nature of the transition between the acid and the neutral pH regimes, the photocycle kinetics were measured at the following fifteen pH values: 4.6, 4.8, 5.1, 5.4, 5.7, 6.0, 6.3, 6.6, 6.75, 6.9, 7.35, 7.7, 7.9, 8.1 and 8.4. With excitation at 430 nm, time traces were collected at the seven wavelengths 340, 370, 390, 410, 450, 490, and 500 nm over the time range from 50 ns to 50 s. Results for selected wavelengths are shown in Figure 3.3. Note that the panels of Figure 3.3 have very different vertical scales, and correspondingly different signal to noise ratios. The smallest pH-induced absorbance changes are at 500 nm. The initial absorbance change is almost pH independent at every wavelength, suggesting that the amount of \( I_1 \) formed is independent of pH in this range. At each pH, the absorbance changes at all wavelengths could be fitted simultaneously with a sum of three exponentials. The first time constant was virtually constant in this pH range, varying between 200 and 350 µs. The second time constant varied between 1.3 (pH 8.4) and 10.6 ms (pH 5.1), and is pH dependent. As we saw above, the first transition is due to the decay of \( I_1 \) to \( I_2 \) and the second transition is due to the decay of \( I_1/I_2 \) to the \( I_1/I_2/I_2' \) equilibrium. The data of Figure 3.3 show that the third time constant, the return of the \( I_1/I_2/I_2' \) equilibrium to P, is also strongly pH dependent, slowing down with decreasing pH. The pH dependent rate constants \( k_2 \) and \( k_3 \) are plotted in Figure 3.5B and D respectively.

Some preliminary conclusions on the pH dependence of the \( I_1, I_2, I_2' \) intermediate populations may be drawn by inspection of these data. At 340 nm, the extinction coefficient of \( I_2' \) is larger than that of \( I_2 \) (Figure 3.2A). Although the sequence of time traces is not entirely regular, the absorbance at 340 nm around 10 ms (Figure 3.3A) seems to increase with pH, suggesting an increase in the relative amount of \( I_2' \). At 370 nm, the extinction coefficient of \( I_2 \) is larger than that of \( I_2' \) (Figure 3.2A). The decrease in absorbance at 370 nm
with pH in the ms time range (panel B of Figure 3.3), may thus be interpreted as a decrease in the relative amount of I₂.

**Figure 3.3:** pH dependence of the transient absorbance changes after excitation at 430 nm at various wavelengths. (A): 340 nm (characteristic for I₂'), (B): 370 nm (characteristic for I₂), (C): 390 nm (characteristic for I₁'), (D): 410 nm (characteristic for I₁'), (E): 450 nm (characteristic for P) and (F): 500 nm (characteristic for I₁). The color code for the pH values in each panel are: black, pH 8.4; red, pH 6.9; green, pH 6.6; blue, pH 6.0; light blue, pH 5.7; pink, pH 5.4; dark blue, pH 4.8. Conditions: 50 mM MES, 50 mM KCl, 20°C. PYP concentration: 53 μM.

At 390 nm, the difference in extinction coefficient between I₂ and I₂' is even larger. This wavelength is therefore diagnostic for the I₂ to I₁' transition and for pH effects on this
transition. Panel C of Figure 3.3 shows the increase in absorbance below 1 ms due to the I₁ to I₂ transition (the extinction coefficient of I₂ is larger than that of I₁ at this wavelength, see Figure 3.2A). The amount of I₂ formed apparently decreases with increasing pH judging from the amplitude of the absorption change below 1 ms in Figure 3.3C. Around 2 to 3 ms there is a large decrease in absorbance due to the I₂ to I₂' transition. The amplitude of this transition increases with pH, suggesting that more I₂' is formed at alkaline pH. Panel D shows time traces at 410 nm. This wavelength is appropriate for monitoring the I₁' intermediate which occurs at alkaline pH (section 3.2.1). These traces indicate that this intermediate is absent in this pH range. The traces at 500 nm (Panel F) are characteristic for I₁. They suggest that, with increasing pH, more I₁ remains after the I₁/I₂ to I₂' transition. This is also supported by the traces at 450 nm (panel E) indicating that the ground state depletion decreases with pH.

To obtain the time courses of the intermediate populations, it was assumed that the spectra of I₁, I₂ and I₂' of Figure 3.2A are pH independent and that no other intermediates contribute in the pH range from 4.6 to 8.4. The Eq. 2.18 was then used to calculate the time traces nᵢ(t) for each intermediate at each pH value from the absorbance changes ΔA(λᵢ, t) and the spectra Aᵢ(λ) by matrix inversion. The time dependencies of the populations of I₁, I₂ and I₂' at seven of the fifteen pH values are shown in panels A, B and C of Figure 3.4. They confirm what was suggested by the data of Figure 3.3: I₁ decays partially to I₂; I₁ and I₂ then partially decay to I₂'; beyond 10 ms I₁, I₂ and I₂' are in equilibrium and return together to P. Figure 3.4D shows that the sum of the populations is approximately constant in time and equal to the fraction cycling. Whereas the population of I₁ in equilibrium with I₂ and I₂' is only slightly pH dependent (see traces of Figure 3.4A around 10 ms), the concentrations of I₂ and I₂' show a strong and opposite pH dependence. With increasing pH, the amount of I₂' increases at the expense of a corresponding decrease in the I₂ population.

To quantify the observed pH dependence of the equilibrium populations, the relative concentrations of the intermediate populations corresponding to the different transitions were derived from the \( \mathbf{C} \) matrix of eq 2.31. The columns of this matrix, as shown in eq 2.30, contain the relative concentrations of the intermediates in the difference spectra \( \mathbf{B} \). For every pH, the amplitude spectra were derived from the simultaneous fit of the measured transient absorbance changes at 340 nm, 370 nm, 390 nm, 410 nm, 450 nm, 490 nm and 500 nm of Figure 3.3, with a sum of three exponentials. These amplitude spectra were arranged, with increasing order of the transitions, in the columns of matrix \( \mathbf{B} \). Extrapolated difference
spectra $\tilde{B}$ matrix of eq 2.31 was obtained from the $B$ matrix by adding the columns as defined in the eq 2.29.

The corresponding elements of the $\tilde{C}$ matrix defined by eq 2.30 were acquired by matrix inversion of eq 2.31 where $A-A_p$ was derived from Figure 3.2A. The columns of $\tilde{C}$ matrix defined in eq 2.30 represent, in order, the relative concentrations of the $I_1$, $I_2$ and $I'_2$ species in the respective transition. Before the first transition, only $I_1$ is present (i.e. $y_1=0=z_i$) as shown in Figure 3.4A-C ($\sim 10 \mu s$). The first and second row of the second column ($x_2$ and $y_2$) of the $\tilde{C}$ matrix represent the respective relative concentrations of the $I_1$ and $I_2$ intermediates, before the second transition. The pH dependence of these values are plotted in Figure 3.5A. The solid curves are the results of a simultaneous fit with the Henderson-Hasselbalch equation. The fit parameters were $pK_a \sim 7$ and $n \sim 0.97$ indicating that $I_1$ and $I_2$
are in a pH-dependent equilibrium. Before the second transition, I$_2'$ makes a minimal contribution, as shown by Figure 3.4C (i.e. $z_2$=0).

**Figure 3.5:** (A) pH dependence of the equilibrium concentrations of the I$_1$(●) and I$_2$(○) intermediates derived from the $\tilde{C}$ matrix as described in the text. The solid curves are the simultaneous fits of these titration curves with the Henderson-Hasselbalch equation with pK$_a$ = 7 and $n \sim 0.97$. (B) pH dependence of the decay rate $k_2$ of I$_1$/I$_2$ equilibrium to I$_1$/I$_2$/I$_2'$ equilibrium. For every pH, the decay rates $k_2$ and $k_3$ were derived from the simultaneous fit of the measured transient absorbance changes at 340 nm, 370 nm, 390 nm, 410 nm, 450 nm 490 nm and 500 nm with a sum of three exponentials. The solid curve is the fit of the decay rate with the Henderson-Hasselbalch equation with pK$_a$ = 6.7, $n \sim 0.95$. (C) pH dependence of the equilibrium concentrations of the I$_2$(○) and I$_2'$ (●) intermediates derived from the $\tilde{C}$ matrix as described in the text. The solid curves are the simultaneous fits of these titration curves with the Henderson-Hasselbalch equation with pK$_a$ = 6.3 and $n \sim 0.98$. (D) pH dependence of the decay rate $k_3$ of the ground state recovery. The solid curve is the fit of the decay rate with the Henderson-Hasselbalch equation with pK$_a$ = 6.3, $n \sim 0.84$.

The formation of the I$_1$/I$_2$ equilibrium before the second transition is directly evident from the 390 nm traces of the Figure 3.3C where the height of the hill at about $\sim$ 500 µs increases with decreasing pH. This is due to the I$_1$ to I$_2$ transition (I$_2'$ does not yet contribute to the cycle) with I$_2$ decreasing at higher pH. The next transition to the I$_1$/I$_2$/I$_2'$ equilibrium from the I$_1$/I$_2$ equilibrium may also be discerned from the same Figure. Moreover, this is mirrored in the I$_2$ time courses with the bump between 500 µs and 1 ms, Figure 3.4B. The I$_1$/I$_2$ equilibrium is also evident from the $\tilde{B}_4$ (positive absorbance for $\lambda \geq 500$ nm and around $\lambda \sim 360$ nm) which is the extrapolated difference spectrum before the second transition. The decay of the I$_1$/I$_2$ equilibrium during the second transition could not be visually inspected.
from the I₁ time course in Figure 3.4A, or from the 500 nm trace of Figure 3.3F. This is because I₁ continues to decay in both of the first and second transitions.

The decay rate corresponding to the second transition slows down upon decreasing pH, Figure 3.3. The rate constant k₂ for this transition was determined from a global fit of the data of Figure 3.3 at all seven wavelengths. Its pH dependence is plotted in Figure 3.5B. A fit with the Henderson-Hasselbalch equation results in a pKₐ of ~ 6.7 and n ~ 0.95. The apparent decay rate corresponding to the second transition thus seems to be proportional to the I₁ population in the I₁/I₂ equilibrium, compare Figure 3.5A and B. This relationship is expected in the framework of a simple model presented in the discussion (scheme I).

The second and third row of the third column (y₃ and z₃) of the C̃ matrix represent the respective relative concentrations of I₂ and I₂' intermediates, before recovery. The pH dependence of their concentrations is plotted in Figure 3.5C. The x₃ value represents the relative I₁ population before the third transition (~ 10 ms) and is weakly pH dependent. This is consistent with the observation presented in Figure 3.4A. The corresponding relative concentrations of I₂, I₂' intermediates plotted in Figure 3.5C are very similar to the corresponding values of the I₂ and I₂' intermediates read off at 10 ms of Figure 3.4B and C respectively (vertical dashed lines), as it should be. The solid curves are the results of a simultaneous fit with the Henderson-Hasselbalch equation. The fit parameters were pKₐ ∼ 6.4 and n ∼ 0.98. I₂ and I₂' are thus in a pH-dependent equilibrium.

The ground state recovery slows down with decreasing pH, Figure 3.3. The rate constant k₃ for this recovery is plotted in Figure 3.5D. A fit with the Henderson-Hasselbalch equation results in a pKₐ of ~ 6.3 and n ~ 0.8 . The apparent decay rate for the dark-state recovery thus seems to be proportional to the I₂' population in the I₁/I₂/I₂' equilibrium. This relationship is also described later in the discussion with a simple model (scheme II).

In summary, the I₁/I₂ equilibrium is formed from I₁ during the first transition with about ~ 350 µs life time, and is pH dependent with pKₐ ~7. The I₁/I₂ equilibrium decays to another pH dependent equilibrium I₁/I₂/I₂' in the second transition. During recovery, the I₁/I₂/I₂' equilibrium decays to P with a pKₐ ∼ 6.3. A detailed photocycle model based on these observations is shown in Figure 3.6. We note that the transitions of the Figure 3.5A and C are incomplete. The possible reasons for this observation are presented later in the discussion.

3.1.3 Discussion

From measurements of the pH dependence of the photocycle and photoreversal (see section 4.2) kinetics of PYP between 4.6 and 8.4, the following results were obtained: 1) the
spectra of the signaling state $I_2'$ (350 nm) and its precursor $I_2$ (370 nm) differ by about 20 nm, 2) at about 500 µs, $I_1$ and $I_2$ are in pH dependent equilibrium with pKa ~7, 3) the decay rate of the second transition is pH dependent with pKa ~6.7, 4) from several ms (formation of $I_2'$) to the end of the cycle the three intermediates $I_1$, $I_2$ and $I_2'$ are in equilibrium, 5) the pKa of the pH-dependent equilibrium between $I_2'$ and its precursor $I_2$ is ~ 6.4 from photocycle kinetics and ~ 6.1 from photoreversal kinetics (see section 4.2). The pH is thus an important parameter that controls the amount of receptor in the active state. This is analogous to the case of the photoreceptor rhodopsin where the equilibrium between the signaling state $M_{II}$ and its precursor $M_I$ is also strongly pH dependent [Dickopf, 1998]. In [Borucki, 2005] we showed that the $I_2/I_2'$ equilibrium also depends on the salt concentration.

The existence of two distinguishable $I_2$ intermediates with protonated chromophore was first demonstrated by time-resolved FTIR [Xie, 2001], [Brudler, 2001]. $I_2$ decays to $I_2'$ in about 2 – 3 ms [Xie, 2001], [Brudler, 2001]. This transition to the signaling state $I_2'$ is characterized by a global conformational change [Xie, 2001], [Brudler, 2001]. Here, we showed that this transition may also be monitored by transient electronic absorption spectroscopy and determined the absorption spectra of $I_2$ and $I_2'$ by the extrapolated difference method [Borucki, 1999]. The $\lambda_{\text{max}}$ values of $I_2$ and $I_2'$ are 370 ± 5 and 350 ± 5 nm, respectively. Absorption spectra for $I_2$ and $I_2'$ at pH 8.1 were presented in [Hendriks, 2003]. No $\lambda_{\text{max}}$ values were provided, but the spectrum of $I_2$ was said to be “slightly red-shifted” with respect to $I_2'$, in agreement with our results. The spectrum of $I_2$ presented in [Hendriks, 2003] is so noisy that it is difficult to estimate $\lambda_{\text{max}}$. The poor quality of this spectrum is probably due to the fact that at pH 8.1 the contribution of $I_2$ in the equilibrium is very low, as we showed here (Figure 3.5C). Our $I_2$ and $I_2'$ spectra are comparable to those presented in [Otto, 2005], [Yeremenko, 2006] and [Shimizu, 2006], which were obtained by different methods and published after conclusion of our experiments.

Using these spectra and assuming that they are pH independent in the pH range from 4.6 to 8.4, we obtained the time dependence of the concentrations of $I_1$, $I_2$ and $I_2'$ (Figure 3.4). At about 500 µs, $I_1$, $I_2$ are in equilibrium and decay together to the $I_1$, $I_2$ and $I_2'$ equilibrium during the second transition, Figure 3.4A-C. The equilibrium intermediate populations of $I_1$ and $I_2$ are pH dependent with a pKₐ of ~ 7, Figure 3.5A. Below the pKₐ, $I_2$ is the major species, above the pKₐ the opposite holds.
3.1.3 Discussion

The decay rate $k_2$ of the second transition, is also pH dependent with a $pK_a$ of 6.7, i.e. very close to that for the $I_2/I_2'$ equilibrium as shown in Figure 3.5A. This relationship might be explained using the scheme I, as discussed in section 2.5.2.

During the second transition $I_1$ and $I_2$ decay to $I_2'$, forming the $I_1/I_2/I_2'$ equilibrium. Under the assumption that the $I_1/I_2$ equilibrium is formed (scheme I) much faster than the respective individual microscopic rates $k'$ and $k''$ for decay of $I_1$ and $I_2$ to $I_2'$, the apparent rate $k_2 \sim [I_1]$, when $k' \gg k''$. Analysis of this reaction scheme I, as described in section 2.5.2, shows that the apparent rate constant for the decay rate corresponding to the second transition ($k_2$ of Figure 3.5B) has the observed sigmoidal pH dependence, and a $pK_a$ equal to that for the $I_1/I_2$ equilibrium. The observed pH dependence of the recovery rate is thus a consequence of the pH dependence of the intermediate equilibria. Since the protonation of the chromophore changes in this equilibrium, the simplest interpretation is that this $pK_a \sim 7/6.7$ might be due to the phenol group of chromophore. This value of $pK_a$ lies within the range of the chromophore $pK_a$ inside the protein ($\sim 2.8$) and free in the solution ($\sim 9$). The $I_1/I_2$ equilibrium is also observed in the E46Q mutant with a $pK_a \sim 8.2$ [Borucki, 2003], and also assigned to the phenolate oxygen of the chromophore.

The $I_1$ to $I_2$ transition is incomplete at high pH (Figure 3.5A). This might be due to one of the following reasons. First, the time constant of the first ($\sim 370 \mu s$) and second transition (about 1-10 ms) are not well separated. So, the $I_1/I_2$ equilibrium might not be formed completely in the $I_1$ to $I_2$ decay during the first transition. Additionally, we note that this equilibrium decays further to the another equilibrium $I_1/I_2/I_2'$ in the second transition. A method to separate these two time constants further (e.g. by a change of the temperature) would help to confirm this argument. Second, there is a $I_1'$ contribution at alkaline pH (above pH 8), replacing $I_2$ at acid/neutral pH, Figure 3.11. Both $I_1$ and $I_1'$ increase with increasing pH in the alkaline pH range. This is reflected by the increase of $I_1/I_2$ at pH $> 8$ (Figure 3.5A), leading to the incomplete titration curves in this pH region. Third, since the $I_1'$ and $I_2$ contributions are small at pH 8-8.5 and the cycle is also the fastest, a precise determination of the relative contributions of all four intermediates $I_1$, $I_1'$, $I_2$ and $I_2'$ might not be possible. Here, it is assumed that no $I_1'$ contributes in this pH range.
From about 5 ms onwards, $I_1$, $I_2$ and $I_2'$ are in equilibrium and decay together to the initial dark state $P$, Figure 3.4A-C. The equilibrium intermediate populations of $I_2$ and $I_2'$ are pH dependent with a pK_a of $\sim 6.4$, Figure 3.5C. Below the pK_a, $I_2$ is the major species, above the pK_a the opposite holds.

As is well known (e.g. from [Genick, 1997]), the rate $k_3$, for the ground state recovery, is also pH dependent with a pK_a of 6.3, i.e. within experimental error equal to that for the $I_2/I_2'$ equilibrium (6.4 and 6.1, from single flash photolysis and photoreversal measurements (section 4.2), respectively). Thus, $k_3$ seems to be proportional to the $I_2'$ population (compare Figures 3.5C and 3.5D). Such a proportionality might be explained with the scheme II as described in section 2.5.2, and is expected under the following conditions: 1) the equilibration rates between $I_1$, $I_2$ and $I_2'$ are rapid compared to the microscopic rates of return from each intermediate to the ground state, 2) the latter are pH independent, and 3) the rate from $I_2'$ to $P$ is much larger than from the other intermediates ($k'' \gg k', k''' \gg k'''$). A similar model is also proposed to explain the pH dependence of the rate of ground state recovery at alkaline pH, section 3.2.2. At alkaline pH, the $I_1$, $I_1'$, and $I_2'$ intermediates are in equilibrium (section 3.2.2). The pK_a of the $I_1'$ to $I_2'$ equilibrium is $\sim 9.9$, the ground state recovery rate constant $k_3$ has a pK_a of 9.7, and is proportional to the $I_2'$ population. There is thus a striking similarity between the behaviour at high and low pH. For both branches of the bell-shaped pH dependence of $k_3$, it seems that $k_3$ is proportional to $[I_2']$. This proportionality is not exact however, since $k_3$ approaches zero at low pH, whereas $[I_2']$ approaches a constant value unequal to zero. A more detailed model thus seems to be required. Nevertheless this symmetry between low and high pH behaviour is worth pointing out and the underlying model provides a lowest order explanation.

The proposed reaction scheme for the kinetics and equilibria of the photocycle in this pH range combining the results of the scheme (I) and (II) and the photoreversal results (see sections 4.1 and 4.2) is presented in Figure 3.6.
The pH dependence of the absorption spectrum of a photostationary mixture of P, I₃ and I₃ʹ produced by background illumination was recently analysed by SVD [Otto, 2005], [Shimizu, 2006]. It was shown that I₃ and I₃ʹ are in a pH dependent equilibrium with a pKₐ of 6.3, and that the spectrum of the high pH species I₃ʹ is blue shifted with respect to that of the low pH species I₃ [Otto, 2005], [Shimizu, 2006]. The I₃ and I₃ʹ intermediates also differ with regard to the fluorescence lifetime of the single tryptophan of PYP, W119 [Otto, 2005]. In I₃, the lifetime is long (0.82 ns). In I₃ʹ, the lifetime is much shorter (0.04 ns). Using background illumination, it was shown that the fluorescence decay in the photostationary state is pH dependent with I₃ dominating at low pH and I₃ʹ at high pH [Otto, 2005]. The pKₐ was ~ 6.3. Combining the pH dependence of the fluorescence amplitudes with that of the photostationary absorption, absorption spectra were calculated for the I₃ and I₃ʹ species. These had λₘₐₓ values of ~370 and ~350 nm for I₃ and I₃ʹ, respectively [Otto, 2005], in good agreement with the results reported in this work.

Figure 3.6: Proposed model for the kinetics of the photoregion and photoreversion (described in sections 4.1 and 4.2) of PYP in the pH range from 4.6 to 8.4. Note the equilibria between I₁, I₂ and I₂ʹ. For each intermediate, the chromophore configuration state: cis or trans is denoted in the superscript. The I₂cis and I₂′cis intermediates are in a pH dependent equilibrium and photoreverse to P* (370 nm) with exponential time-constants of 57 and 380 µs (section 4.1.2). The pKₐ of the I₂cis / I₂′cis equilibrium is 6.4. Reisomerization from I₁cis to I₁trans is with a time constant < 1 µs (section 4.1.4). The I₁cis and I₂cis intermediates are in a pH dependent equilibrium with pKₐ of ~7. These three intermediates I₁, I₂ and I₂ʹ decay together to P. For each of the intermediates, protonation states of the chromophore (pCA) and the residue E46 are represented by 0, protonation and –, deprotonation in the superscript. For clarity the short-lived intermediates I₀ and I₀⁺ between P* and I₁ are not shown. The corresponding values of the absorbance maxima are indicated in the brackets.

These results were recently confirmed in [Shimizu, 2006]. Analysis of the photostationary absorption spectra by a scaled subtraction procedure yielded a pKₐ of 6.4 and λₘₐₓ values of 367 and 356 nm for I₂ and I₂ʹ, respectively [Shimizu, 2006]. These authors showed moreover from CD and small angle X-ray scattering experiments that the
global structural transition occurs between these two intermediates with a pKₐ of 6.4. Together with the kinetics results from time-resolved absorption spectroscopy presented here, these complementary methods lead to a comprehensive picture of the I₂ to I₂' equilibrium.

In a related study [Yeremenko, 2006] spectra and time courses of the photocycle intermediates were presented at pH 6.5 in solution and crystals of WT and the mutant E46Q. The I₁, I₂ and I₂' spectra of Figure 3.2A are comparable to those presented in [Yeremenko, 2006]. Moreover, the time constants associated with various transitions are also similar. However, the time courses corresponding to different intermediates differ significantly from our work (Figure 3.4A-C). In [Yeremenko, 2006] I₁ decays partially to I₂ during the first transition, which is similar to that shown in Figure 3.4A,B. During the second transition, I₁ and I₂ decay completely to I₂', which is different to what is observed here (Figure 3.4B). At pH 6.5, from about ~10 ms onwards, only I₂' contributes in the photocycle [Yeremenko, 2006]. In contrast in our work, at pH 6.5 which is near the pKₐ ~6.4 between I₂ and I₂', I₂ makes a significant contribution (about 50%). This pH dependent equilibrium is also established in [Otto, 2005] by measurement of the tryptophan fluorescence, in [Shimizu, 2006] by CD measurements, and in our own separate investigation by measurements of the photo reversal kinetics using double flash excitation (see section 4.2). The discrepancy is probably due to the data analysis method used: target analysis [Yeremenko, 2006]. In target analysis, different pre-defined photocycle models are tested to fit the measured data [Yeremenko, 2006], where rate constants and the spectra are the input parameters. Therefore, this analysis accepts many possible parameters leading to many equally likely photocycle models. In contrast, in our study, first of all the spectra of the intermediates contributing to the photocycle were determined from the measured time traces using only two well established plausible assumptions, which is the key information to elucidate the kinetics mechanism from the transient absorption measurement. Knowledge of the spectra of the intermediates leaves not too many possibilities for the photocycle model. Moreover, the time courses of the intermediates determined in this way from the measured absorbance changes and the spectra of intermediates (eq. 2.18) indicate precisely the interconversion of the intermediates during the photocycle (Figure 3.2C).

An important question concerns the group responsible for the pKₐ of ~6.4. The similar pKₐ for the recovery rate k₃ is commonly attributed to the carboxyl group of E46 [Demchuk, 2000], [Meyer, 2003]. We now need to discuss this pKₐ in the context of the underlying I₂/I₂' equilibrium. What is the mechanism whereby the change in protonation of
E46 shifts the equilibrium from I$_2$ to I$_2'$? In I$_2$ the chromophore is already protonated and has moved away from E46 towards the surface [Genick, 1997A]. If E46 is the internal proton donor for the chromophore, its carboxyl group is presumably already deprotonated in I$_2$, in accordance with some observations from time-resolved FTIR [Xie, 2001]. In I$_2'$ the chromophore remains protonated, but the protein structure is changed in a major way. It is unclear how the deprotonated E46 could affect the I$_2$/I$_2'$ conformational equilibrium. If however, E46 is not the internal proton donor, and the chromophore is protonated from the external medium, as suggested [Borucki, 2002], E46 could remain protonated in I$_2$ and be deprotonated in I$_2'$. In other time-resolved FTIR measurements [Brudler, 2001] a positive band was observed at 1759 cm$^{-1}$ with a risetime of 113 μs (formation of I$_2$) and assigned to an environmental shift of the protonated E46. The authors of [Brudler, 2001] were not aware of the I$_2$/I$_2'$ equilibrium and concluded from the fact that the amplitude of the positive band was significantly smaller than that of the negative band due to the initial dark state, that only a fraction of the molecules cycling had a protonated E46 in I$_2$. Their experiments were however performed in buffer at pH 7. At this pH the I$_2$/I$_2'$ equilibrium is far on the side of I$_2'$ (see Figure 3.5C), so that only a minority of molecules would have been in the I$_2$ state. It is thus consistent with the experimental results of [Brudler, 2001] to conclude that in I$_2$ the carboxyl group of E46 is protonated.

In fact, recent photostationary FTIR measurements showed that E46 is at least partially protonated in I$_2$ [Shimizu, 2006]. A role of E46 in controlling the I$_2$/I$_2'$ equilibrium is thus plausible. In the absence of the carboxyl group, in the mutant E46Q, the conformational change in I$_2'$ is much smaller or absent [Xie, 2001] and the absorption maximum at pH 7 is at 368 nm [Imamoto, 2001A], i.e. I$_2$-like. These results suggest that in the absence of E46 the I$_2$/I$_2'$ equilibrium is predominantly or entirely on the side of I$_2$ ([Borucki, 2003], [Shimizu, 2006]) and further support the idea that E46 is responsible for the wild type pK$_a$ of 6.4. We note that this reinterpretation of the FTIR results is consistent with a mechanism of chromophore protonation from the external medium [Borucki, 2002]. Following this model, the protonation states of the chromophore (pCA) and the residue E46 are represented by 0, protonation and −, deprotonation in the superscript in Figure 3.6, for each of the intermediate.

Another residue that might be involved is H108. In [Hendriks, 1999A] the pH dependence of the steady-state proton uptake was investigated. From the observed pH dependence, a pK$_a$ of 6.6 was obtained, which was attributed to histidine 108. This residue is located on the central β-scaffold (Figure 1.7, blue sticks) and may be involved in the
interaction between the α-scaffold and the N-terminal domain (Figure 1.7). It was recently
postulated, on the basis of the observation that the I₂ to I₂′ transition is blocked at low salt
concentrations, and that the loss of this interaction is a prerequisite for the formation of I₂′
[Borucki, 2005].

Recently two forms of I₁ could be distinguished on the basis of their resonance
Raman spectra, which are in a pH-dependent equilibrium with a pKₐ of ~ 6.2 [Unno, 2004].
The low pH form (I₁) lacks the hydrogen bond with E46, whereas the high pH form (I₁h)
has both hydrogen bonds. It is possible that the pH dependence observed here for the
I₂/I₂′ equilibrium is due to the pH dependence of the preceding I₁/I₁h equilibrium.

3.2 Photocycle Kinetics at Alkaline pH

The intermediate spectra were determined at pH 10. Using these spectra the
corresponding pH dependent time courses of the photocycle intermediates in the pH range 8
to 11 were calculated. A photocycle model is proposed from these observations.

3.2.1 Spectra and Time Courses of Intermediates at pH 10

The intermediate spectra at pH 10 were determined by two independent methods:
extrapolated difference method and scaled subtraction method.

3.2.1.1 Extrapolated Difference Method

Panels A and B of Figure 3.7 allow a comparison between the transient absorption
changes at pH 10 (A) and pH 6 (B) at a number of selected wavelengths. The data show
striking differences between these two pH values in the time range between 200 µs and 2 ms,
for wavelengths between 390 and 420 nm. It will be argued that this difference at pH 10 is
due to the transient formation and decay of an intermediate called I₁′ which absorbs
maximally around 425 nm, and has rise- and decay-times of 330 µs and 1 ms, respectively
(the first two vertical dashed lines of panel A). Comparing the trace in panel A at 340 nm
(rise of I₁′ intermediate) with those at 390 - 420 nm, we note that the additional contribution
in the latter traces develops prior to the rise of the I₂′ intermediate (about 1 ms).

The transient absorbance changes at pH 10 were measured at 33 wavelengths, ranging
from 330 to 510 nm, in the time range from 100 ns to 5 s. Only eight of these traces are
shown in Figure 3.7A. The complete data set was subjected to SVD analysis as described in
section 2.3.2. The first six singular values were: 11.8, 2.6, 0.28, 0.071, 0.059 and 0.051.
3.2.1 Spectra and Time Courses of Intermediates at pH10 (Extrapolated Difference Method)

Since the contributions from $s_4$, $s_5$ and $s_6$ are within the noise level, we consider only the first three components as significant. This suggests the presence of only three spectrally distinguishable intermediates. The three weighted time traces from SVD were fitted simultaneously starting at 8 µs with a sum of three exponentials with time constants $\tau_1 = 330$ µs, $\tau_2 = 1$ ms and $\tau_3 = 830$ ms. The solid lines in Figure 3.7A represent these fit curves for the individual time traces. From the fit to the SVD time traces and the corresponding basis spectra, the amplitude spectra $B_i(\lambda)$ were calculated using eq 2.27. These are presented in Figure 3.7C.

![Figure 3.7](image)

**Figure 3.7**: (A) Transient absorption changes after excitation at 430 nm at wavelengths varying from 330 to 510 nm. For clarity, only the traces at the indicated wavelengths are shown (8 out of 33 wavelengths measured). The vertical dashed lines indicate the time constants for a global fit to the weighted SVD time traces with a sum of three exponentials. $\tau_1 = 330$ µs is the rise time of $I_1'$, $\tau_2 = 1$ ms is the rise time of $I_2'$, and $\tau_3 = 830$ ms is the return to P. The solid lines, only distinguishable from the data in the µs-time range, are the fits. Conditions: pH 10, 20 °C, 50 mM KCl and 20 mM Tris. PYP concentration: 58 µM. (B) The corresponding data at pH 6. PYP concentration: 43 µM. (C) Amplitude spectra $B_i(\lambda)$ calculated from the amplitudes of the exponential fits to the SVD time traces and the corresponding basis spectra of the data in (A). The three amplitude spectra correspond to the following time constants: $\tau_1 = 330$ µs ($\bullet$), $\tau_2 = 1$ms ($\square$), $\tau_3 = 830$ ms ($\circ$). The dotted line is a scaled and inverted ground-state spectrum. (D) Extrapolated difference spectra obtained from the amplitude spectra of (C) according to eq 2.29: $\tilde{B}_1 (\triangle), \tilde{B}_2 (\diamond), \tilde{B}_3 (\bigcirc)$.

The amplitude spectrum $B_1(\lambda)$ associated with the 330 µs life time, has a positive peak near 465 nm and negative peak near 420 nm with no contribution in the UV (350 nm). The data thus clearly show that in this transition $I_1$ is converted to $I_1'$ without the formation of
I_2'. This conclusion follows directly from inspection of the data (i.e., the amplitude spectrum B_1(\lambda)) and is model-independent. The amplitude spectrum B_2(\lambda) associated with the 1 ms lifetime has a very broad positive contribution between 380 and 500 nm and a negative contribution in the UV (350 nm). B_2(\lambda) thus strongly suggests that in this later transition an I_1/I_1' equilibrium decays to I_2'. Note that the scaled ground state spectrum in Figure 3.7C(…) does not fit to B_3(\lambda) for \lambda > 410 nm, indicating the presence of I_1 and I_1' in equilibrium with I_2' before recovery.

The three amplitude spectra, B_1, B_2 and B_3, were used to construct the \( \tilde{B} \) matrix according to eq 2.29. \( \tilde{B}_1 \) is the sum of amplitude spectra B_1, B_2 and B_3 (arranged in columns). \( \tilde{B}_2 \) is the sum of amplitude spectra B_2 and B_3, and \( \tilde{B}_3 \) is same as B_3. The three columns, \( \tilde{B}_1, \tilde{B}_2 \) and \( \tilde{B}_3 \), representing the extrapolated difference spectra are presented in Figure 3.7D. \( \tilde{B}_1 \) is the initial absorbance change right after the flash, and suggests that the initial bleach led to the formation of the I_1 intermediate (positive absorbance change near 480 nm). \( \tilde{B}_2 \) is the difference spectrum with respect to the ground state after the first transition. Inspection of Figure 3.7D shows that below 390 nm \( \tilde{B}_2(\lambda) \) is zero, i.e., has no contribution from intermediates absorbing in the UV like I_2'. Inspection of B_1(\lambda), B_2(\lambda) and \( \tilde{B}_2(\lambda) \) thus allows the conclusion that I_1' is the direct decay product of I_1. We note that this conclusion was already evident from the experimental time traces at 390, 400, 410 and 420 nm of Figure 3.7A.

We now use the second constraint described in Materials and Methods (section 2.4.1), that I_2' does not absorb beyond 410 nm, and consider eq 2.34 only in the range \( \lambda > 410 \) nm; i.e., we drop the rows for the shorter wavelengths. Then the third column of the reduced matrix \( \tilde{A} \), corresponding to the spectrum of I_2', is the null vector: \( (A)_3 = 0 \). Since \( (A)_3 \) is zero, one can solve eq 2.34 for \( (\tilde{C}^{-1})_3 \). In this way, we determine the third column of \( \tilde{C}^{-1} \). Provided that the number of wavelength points is larger than 3, eq 2.34 is an overdetermined system of linear equations, for which the least squares solution may be found by multiplication with the pseudoinverse of \( \tilde{B} \) from the left and rearranging, which is eq 2.36. Using the well established ground state spectrum \( \tilde{A}_p \) and the measured \( \tilde{B} \) spectra, we find from eq 2.36, for the three elements of the third column of \( \tilde{C}^{-1} \): + 0.103, - 4.52, 7.56. Using the first constraint, the sum of these matrix elements equals \( \eta^{-1} \). In this way, the fraction of cycling molecules, \( \eta = 0.318 \) is determined. Finally, using \( \tilde{B} \) and \( \tilde{A}_p \) for the whole spectral
3.2.1 Spectra and Time Courses of Intermediates at pH10 (Extrapolated Difference Method)

range allows us to calculate the spectrum of $I_2'$ from $(\mathbf{C}^{-1})_3$ using eq 2.34. The result is shown in Figure 3.8A (■).

Further progress can be made by noting that only $I_1$ contributes to $\mathbf{B}_1$ (Figure 3.7D). Thus the elements $\mathbf{C}_{21} = y_1$ and $\mathbf{C}_{31} = z_1$ of $\mathbf{C}$ defined by eq 2.30 are given by $y_1 = z_1 = 0$. This allows us to calculate the elements of the first column of $\mathbf{C}^{-1}$ of eq 2.33. The result is $\mathbf{C}^{-1}_{11} = 1/x_1$, $\mathbf{C}^{-1}_{21} = 0$, $\mathbf{C}^{-1}_{31} = 0$. Since the sum of these elements equals $1/\eta$ (conservation constraint, eq 2.35), we have $x_1 = \eta = 0.318$. With $(\mathbf{C}^{-1})_1$ now completely known, we can calculate the spectrum of $I_1$ from $(\mathbf{C}^{-1})_1$, $\mathbf{B}$ and $\mathbf{A}_p$ using eq 2.34. The result is shown in Figure 3.8A (□).

Figure 3.8: (A) Intermediate spectra of $I_1$ (□), $I_1'$ (○) and $I_2'$ (■) calculated from the extrapolated difference spectra of Figure 3.7D. ●: spectrum of the dark state $P$ for comparison. Vertical dashed lines indicate the wavelengths of the blue (430 nm), violet (355 nm) and green (500 nm) excitation flashes used. (B) $I_1'$ spectra for various allowed values of $y_2$ as described in the text. $y_2 = 0.05$ (●), $y_2 = 0.07$ (■), $y_2 = 0.09$ (▲), $y_2 = 0.13$ (○), $y_2 = 0.21$ (□) and $y_2 = 0.3$ (△). (C) Time-courses of the relative concentrations of $I_1$ (—), $I_1'$ (---), and $I_2'$ (....) calculated according to eq 2.18 with $y_2 = 0.13$. The time-courses of the sum of the relative concentrations of $I_1$, $I_1'$ and $I_2'$ is indicated by ——.. The vertical dashed lines indicate the time constants from the global SVD fit of Figure 3.7A.

To calculate the spectrum of the third spectral species, $I_1'$, we proceed in a similar fashion. From Figure 3.7D and as discussed above, $\mathbf{B}_2$ has no contribution from $I_2'$. Thus $z_2 = 0$. The elements $x_2$, $y_2$, $z_2$ of the second column of $\mathbf{C}^{-1}$ can now be expressed in terms of $x_2$, $y_2$, and $\eta$ with the help of $\mathbf{C}^{-1}\mathbf{C} = \mathbf{I}$ as shown in eq 2.39. Using the conservation constraint eq 2.35, $x_2 + y_2 = \eta$, we finally obtain for the elements of the second column of $\mathbf{C}^{-1}$ of eq
Chapter 3 Photocycle Kinetics Mechanism of PYP

2.40, $\tilde{C}_{12}^{-1} = - (\eta - y_2)/\eta y_2$, $\tilde{C}_{22}^{-1} = 1/y_2$, $\tilde{C}_{32}^{-1} = 0$. So, all elements of $\tilde{C}^{-1}$ are determined now, the only free parameter remaining is $y_2$. Since $x_i$, $y_i$ and $z_i$ can only assume positive values and $x_2 + y_2 = \eta$, $y_2$ is restricted to values between 0 and $\eta$. The spectrum of $I_{1'}$ can now be calculated from $(\tilde{C}^{-1})_2$, $\tilde{B}$ and $\tilde{A}_p$ using eq 2.34. The results are shown in Figure 3.8B for six values of $y_2$ from 0.05 to 0.3 (−$\eta$). Since the extinction coefficient has to be positive, physically meaningful absorption spectra are only obtained for $y_2 \geq 0.13$. Of the spectra with $y_2 \geq 0.13$ in Figure 3.8B we pick the one associated with $y_2 = 0.13$, since it has a spectral bandwidth which is most similar to that of the other intermediates and P. As shown in Figure 3.8B, for $y_2$ considerably larger than 0.13, the spectral bandwidth becomes much larger than for P and $I_1$, which is unlikely to be correct. The spectrum of $I_{1'}$ for $y_2 = 0.13$ is redrawn in Figure 3.8A (○). Its $\lambda_{\text{max}}$ value is at about 425 nm. We note this spectrum is confirmed by an independent analysis method (scaled subtraction method) presented in the next section.

Since the spectra of intermediates are determined, the time courses of the intermediates may be calculated using eq 2.18. The time-dependence of the relative concentrations of the $I_1$, $I_{1'}$ and $I_{2'}$ intermediates are shown in Figure 3.8C. As expected from the data (Figure 3.7A), $I_1$ partially decays to $I_{1'}$ in 330 $\mu$s. $I_1$ and $I_{1'}$ then further decay around 1 ms to an $I_0/I_1'/I_2'$ equilibrium. This equilibrium finally decays to P in 830 ms. Also shown is the sum of the relative concentrations of these intermediates (dash-dot line). To a very good approximation, this sum is constant over the entire time range prior to the decay to P, as it should be. Its value is very close to $\eta = 0.318$, the fraction cycling, showing the internal consistency of the analysis.

The matrix containing the relative contributions of the intermediates, $\tilde{C}$ of eq. 2.30, its inverse, $\tilde{C}^{-1}$ of eq. 2.33, and the coefficient matrix, $C$ of eq. 2.17, calculated in this analysis are

$$\tilde{C} = \begin{pmatrix} 0.318 & 0.188 & 0.108 \\ 0 & 0.13 & 0.078 \\ 0 & 0 & 0.132 \end{pmatrix},$$

$$\tilde{C}^{-1} = \begin{pmatrix} 3.144 & -4.55 & 0.103 \\ 0 & 7.69 & -4.52 \\ 0 & 0 & 7.563 \end{pmatrix}$$
3.2.1 Spectra and Time Courses of Intermediates at pH10 (*Scaled Subtraction Method*)

\[
C = \begin{pmatrix}
0.13 & 0.08 & 0.108 \\
-0.13 & 0.052 & 0.078 \\
0 & -0.132 & 0.132
\end{pmatrix}
\]

respectively.

### 3.2.1.2 Scaled subtraction method

The method presented in the previous section for the spectra and time courses of the intermediates is rigorous but not intuitive. A less rigorous but more transparent method is presented below which confirms that the spectra of intermediates \(I_1, I_1',\) and \(I_2'\) presented in Figure 3.8A are correct.

**Figure 3.9:** (A) In the scaled subtraction method, the \(I_1\) spectrum of Figure 3.8A is scaled down (**) in such a way that its long wavelength shoulder (\(\lambda \geq 485\) nm) fits optimally to the corresponding shoulder of the amplitude spectrum \(B_1(\lambda)\) (**). The spectrum of \(I_1'\) (○) was obtained by subtracting the ** spectrum from the ● spectrum and inverting this difference. The \(I_1\) spectrum is also scaled down (■) to match its long wavelength shoulder (\(\lambda \geq 485\) nm) with that of the \(B_3(\lambda)\) (□) amplitude spectrum. The amplitude spectrum plotted in 3C (□) was obtained by subtracting the ■ spectrum of this panel from the □ spectrum. (B) The \(B_3\) amplitude spectrum (○) has to be corrected for the contribution of the ground state spectrum, \(P\) in addition to \(I_1\), because the rise of \(P\) is the final step of photocycle. The down scaled \(I_1\) spectrum (**) was subtracted from \(B_3\) (○) amplitude spectrum which is: ■ spectrum, and has contributions from \(I_1',\) \(I_1'\) and \(P\). The scaled and inverted dark spectrum, \(P\) (★) overlaps \(\lambda \geq 465\) nm region of this spectrum. The amplitude spectrum plotted in 3C (○) was obtained by subtracting scaled and inverted dark spectrum, \(P\) (★) from ■ spectrum of this panel. (C) The open squared spectrum (□) obtained from the \(B_3\) amplitude spectrum, in A represents the partial decay of \(I_1'\) and rise of \(I_2'\). The \(I_1'\) spectrum is scaled down (**) in such a way that its long wavelength shoulder (\(\lambda \geq 430\) nm) fits optimally the corresponding shoulder of the □ amplitude spectrum. The \(I_2'\) spectrum (■) was obtained by subtracting the ** spectrum from the □ spectrum and inverting this difference. The ○ spectrum of this panel is obtained from the \(B_3\) amplitude spectrum in B, which represents the mixture of \(I_1'\) and \(I_2'\) spectra. Also shown is the down scaled \(I_1'\) spectrum (●), where its shoulder (\(\lambda \geq 430\) nm) fits with the shoulder of ○ amplitude spectrum.
None of the amplitude spectra of Figure 3.7C: $B_1$ (330 µs, ●), $B_2$ (1 ms, □) and $B_3$ (830 µs, ○) scale to the dark spectrum even in the spectral range $\lambda \geq 450$ nm. Rather all these amplitude spectra show $I_1$ contributions (positive for $\lambda \geq 500$ nm). So the contribution of the $I_1$ spectrum must be subtracted from these amplitude spectra. The resulting amplitude spectrum after correction of the $I_1$ contribution is either the pure $I_1'$ spectrum (from $B_1$) or a mixture of the $I_1'$ and $I_2'$ spectra (from $B_2$ and $B_3$) as shown below.

The scaled subtraction method requires accurate knowledge of the $I_1$ spectrum. It is an experimental fact that the $I_1$-P difference spectrum (absorbance difference $\sim 1$ µs; e.g. Figure 3.1C(A) and 3.7D(A)) is almost independent of pH, i.e. the amount of $I_1$ in the µs time range is pH independent. This can be taken as the measure of the amount of the cycling molecules. Thus, the $I_1$ spectrum at pH 6 can be taken as the $I_1$ spectrum at pH 10. As we observed here, at alkaline pH $I_1'$ contributes in the ms range which makes it difficult to calculate the excitation efficiency of the blue flash. This is simpler at pH 6 where $I_2'$ contributes on the ms time scale and doesn’t absorb for $\lambda \geq 430$ nm, thus making it possible to scale the dark spectrum for $\lambda \geq 430$ nm to the difference spectrum of the transient absorbance changes of the normal cycle measured at 10 ms for same wavelength range. The spectrum of $I_1$ can be calculated from the 1 µs difference spectrum of the normal cycle by adding this scaled amount of the ground-state spectrum. For further analysis, the $I_1$ spectrum of Figure 3.8A is used.

The starting point for this procedure is the amplitude spectrum $B_1(\lambda)$ (Figure 3.9A, ●) for the 330 µs transition from $I_1$ to $I_1'$. We assume that this transition only involves the $I_1$ and $I_1'$ intermediates. This is strongly supported by the fact that this amplitude spectrum contains a positive peak around 465 nm presumably due to $I_1$, a negative peak around 420 nm presumably due to $I_1'$ and has zero absorbance around 355 nm where $I_2'$ absorbs. For a sequential unidirectional transition between $I_1$ and $I_1'$, $B_1(\lambda)$ should equal their difference spectrum and experimentally this seems to be the case. This allows us to remove the contribution of $I_1$ from $B_1(\lambda)$, thereby obtaining the spectrum of $I_1'$. This procedure is shown in Figure 3.9A. The spectrum of $I_1$ is scaled (⃣) so that its long wavelength shoulder matches optimally with the $B_1(\lambda)$ spectrum, which assumes that $I_1'$ does not absorb in the matching region. As we see from Figure 3.9A, the match is excellent for $\lambda \geq 485$ nm when the spectrum of $I_1$ is scaled by a factor of 0.132. Therefore, the extinction coefficient of $I_1'$ is zero beyond 485 nm. Thus, the matching criterion is equivalent to this constraint on the $I_1'$ spectrum.
3.2.1 Spectra and Time Courses of Intermediates at pH10 (Scaled Subtraction Method)

completely for the $I_1$ contribution, leaving the $I_1'$ spectrum ($\circ$), which is shown in Figure 3.9A. The negative sign of the $I_1'$ spectrum obtained after subtraction of the $I_1$ contribution from $B_1(\lambda)$ indicates the rise of this intermediate in first transition. When this $I_1'$ spectrum is scaled up by dividing with a factor of 0.132 the result is almost identical to the $I_1'$ spectrum of Figure 3.8A($\circ$) obtained by the matrix method. This shows that the $B_1$ amplitude spectrum represents the partial decay of $I_1$ (a factor 0.132 of $I_1$ from Figure 3.8A) to $I_1'$ (a factor of -0.132). This agreement is not fortuitous, since the constraint of requiring the extinction coefficient to be zero beyond 485 nm is equivalent to the choice $y_2 = 0.13$ (see Figure 3.8B). The corresponding factors of $I_1$ and $I_1'$ acquired in this analysis are similar to those arranged in the first column of the $C$ matrix of the previous section (extrapolated difference method), also arranged in $C$ matrix of eq 3.1 below. The rows of $C$ matrix defined by eq 2.17, correspond to the relative contributions of a particular intermediate, so that, first, second and third rows correspond to the relative contributions of $I_1$, $I_1'$ and $I_2'$ intermediates respectively. Similarly, the column of $C$ matrix corresponds to the relative contributions of the all intermediates during a particular transition, so that, first, second and third column represent the relative contribution of all $I_1$, $I_1'$ and $I_2'$ intermediates during the 370 µs, 1 ms and 830 ms transition respectively.

Next, it will be shown that the amplitude spectrum $B_2(\lambda)$ (Figure 3.9A, □) for the 1 ms transition is due to the partial decay of $I_1$ and $I_1'$ to $I_2'$. We assume that this transition involves the $I_1$, $I_1'$ and $I_2'$ intermediates only. This is strongly supported by the fact that this amplitude spectrum contains a broad positive absorption band around 400-500 nm presumably due to $I_1$ and $I_1'$, a negative peak around 350 nm presumably due to $I_2'$. It will be shown below that there is no dark recovery during this transition. This implies that there is zero contribution of dark state in this amplitude spectrum. For a sequential unidirectional transition between $I_1$ and $I_1'$ to $I_2'$, $B_2(\lambda)$ should equal their difference spectrum. Thus the $I_1$ contribution might be removed from $B_2(\lambda)$, obtaining in that way, the amplitude spectrum containing the contributions from the remaining intermediates $I_1'$ and $I_2'$. This is also shown in Figure 3.9A. The spectrum of $I_1$ is down scaled (■) so that its long wavelength shoulder matches with the $B_2(\lambda)$ spectrum (□). The match is excellent for $\lambda \geq 485$ nm when the spectrum of $I_1$ is scaled by a factor of 0.08. This factor is described below. Subtracting this amount of $I_1$ should correct completely for the $I_1$ contribution, leaving the contributions from the remaining intermediates $I_1'$ and $I_2'$, which is plotted in Figure 3.9C (□). Moreover, there is no contribution from the dark spectrum. This is supported by the fact that this amplitude
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The spectrum of Figure 3.9C (□) contains a positive peak around 430 nm presumably due to the decay of \( I'_1 \), a negative peak around 350 nm presumably due to the rise of \( I'_2 \) and has zero absorbance beyond 480 nm where P absorbs. For a sequential unidirectional transition between \( I'_1 \) and \( I'_2 \), □ amplitude spectrum should equal their difference spectrum. This allows to remove the contribution of \( I'_1 \) from the □ amplitude spectrum, thereby obtaining the spectrum of \( I'_2 \). This is shown in Figure 3.9C where the spectrum of \( I'_1 \) is down scaled (⊗) so that its long wavelength shoulder matches with the □ amplitude spectrum, which assumes that \( I'_1 \) does not absorb in the matching region. This match is excellent for \( \lambda \geq 430 \) nm, when the spectrum of \( I'_1 \) is scaled by a factor of 0.055. Therefore, the extinction coefficient of \( I'_2 \) is zero beyond 430 nm. This factor is described below. Subtracting this amount of \( I'_1 \) from the □ amplitude spectrum and inverting this difference should correct completely for the \( I'_1 \) contribution, leaving the \( I'_2 \) spectrum (■) of Figure 3.9C. The negative sign of the spectrum obtained from the subtraction indicates the rise \( I'_2 \). This \( I'_2 \) spectrum is scaled up by dividing with a factor of 0.135 (the total of 0.08 factor of \( I_1 \) and 0.055 factor of \( I'_1 \)). The resulting spectrum is almost identical to \( I'_2 \) spectrum of Figure 3.8A(■) obtained by the matrix method. This shows that the \( B_2 \) amplitude spectrum is due to the partial decay of \( I_1 \) (a factor of 0.08) and \( I'_1 \) (a factor of 0.055) to \( I'_2 \) (a factor of -0.135). No direct recovery to the dark state is observed during the second transition. The corresponding factors of \( I_1, I'_1 \) and \( I'_2 \) acquired from the \( B_2 \) amplitude spectrum are close to those in the second column of the \( C \) matrix of the previous section (extrapolated difference method), and are arranged in the \( C \) matrix of eq 3.1 below.

The \( I'_1 \) and \( I'_2 \) spectra can also be acquired from the \( B_3 \) amplitude spectrum of Figure 3.9B (○). In this case, the contribution of ground state spectrum P has to be subtracted in addition to the \( I_1 \) contribution. The amount of P (★) is the same as the amount of P added to calculate the \( I_1 \) spectrum from the \( I_1\)-P difference spectrum (absorbance difference at 1 µs), which is the excitation efficiency (0.31) times the spectrum of the dark state of Figure 3.8A (●). It will be shown that the amplitude spectrum \( B_3 \) for the 830 ms transition is due to the decay of \( I_1, I'_1 \) and \( I'_2 \) to P. This is evident from the fact that this amplitude spectrum has a positive absorption near 480 nm and 350 nm, presumably due to \( I_1 \) and \( I'_2 \) respectively, and a negative peak around 450 nm presumably due to P.

The ■ spectrum of in Figure 3.9B is obtained by subtracting the \( I_1 \) contribution (⊗) from the \( B_3 \) amplitude spectrum (○). The inverted dark spectrum P(★) is scaled in such a way that it matches the ■ spectrum for \( \lambda \geq 465 \) nm. The ■ spectrum is due to the rise of P (the
negative peak around 450 nm), the decay of I$_2^\prime$ (a positive absorption around $\lambda \sim$ 350 nm) and I$_1^\prime$ (a mismatch around $\lambda \sim$ 420 nm). Here, only the amount of I$_1$ (a factor of 0.1) was varied and the other two spectra B$_3$ and P($\star$) are fixed. The criterion for selecting the I$_1$ contribution in the B$_3$ amplitude spectrum is critical, where the I$_1$ spectrum is scaled down to overlap in the $\lambda \geq$ 500 nm range. In this spectral region not many measured points are available. However, further optimisation of this factor to 0.1 is attained from successive step of subtraction where the I$_1$ amplitude spectrum overlaps the P($\star$) spectrum in the $\lambda \geq$ 465 nm range. In this region, many measured points are available making it easier to optimise. The ground state spectrum P($\star$) was also subtracted from the I$_1$ amplitude spectrum. The result is a mixture of the I$_1^\prime$ and I$_2^\prime$ spectra as shown in Figure 3.9C ($\bigcirc$). The two positive maxima around ~ 430 nm and ~ 360 nm are presumably due to I$_1^\prime$ and I$_2^\prime$, and the $\bigcirc$ spectrum is their sum. The removal of the contribution of I$_1^\prime$ from the $\bigcirc$ spectrum results in the I$_2^\prime$ spectrum as shown in Figure 3.9C, where the spectrum of I$_1^\prime$ is scaled down ($\bullet$) so that its long wavelength shoulder matches with $\bigcirc$ amplitude spectrum in the $\lambda \geq$ 430 nm region. Subtracting this amount of I$_1^\prime$ (with a factor of 0.077) corrects for the I$_1^\prime$ contribution leaving the I$_2^\prime$ spectrum. This I$_2^\prime$ spectrum is very similar to the I$_1^\prime$ spectrum of Figure 3.9C ($\blacksquare$) (data not shown). Taken together these subtraction steps show that the B$_3$ amplitude spectrum has contributions from the decay of I$_1$ (a factor of 0.1), I$_1^\prime$ (a factor of 0.077) and I$_2^\prime$ (a factor of 0.135) and the rise of P. The corresponding factors of I$_1$, I$_1^\prime$ and I$_2^\prime$ acquired from the B$_3$ amplitude spectrum are similar to those in the third column of the C matrix calculated in the extrapolated difference method (section 3.2.1.1).

Thus, the spectra of the intermediates I$_1$, I$_1^\prime$ and I$_2^\prime$ contributing to the photocycle are determined using the scaled subtraction method. The corresponding C matrix acquired from this method is:

$$
C = \begin{pmatrix}
0.132 & 0.08 & 0.1 \\
-0.132 & 0.055 & 0.077 \\
0 & -0.135 & 0.135
\end{pmatrix}
$$

which should be compared with the C matrix of the previous section (extrapolated difference method). The agreement is excellent.

From the known spectra of the intermediates, the time courses can be calculated using eq. 2.18. Thus the two methods, extrapolated difference method and scaled subtraction method, are equivalent.
3.2.2 pH Dependence

To learn more about the nature of the transition between the neutral and alkaline pH regimes, the photocycle kinetics was measured at the seven pH values 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0. With excitation at 430 nm, time traces were collected at 14 wavelengths from 370 to 500 nm in steps of 10 nm.

Results for selected wavelengths are shown in Figure 3.10. Note that the panels of Figure 3.10 have very different vertical scales, and correspondingly different signal to noise.
3.2.2 pH Dependence

The smallest pH-induced absorbance changes are at 500 nm. The initial absorbance change is almost pH independent at every wavelength, suggesting that the amount of I₁ formed is pH independent.

At each pH, the absorbance changes at all wavelengths could be fitted simultaneously with a sum of three exponentials. The first two time constants were virtually independent of pH. The first time constant varied between 200 and 370 µs in this pH range. For the second time constant, the range was 720 µs to 1.3 ms. As we saw above, the first transition is due to the decay of I₁ to I₁', the second transition due to the decay of I₁/I₁' to the I₁/I₁'/I₂' equilibrium. The data of Figure 3.10 show that the third time constant, the return of the I₁/I₁'/I₂' equilibrium to P, is strongly pH dependent, slowing down with pH. The amplitudes of the time traces around 10 ms, when the I₁/I₁'/I₂' equilibrium is well established, may be used to draw conclusions on the pH dependence of the equilibrium intermediate populations.

Panel A of Figure 3.10 shows that the I₁' population decreases with pH. From panel E we may conclude that the fraction of molecules in I₁ in the ms time range increases with pH. The traces at 450 nm, panel D, indicate that the ground state depletion decreases with pH, consistent with the reduced amount of I₁'. The traces at 390 and 410 nm (panels B and C) are characteristic for I₁'. Here again the pH dependence is consistent with an increase of the I₁/I₁' equilibrium contribution and a decrease in the amount of I₂' with pH.

To obtain the time courses of the intermediates the data at the five pH values from 8.0 to 10.0 in steps of 0.5 were measured at 14 wavelengths from 370 to 500 nm in steps of 10 nm. Since hydrolysis occurs at high pH [Meyer, 2003], fewer data points were collected at pH 10.5 and 11 to reduce the measuring time. At these pH values, data were collected at only 8 wavelengths from 370 to 500 nm. The spectrally superior data at the five pH values between 8 and 10 were arranged by truncating the data matrix measured at each pH (time traces are arranged in the rows), and forming a large matrix as described in section 2.5.1. The number of rows in this large matrix is the number of wavelengths and the number of columns is the sum of the time points from the whole pH range. This combined matrix is subjected to a SVD analysis (eq 2.41). This assumes that the same spectral species contribute over the whole pH range. We then have:

$$(\Delta A_{\text{pH}8}, \ldots, \Delta A_{\text{pH}10}) = U^T D (s) (V_{\text{pH}8}, \ldots, V_{\text{pH}10})$$

The first five singular values were: 17.03, 2.54, 0.45, 0.16, 0.12. Since the contributions from s₄ and s₅ are within the noise level, only the first three components are considered as significant. This suggests again the presence of only three spectral components,
presumably I₁, I₁' and I₂'. The time traces of the three SVD components V₁ at pH 10 (where significant amounts of I₁' and I₁ are present), weighted with the corresponding singular values sᵢ, were fitted simultaneously with a sum of three exponentials. From the amplitudes of this fit and the basis spectra Uᵢ, the three amplitude spectra Bᵢ(λ) were calculated as described by eq 2.27. Finally the extrapolated difference method was used to calculate the spectra of the intermediates (I₁, I₁', I₂') from these amplitude spectra. The spectra of I₁, I₁' and I₂' obtained in this way (not shown) are, as expected, very similar to those from the separate measurements at pH 10 (Figure 3.8A), but they have the advantage that they are the best average spectra over this whole pH range.

The time courses of the intermediate concentrations nᵢ(t) at each pH were then calculated from ΔA(λ, t) at all 14 wavelengths and the intermediate spectra by using eq 2.18. For the two pH values 10.5 and 11.0, where data at only 8 wavelength values were collected,

Figure 3.11: Time courses of the relative concentrations of the I₁ (A), I₁' (B) and I₂' (C) intermediates at various pH values calculated from the combined SVD as explained in the text. (D): time course of the sum of the populations of I₁, I₁' and I₂'. Color code as in Figure 3.10.
which were not included in the joint SVD, the same intermediate spectra were used to calculate their time courses, again from eq 2.18.

The resulting time traces are shown in Figure 3.11. The fractions of the molecules in \( I_1 \), \( I'_1 \) and \( I'_2 \) have the time dependence we expect from the data and from our previous results at pH 10 (Figure 3.8C). The sum of the fractions is plotted in Figure 3.11D. This sum is practically constant in time until the ground state recovery, as required. Moreover, this plateau value is virtually pH independent and equal to the fraction cycling which is similar to that of the previous experiments at pH 10 (Figure 3.8C).

Figure 3.12: pH dependence of the equilibrium concentrations of the \( I_1 \), \( I'_1 \) and \( I'_2 \) intermediates at 10 ms, derived from Figure 3.11A, B and C, respectively. The dotted curves are simultaneous fits of these titration curves with \( pK_a \sim 9.9 \) and \( n \sim 0.96 \). The solid curves are individual fits with the Henderson-Hasselbalch equation. (A): \( I_1 \), \( pK_a = 9.3 \), \( n = 1.21 \); (B): \( I'_1 \), \( pK_a = 10.0 \), \( n = 0.9 \); (C): \( I'_2 \), \( pK_a = 9.8 \), \( n = 1.06 \). (D): pH dependence of the decay rate \( k_3 \) for the ground state recovery. Solid curve is fit with Henderson-Hasselbalch equation and \( pK_a = 9.7 \), \( n = 0.87 \).

The pH dependence of the time traces in panels A to C not only confirms what was already suggested by the data at various wavelengths (Figure 3.10), but allows a quantitative analysis of the pH dependence of the intermediate equilibria. From around 5 ms to the ground state recovery, the intermediate concentrations are constant in time and the
intermediates are in equilibrium. The fractions in equilibrium are clearly pH dependent. The main effect is that the fraction in I′ increases with pH at the expense of I′. The increase in I is smaller. The population values at 10 ms (vertical dashed lines in panels A to C of Figure 3.11) were used as a measure for the equilibrium values. The pH dependence of the equilibrium values of the intermediate concentrations at 10 ms is plotted in Figure 3.12. The dotted curves are simultaneous fits of the data of panels A, B and C with the Henderson-Hasselbalch equation with pK_a = 9.9 and n = 0.96. The solid curves are individual fits with the Henderson-Hasselbalch equation. The fit parameters are: I_1, pK_a = 9.3, n = 1.21; I_1′, pK_a = 10.0, n = 0.9; I_2′, pK_a = 9.8, n = 1.06. The global fit is excellent for I_2′ and I_1′, but only adequate for I_1. Panel D of Figure 3.12 shows the pH dependence of the rate constant for the recovery to the ground state P. This sigmoidal curve yielded a pK_a of 9.7 and a Hill coefficient of 0.87. The high pH end value for this fit was fixed at zero.

From Figure 3.12, we conclude that the populations of I_1 and I_1′ increase with pH, whereas the population of I_2′ simultaneously decreases. One possible scheme (III), which can explain the observed common pH dependence of the equilibrium populations and the recovery rate (Figure 3.12D) involves a rapid equilibrium between I_1, I_1′ and I_2′. Rapid means that the equilibration rates between I_1, I′_1 and I′_2 are fast with respect to the recovery rates k′, k″ and k‴ (see section 2.5.2 or scheme (III) below). The I_1/I_1′ equilibrium is assumed to be pH independent, this guarantees that I_1 and I_1′ increase in parallel. The I_1′/I_2′ equilibrium is pH dependent and the rate constants for the return to P are pH independent.

Analysis of this reaction scheme shows that the apparent rate constant for the ground state recovery (k_3 of Figure 3.12D) has the observed sigmoidal pH dependence with the highest rate at pH 8 and equal to k′, and a pK_a equal to that for the I_1′/I_2′ equilibrium. The observed pH dependence of the recovery rate is thus a consequence of the pH dependence of the intermediate equilibria. The data in Figure 3.12D with k_3 almost zero at pH 11 suggest that k′ >> k″, k‴. Since the protonation of the chromophore changes in this equilibrium, the
simplest interpretation is that this pKₐ of ~ 9.9 represents the surface exposed chromophore. The I₁’ intermediate thus appears to be the alkaline form of I₂’.

3.2.3 Discussion

From measurements of the pH dependence of the photocycle kinetics of PYP between 8 and 11, the following results were obtained: 1) the spectrum of I₁’ with a λ_max at about ~ 425 nm, 2) I₁’ is the decay product of I₁, 3) from several ms (formation of I₂’) to the end of the cycle the three intermediates I₁, I₁’ and I₂’ are in equilibrium, 4) the pKₐ of the pH-dependent equilibrium between I₂’ and its precursor I₁’ is ~ 10. The pH is thus an important parameter that controls the amount of receptor in the active state, i.e. I₂’.

Evidence for the I₁’ intermediate was also obtained from the B₁ amplitude spectrum of the photoreversal kinetics (see section 4.3). An I₁’-like intermediate was also detected at alkaline pH in the mutant Y98Q [Borucki, 2005]. The absorption maximum of the spectrum of I₁’ at about ~ 425 nm, lies in between the λ_max values of I₁ and I₂’. At neutral pH, three intermediates are required, in the sequence I₁, I₁’, and I₂’ (Figure 3.2C). In I₂’ the chromophore is believed to be exposed and protonated. Since at sufficiently high pH, the exposed chromophore can no longer be protonated, the I₁’ intermediate may be regarded as the alkaline form of I₂’ with deprotonated chromophore but similar structure. Indeed, a wavelength maximum of 425 nm is consistent with a deprotonated partially exposed chromophore. For both the cis and trans forms, a shift of about 58 nm is observed between the λ_max values of the protonated and deprotonated bound exposed chromophore [Imamoto,
The electronic spectra of I\textsubscript{2} and I\textsubscript{2}' at pH 7 can be distinguished and the $\lambda_{\text{max}}$ values for I\textsubscript{2} and I\textsubscript{2}' are about 372 and 352 nm respectively (Figure 3.2A, [Otto, 2005]). Thus, the deprotonated forms of I\textsubscript{2} and I\textsubscript{2}' may be expected to have their $\lambda_{\text{max}}$ values near 430 and 410 nm, respectively. The observed value of 425 nm is therefore reasonable and it seems likely that I\textsubscript{1}' has a deprotonated chromophore. This question could be settled by time-resolved vibrational spectroscopy.

Concerning the intermediate nomenclature, we labeled the 425 nm alkaline species, I\textsubscript{1}'\textsuperscript{′}. The subscript one was chosen since its chromophore is deprotonated, and a prime was added to distinguish it from its precursor I\textsubscript{1}. We note that for wild type at neutral pH, heterogeneity in I\textsubscript{1} has been detected by FTIR [Imamoto, 2002], resonance Raman [Unno, 2004] and X-ray diffraction [Ihee, 2005]. In those cases, the I\textsubscript{1}-like intermediates had chromophores that were still hydrogen bonded in the binding pocket, whereas I\textsubscript{1}' at alkaline pH refers most likely to a state with a surface exposed chromophore. For the mutant E46Q, we previously introduced an intermediate I\textsubscript{1}' between I\textsubscript{1} and I\textsubscript{2} which had a surface exposed chromophore and was in a pH-dependent equilibrium with I\textsubscript{2} [Borucki, 2003]. In that case we had no evidence however that this intermediate absorbed at 425 nm in the pH-range studied.

The results presented here for the photocycle showed that at pH 10, I\textsubscript{1} decays partially to I\textsubscript{1}' (330 µs). I\textsubscript{1} and I\textsubscript{1}' then decay further to I\textsubscript{2}' (1 ms). The I\textsubscript{1}/I\textsubscript{1}'/I\textsubscript{2}' equilibrium finally decays to P in 830 ms. The proposed reaction scheme for the kinetics of the photocycle and the photoreversal (described in section 4.3) at pH 10 is presented in Figure 3.13.

The spectra of I\textsubscript{1}, I\textsubscript{1}' and I\textsubscript{2}' (Figure 3.8A) as well as their time-courses were obtained from the transient absorbance data with the help of the extrapolated difference method [Borucki, 1999]. The assumptions used in this procedure are plausible and explained in detail in Materials and Methods: 1) the absorbance of I\textsubscript{2}' is zero beyond 410 nm, 2) the sum of the relative intermediate contributions to the extrapolated difference spectra is constant and equals the fraction cycling. This method was previously successful with bacteriorhodopsin where in addition constraints from linear dichroism were used [Borucki, 1999]. It is validated for PYP by the fact that the spectra of I\textsubscript{2}' and I\textsubscript{1} are in excellent agreement with the corresponding spectra at acid/neutral pH of Figure 3.2A, that the sum of the relative intermediate concentrations remains constant during the cycle until the recovery of the groundstate (Figure 3.8C) and that an independent analysis (scaled subtraction) led to the same spectrum for I\textsubscript{1}′.
The spectrum of the UV intermediate in Figure 3.8A was assigned to I\textsuperscript{1′} and not to I\textsubscript{2}, since its wavelength maximum is at 355 nm. The spectra of I\textsubscript{2} and I\textsubscript{2′} are known to have their maxima at 372 nm and 352 nm respectively (Figure 3.2A, [Otto, 2005]). At neutral pH, the I\textsubscript{2} and I\textsubscript{2′} intermediates are in equilibrium, as shown in Figure 3.5C. This equilibrium is pH dependent with a pK\textsubscript{a} of 6.2 (Figure 3.5C, [Otto, 2005]). Above this pK\textsubscript{a} the I\textsubscript{2′} intermediate dominates (Figure 3.5C, [Otto, 2005]). At alkaline pH we therefore only expect to observe I\textsubscript{2′}. The absence of an I\textsubscript{2} intermediate at alkaline pH is furthermore supported by the photoreversal experiments which indicate the presence of only one I\textsubscript{2}/I\textsubscript{2′} –like intermediate (section 4.3).

The result for the I\textsubscript{1′} spectrum is also confirmed by an alternative and more subjective method. This scaled subtraction method has the advantage that it is easier to understand. It requires as input an accurate I\textsubscript{1} spectrum. The agreement between the spectra of I\textsubscript{1′} calculated from B\textsubscript{1} using the scaled subtraction method and from the extrapolated difference method was excellent. The amplitude spectra B\textsubscript{2} (1 ms) or B\textsubscript{3} (830 ms) may also be used to extract the I\textsubscript{1′} and I\textsubscript{2′} spectra as described in Figure 3.9.

In previous work approximate spectra for I\textsubscript{1′} were obtained from photostationary light/dark difference spectra at alkaline pH ([Hendriks, 1999A], [Imamoto, 2004], [Hendriks, 2003]). These spectra have to be corrected for the contributions from other intermediates (I\textsubscript{1}, I\textsubscript{2′}), that accumulate at high pH under background illumination. Such measurements suffer moreover from potential artifacts due to the efficient photoreversal of these intermediates (e.g. I\textsubscript{1}) by the background illumination (section 4.1 to 4.3). Due to the very slow recovery rates at alkaline pH this correction is essential.

An analysis similar to a version of scaled subtraction method was performed by Imamoto et. al. [Imamoto, 2004]. The absorbance change was measured at pH 10 with 10 ms time resolution and the obtained difference spectrum was very similar to B\textsubscript{3} of Figure 3.9B(©). The I\textsubscript{1} and dark spectrum contributions were subtracted from this difference spectrum. The resulting spectrum is very similar to the I\textsubscript{1′}/I\textsubscript{2′} spectra of Figure 3.9C(©). A spectrum was presented for PYP\textsuperscript{410}, which has a \( \lambda_{\text{max}} \) value of 410 nm [Imamoto, 2004] and an extinction coefficient that is only half as large as for our I\textsubscript{1′} spectrum of Figure 3.8A. The presented PYP\textsubscript{M} (or I\textsubscript{2′}) and PYP\textsuperscript{410} (or I\textsubscript{1′}) spectra in this publication have similar extinction coefficients at their absorbance maxima. However, we obtained very different results as shown in Figure 3.8A. The reason for this difference is that the I\textsubscript{1′}/I\textsubscript{2′} spectra of Figure 3.9C(©) have to be scaled with different scaling factors (eq 3.1, 0.077 for I\textsubscript{1′} and 0.13
for \( I_2' \) to compare with \( I_1 \) and \( P \) the spectra of Figure 3.8A. Those factors can only be obtained from the scaled subtraction procedure of the first (\( B_1 \)) and second transition (\( B_2 \)) as described in eq 3.1. Thus, the PYP\(^{100} \) spectrum presented in [Imamoto, 2004] is incorrect as these two transitions are not resolved in the measurements presented this publication.

Hendriks et al. [Hendriks, 1999A] acquired a photostationary spectrum of \( pB_{\text{deprot}} \) (deprotonated form of \( I_2' \)) in the presence of background illumination at pH 10.9 and subtracted a scaled ground state spectrum to remove the contribution of this state from the difference spectrum. The spectrum determined in this way had only six wavelength points and a \( \lambda_{\text{max}} \) value of about 420 nm [Hendriks, 1999A]. We observed a very similar spectrum (data not shown), when a scaled ground state spectrum \( P(\ast) \) of Figure 3.9B(\( \odot \)) is subtracted from the \( B_3 \) amplitude spectrum. However, this resulting spectrum is a mixture of \( I_1 \), \( I_1' \) and \( I_2' \) intermediates as shown in Figure 3.9B,C. Thus, the \( pB_{\text{deprot}} \) spectrum presented in [Hendriks, 1999A] is not the pure \( I_1' \) spectrum but rather a mixture of \( I_1 \), \( I_1' \) and \( I_2 \).

Our spectra for \( I_1' \) were obtained from the wavelength dependence of the transient absorption data, do not suffer from the problems described above, and provide the correct \( I_1' \) spectrum.

These measurements provide for the first time kinetic information on the formation of the \( I_1' \) intermediate. The amplitude spectrum \( B_1(\lambda) \) (Figure 3.7C), which is derived directly from the data without any assumptions, clearly proves that in the 330 µs transition \( I_1 \) decays to \( I_1' \), without the formation of \( I_2' \). \( B_2(\lambda) \) shows that in the next transition \( I_1 \) and \( I_1' \) decay to \( I_2' \). These results are model independent. Further analysis, based on the extrapolated difference method and a few plausible assumptions, confirms these results. Both the time traces (Figure 3.7A) and the intermediate time-courses (Figure 3.8C) derived from these data, indicate that \( I_1' \) is the decay product of \( I_1 \) and decays in equilibrium with \( I_1 \) and \( I_2' \) to \( P \). In [Hendriks, 2003] the intermediate \( \text{pB}_{\text{deprot}} \) (equivalent to \( I_1' \)) was introduced between \( I_2' \) and \( P \), and in equilibrium with \( I_2' \). It was concluded that \( \text{pB}_{\text{deprot}} \) (\( I_1' \)) is the decay product of \( \text{pB} \) (\( I_2' \)). Our kinetic data prove, that this is incorrect, and that \( I_1' \) is formed from \( I_1 \). The photocycle model in Figure 4 of [Hendriks, 2003] is thus also incorrect. \( \text{pB}_{\text{deprot}} \) or \( I_1' \) is also presented in [Yeremenko, 2006], in the photocycle model at pH 6.5, although it is mentioned that this state is not characterized in that study. We have shown here that no \( \text{pB}_{\text{deprot}} \) or \( I_1' \) exist at this low pH (Figure 3.3D: 410 nm is the characteristic wavelength for \( I_1' \), Figure 3.12B).
3.2.3 Discussion

Assuming that the spectra of $I_1$, $I_1'$ and $I_2'$ are pH independent, and that no other intermediates contribute, eq 2.18 was used to calculate the time traces of these intermediates at each pH. Then the stationary equilibrium values at 10 ms were used to derive titration curves for the $I_1$, $I_1'$ and $I_2'$ populations (Figure 3.12). The results show that with increasing pH the equilibrium concentrations of $I_1$ and $I_1'$ in the millisecond time range increase at the expense of a corresponding decrease in the $I_2'$ concentration. The common pK$_a$ is ~ 9.9. The pH dependence of the rate constant for the return to the ground state from this equilibrium was sigmoidal with a pK$_a$ of ~ 9.7 (Figure 3.12D), within experimental error equal to the value of the pK$_a$ for the pH dependent change in equilibrium populations. These data were analysed on the basis of scheme (III), in which only the $I_1'/I_2'$ equilibrium is pH dependent. We find that the pH dependence of the recovery rate is a consequence of the corresponding pH dependence of the $I_1'/I_2'$ equilibrium. The pK$_a$ of 9.9 is attributed to the phenol group of the partially exposed chromophore, in agreement with previous assignments ([Demchuk, 2000], [Meyer, 2003]).

These data are analyzed on the basis of the simple kinetic scheme (III) with only one pK$_a$. The titration of Figure 3.12 suggested the presence of a common pK$_a$ of ~ 9.9. The poor fit for $I_1$ (with an individual pK$_a$ of ~ 9.3, Figure 3.12A) is attributed to experimental error (low signal to noise ratio) and the very limited number of pH points. The pH dependence of the equilibrium populations (Figure 3.12) is based on the time traces of Figure 3.11. These depend in turn on the intermediate spectra. It is noted, that some residual freedom remained in these spectra (exact value of the parameter $y_2$), which leads to corresponding uncertainty in the time traces and pH dependencies. For these reasons we do not want to overinterpret our data, and thus limit this analysis to one common pK$_a$. It cannot be excluded however that the pK$_a$ of 9.3 is real and that there are two very close pK$_a$’s. This would require a more complex kinetic scheme, such as discussed for the mutant Y98Q [Borucki, 2005].

It has long been known that the rate constant for the ground state recovery has a bell-shaped pH dependence with pK$_a$’s of 6.4 and 9.4 ([Genick, 1997], [Demchuk, 2000]). In [Demchuk, 2000] these data were analyzed on the basis of an equilibrium model implying that the recovery of P proceeds via a species with deprotonated chromophore and protonated Glu46, i.e. an $I_1$-like species. Since the microscopic rate for the transition from this $I_1$-like state to P was assumed to be pH independent [Demchuk, 2000], the apparent rate constant of the recovery of P follows the accumulation of the $I_1$-like state in the equilibrium that is maximal at ~ pH 8.0. We note that no amplitude data were used in this analysis to confirm the assumed accumulation of the $I_1$-like state. Here it is shown that the accumulation of $I_1$ in
the equilibrium increases from pH 8 to 10, which is inconsistent with the proposed model [Demchuk, 2000]. Moreover, we provide an explanation for the higher pK_a in terms of the pH dependence of the I_1'/I_2' equilibrium.

We determined the equilibrium concentrations of the intermediates I_1, I_1' and I_2' from their time courses and provided direct kinetic evidence for the existence of their equilibria. In [Borucki, 2003] we first demonstrated the equilibrium between I_1 and I_2' at pH 11. Recently the equilibrium concentrations of these intermediates at alkaline pH were obtained from photostationary absorption spectra in the presence of background illumination [Imamoto, 2004]. The pH dependencies of I_1' and I_2' agree fairly well with our results. A pK_a of 10.2 was obtained for this equilibrium, in good agreement with our value of 9.9. The results for I_1 disagree, however. According to [Imamoto, 2004] the fraction of molecules in I_1 rises with pH up to pH 10.2 and then decreases again. Our data points (Figure 3.12A) agree up to pH 10, but do not decrease at higher pH values. The probable reason of discrepancy is explained above. Moreover, we showed recently, that the ionic strength also affects these equilibria [Borucki, 2005], with increasing salt shifting the I_1'/I_1' equilibrium towards I_2'. Differences in the amounts of salt added during the titration may thus explain the discrepancy. The steady-state data in [Imamoto, 2004] were collected under continuous illumination with light with wavelengths above 430 nm. Another contribution to the discrepancy could thus be photoreversal from I_1 (λ_max ~ 460 nm), which would reduce the I_1 population as described in section 4.1 to 4.3, in particular, at the highest pH values where the return to the initial state is slowest.

Based on their data two very close pK_a’s were introduced in [Imamoto, 2004]. The first one of 10.2 was assigned to the I_1'/I_2' equilibrium, as discussed. The second one of 10.4 was assigned to the I_1/I_1' equilibrium. We note that this assignment is inconsistent since the two distinct pK_a values refer to the protonation of the chromophore, i.e. to only one protonable group. However, the finding of two pK_a values requires the involvement of two protonable groups. Consequently, one of the two pK_a values of [Imamoto, 2004] reflects the protonation of a residue that occurs in an equilibrium between I_1 and I_1’, which both have a deprotonated chromophore. In our scheme (III), which can explain both the equilibrium and kinetic data of Figure 3.12, we have only one pH dependent equilibrium between I_1’ and I_2’, and the I_1'/I_1' equilibrium is pH independent. The existence of the I_1’ intermediate and the absence of the I_2 intermediate at alkaline pH are supported by photoreversal measurements as described in section 4.3.
3.3 Conclusions

The kinetics and intermediates of the photocycle and of the photoreversal were measured by transient absorption spectroscopy in the pH range from 4.6 to 11. SVD analysis of the transient absorption time traces measured in a broad wavelength range (330-510 nm) at pH 7 showed the presence of three spectrally distinguishable species: I₁, I₂ and I₂'. Their spectra were determined by using an advanced data analysis technique, the extrapolated difference method. I₂ and I₂' have absorption spectra, with maxima at 370 ± 5 and 350 ± 5 nm, respectively. Formation of the signaling state is thus associated with a change in the environment of the protonated chromophore, consistent with the observation that the formation of I₂' from I₂ is accompanied by a major conformational change. The signaling state of the photoreceptor photoactive yellow protein is the long-lived intermediate I₂'. The pH dependence of the equilibrium between the transient photocycle intermediates I₂ and I₂' was investigated in acid/neutral pH range (pH 4.6 to 8.4). I₁ decays partially to I₂ in the first transition, and I₂' is formed from I₁ and I₂ during the second transition. I₁, I₂ and I₂' are in equilibrium from ~10 ms onwards, and decay together to P in the third transition.

The time courses of the I₁, I₂ and I₂' intermediates were determined from the wavelength dependent transient absorbance changes in the time range from 50 ns to 50 s at each pH. It was assumed that their spectra are pH-independent. The I₁/I₂ equilibrium at ~500μs is formed from I₁ during the first transition, is pH dependent with a pKₐ of 7, and I₁ as the main species at higher pH. The rate constant k₂ corresponding to the second transition also has a pKₐ of ~6.7. The equality of the pKₐ's of the pH dependence of decay rate and the equilibrium concentrations follows, if we assume that the equilibration rates between the intermediates are much faster than the decay rate and that the equilibrium decays through one of the species, e.g. I₁ here, consequently k₂ ~ [I₁]. After the formation of I₂' (~2 ms), the I₁, I₂ and I₂' intermediates are in equilibrium and decay together to the initial dark state. The equilibrium between I₂ and I₂' is pH dependent with a pKₐ of 6.4 and I₂' is the main species above this pKₐ. The rate constant k₃ for the recovery to the initial dark state also has a pKₐ of ~6.3. This equality of the equilibrium and kinetic pKₐ's suggests that k₃ ~ [I₂'].

Since the habitat of Halorhodospira halophila is distinctly alkaline the kinetics and intermediates of the photocycle and photoreversal were investigated further in the alkaline region from pH 8 to 11. SVD analysis of the data at pH 10 shows the presence of three spectrally distinct species I₁, I₁' and I₂'. The spectra of the intermediates I₁, I₁' and I₂' were obtained in two different ways: extrapolated difference method and scaled subtraction...
method. $I'_1$ absorbs maximally at 425 nm. $I'_1$ probably has a deprotonated chromophore and may be regarded as the alkaline form of $I'_2$. At pH 10, the $I_1$ intermediate decays in ~ 330 µs in part to $I'_1$ before $I_1$ and $I'_1$ decay further to $I'_2$ in ~ 1 ms. From the rise of $I'_2$ (~ 1 ms) to the end of the photocycle the three intermediates $I_1$, $I'_1$ and $I'_2$ remain in equilibrium and decay together to P in ~ 830 ms. Assuming that the spectra of $I_1$, $I'_1$ and $I'_2$ are pH independent, their time courses were determined. On the ms to s time scale they are in a pH dependent equilibrium with a $pK_a$ of ~ 9.9. With increasing pH the $I_1$ and $I'_1$ populations increase at the expense of the amount of $I'_2$. The $I'_1/I'_2$ equilibrium is pH-dependent, while the $I_1/I'_1$ equilibrium is pH-independent. The apparent rate constant for the recovery of P slows down with increasing pH with a $pK_a$ of ~ 9.7. This equality of the equilibrium and kinetic $pK_a$’s suggests that $k_3 \sim [I'_2]$ here too. The $pK_a$ of ~ 9.9 is assigned to the deprotonation of the phenol of the surface exposed chromophore in the $I'_1/I'_2$ equilibrium.

The existence of equilibria between transient photocycle intermediates was demonstrated here for the first time using kinetic methods. Spectra for the photocycle intermediates $I_1$, $I'_1$, $I'_2$ and $I'_2$ were obtained from transient absorption data. In previous work such spectra were obtained from steady-state measurements in the presence of background illumination. Over the whole pH range the rate of recovery $k_3$ is proportional to the concentration of the signaling state. The amount of the signaling state is controlled by the pH and is maximal near pH 8.
Chapter 4

Photocycle Kinetics Mechanism Through Photoreversal Kinetics

Photoreceptors with chromophores isomerizable around a specific double bond can be switched back (reset) from the active (signaling) and to the inactive state by light. They are photochromic. They can usually be photoreversed from most intermediates. In our experiments with PYP, the sample is excited with two flashes applied at a defined delay (the wavelengths of the two flashes lie within the absorption band of the photocycle intermediates). The first flash (normally a blue flash, ~430nm) starts the cycle. A second flash applied during the cycle leads to the photoreisomerization and a rapid return to the initial state. In this way, the concentrations of cycling molecules are reduced via photoreversal. The photoreversal kinetics may be time resolved by triggering the data acquisition system on the second flash.

In this chapter, the results of the photoreversal kinetics measured with double flash excitation are presented. It will be shown that a second violet flash at 355 nm or a second green flash at 500 nm have a specific effect, respectively on the I$_2$/I$'_2$ and I$_1$ intermediates. A photocycle mechanism of WT will be proposed based on these observations. The observations presented in this chapter support the photocycle mechanism derived from measurements with single flash excitation discussed in sections 3.1.3 and 3.2.3.

A relation will be presented to determine the pure photoreversal signal from measurements with single and double flash excitation (section 4.1.1). The dependence of the photoreversal signals on the delay between the flashes, measured with a violet flash as the second flash at pH 6, will be presented in section 4.1.2. This provides information about the temporal variation of the populations of the I$_2$/I$'_2$ intermediates. It is observed that the I$_2$/I$'_2$ intermediates are in equilibrium after about ~20 ms during the cycle. Moreover, the pH dependence of the photoreversal kinetics investigated in the pH range 4.6 to 8.4 (section 4.2), indicates that this equilibrium is pH dependent with a pK$_a$ of ~6.1.
The photoreversal intermediates were further characterized spectrally through the wavelength dependent photoreversal signal measured at a particular delay (section 4.1.3). The photoreversal from I$_1$ is efficient, when selectively excited by a second flash at 500 nm (section 4.1.4) applied with $\sim$ µs time delay. The delay and the wavelength dependence of the photoreversal signals measured at pH 10 (section 4.3) with a second flash at 355 nm indicate that the photoreversal kinetics at this pH differs significantly from the kinetics at pH 6.

4.1 Photoreversal at pH 6

4.1.1 Construction of the Photoreversal Signal from I$_2$ / I$_2'$

Typical transient absorption data for the photoreversal reaction from I$_2$ / I$_2'$ at 340 and 450 nm are shown in panels A and B of Figure 4.1. The delay between the two flashes was 10 ms as indicated by the arrows. At this time, most molecules cycling are in the I$_2$ / I$_2'$ intermediate state, Figure 3.4A-C. Panel C of Figure 4.1 shows the absorption spectra of the three states P, I$_1$, and the I$_2$/I$_2'$ mixture with vertical lines marking the wavelengths of the three excitation flashes.

Panels A and B of Figure 4.1 each contain six time traces labeled BFTB$^1$, DFTB$^1$, DFTV$^1$, BFTV$^1$, VFTV$^1$, and PR$^1$ (photoreversal signal). The first five traces are the data. The photoreversal signal was constructed from the traces DFTV, BFTV, and VFTV as follows. The trace BFTB (black) is the normal single flash trace: blue flash (430 nm) triggered on blue flash. The absorbance increase at 340 nm about 300 µs after excitation is due to the formation of I$_2$ (Figure 4.1A). The corresponding kinetic component in the ground-state depletion signal is the absorbance decrease at 450 nm due to the I$_1$ to I$_2$ transition (Figure 4.1B). The photoreversal reaction is evident from the traces labeled DFTB (red) in panels A and B of Figure 4.1. DFTB stands for double flash triggered on the blue flash. At 340 nm (Figure 4.1A) there is a positive spike followed by a large unresolved drop in absorbance at the delay time of 10 ms (marked by arrow). At 450 nm (Figure 4.1B), there is a large unresolved increase in absorbance. Together, these effects are the signature of photoreversal from I$_2$/I$_2'$ to P; the concentration of I$_2$/I$_2'$ (absorbing around 350 nm) drops and simultaneously the concentration of the initial dark state P (absorbing around 450 nm) increases. The kinetics of the photo-back-reaction is clearly fast compared to the I$_2$ to I$_1'$ transition and cannot be resolved by using a logarithmic time base when triggering on the first (blue) flash. The photoreversal from I$_2$ can be resolved, however, by triggering on the second (violet, 355 nm) flash. This is shown in the traces labeled DFTV (green) in panels A and B of Figure 4.1: double flash triggered on
the violet flash. Note that when the trigger is on the second flash, the zero in the linear time basis (origin of the horizontal axis of Figure 4.1A,B) is the second flash.

Figure 4.1 Absorbance changes at 340 nm (A) and 450 nm (B) after single (430 or 355 nm) or double flash excitation [430 nm followed by 355 nm 10 ms later (arrow)]. Each trace is the average of 10 flashes. Note the logarithmic time scale. The double flash traces labeled DFTB and DFTV are triggered on the first flash (430 nm) and on the second flash (355 nm), respectively. If the trigger is on the second flash, that flash sets the zero time point on the horizontal time axis. Single flash signals with either blue (B, 430 nm) or violet (V, 355 nm) excitation and triggered on the first (BFTB) or second flash (BFTV, VFTV) are displayed as well. These are needed to construct the photoreversal signal (PR) according to eq 4.2, with $f_1 = 0.76$ and $f_2 = 0.73$. Conditions: WT, pH 6, 20 °C, 20 mM Tris, and 50 mM KCl. PYP concentration, 64µM. (C) Absorption spectra of the I$_1$/I$_2'$ mixture were calculated from the single flash data of Figure 4.4B as described in the text.

At the time of the second flash, there exists a mixed population of the intermediates I$_1$, I$_2$, and I$_2'$ as well as the remaining population of PYP molecules that were not excited by the first flash, Figure 3.4A-C. The violet flash is able to excite all of these species (see overlapping intermediate spectra in panel C of Figure 4.1 or Figure 3.2A. The spectra of Figure 4.1C were calculated from the data of Figure 4.4B, as described below). Except for the very short delays, the photoreversal from I$_1$ can be ignored (see below). Therefore, we need only to correct for the fraction of PYP molecules that were not excited by the first, blue flash but were excited by the second, violet flash. The traces labeled VFTV, violet flash triggered on the violet flash, show that the excitation of the PYP sample by a single flash, at 355 nm, is quite efficient. Moreover, these traces have the same kinetics as for excitation at 430 nm and contribute in the same time range as the photoreversal changes (compare DFTV with VFTV).
Thus, the double flash signal DFTV has to be corrected for the contribution from those molecules that were not excited by the first, blue flash but by the second, violet flash. To make this correction, we need to know $f_1$, the fraction of the initial PYP population that was not excited by the first flash and remains in the ground state. Since $f_1$ equals 1 minus the probability of cycling induced by the first flash, $f_1$ was determined by measuring the amplitude of the ground-state depletion signal at 450 nm and comparing it with the dark absorption at 450 nm. In this way, typical values for $f_1$ of 0.76 were obtained. This means that, with our laser system, the first flash drove 24% of the molecules through the photocycle. The remaining 76% could be excited by the second, violet flash. The dependence of $f_1$ on the delay is shown in Figure 4.2B. Since no significant thermal relaxation from I$_2$ back to the ground state occurs up to about 10 ms, $f_1$ remains constant up to that time (Figure 4.2B). With delays beyond this value, a growing number of molecules return to the initial state, thus $f_1$ increases to a final value of 1.

![Figure 4.2](image.png)

Figure 4.2 (A) Correction factor $f_2(t)$ calculated according to eq 4.1 for delays of 1 µs, 500 µs, and 10 ms. The plateau values are plotted in panel B for various delays. (B) Correction factors $f_1$ and $f_2$ for various values of the time delay between the flashes. $f_1$ is the fraction of PYP molecules not excited by the first flash. $f_2$ is the fraction of cycling PYP molecules not excited by the second flash.

To obtain the true photoreversal signal, the double flash absorption change also has to be corrected for the contribution from the first flash of those molecules in the cycle that are not excited by the second flash and thus complete the thermal cycle. The trace BFTV (blue flash triggered on violet flash) corresponds to a single blue flash excitation triggered at the time point where the second, violet flash would have occurred in the double flash excitation. This trace has to be weighted by $f_2$, the fraction of cycling PYP molecules not excited by the second flash. $f_2$ was determined in a similar way as $f_1$ from the amplitudes of the depletion signal at 450 nm at times after completion of the photoreversal kinetics. Its calculation is
4.1.1 Construction of the Photoreversal Signal from I$_2$/I$_2'$. 

Based on the assumption, borne out by the experimental data, that the photoreversal kinetics are faster than the thermal decay of I$_2$ to P, $f_2(t)$ is defined as the value of the ratio:

$$f_2(t) = \frac{\Delta A_{\text{DFTV}}(t) - f_1 \Delta A_{\text{VFTV}}(t)}{\Delta A_{\text{BFTV}}(t)}$$

(4.1)

and evaluated at 450 nm.

At times after completion of the photoreversal reaction, the numerator is the amplitude of the depletion signal after photoreversal (DFTV) corrected for the contribution from the molecules that were not excited by the first flash but by the second violet flash (VFTV). The denominator is the amplitude of the depletion signal in the normal single flash photocycle, i.e., in the absence of photoreversal. This ratio is exactly the fraction of molecules cycling that continues along the thermal photocycle after the second flash, i.e., the fraction not excited by the second flash. Using the time traces DFTV, VFTV, and BFTV as well as the value of $f_1$, $f_2(t)$ was calculated according to eq 4.1 as a function of time. In Figure 4.2A, $f_2(t)$ is plotted for delays of 1 µs, 500 µs, and 10 ms. At times after completion of the photoreversal reaction (>~2 ms), $f_2(t)$ reaches a constant time-independent value (see Figure 4.2A). This constant $f_2$ is the fraction required and used in eq 4.2 below. These values depend on the delay (Figure 4.2A) and are plotted in Figure 4.2B. At short delays (<20 µs), $f_2$ is not equal to 1 as one might have expected but to ~0.93. This is due to photoreversal from I$_1$. Evidence for efficient photoreversal from I$_1$ with a second flash at 500 nm will be presented below (section 4.1.4).

We note that, based on the spectrum of I$_1$ in Figure 4.1C, some photoreversal from I$_1$ with excitation at 355 nm is to be expected. Figure 4.2B shows that $f_2$ decreases between delays of 100 µs and 2 ms from about 0.87 to about 0.74. This is due to the growth of the I$_2$/I$_2'$ population in this time interval. The photoreversal signals $\Delta A_{\text{PR}}$ (PR, pink traces) shown in Figure 4.1A,B were obtained from the traces labeled DFTV, VFTV, and BFTV using eq 4.2 and the delay-dependent correction factors $f_1$ and $f_2$ of Figure 4.2B [Druckmann, 1993]:

$$\Delta A_{\text{PR}}(t) = \Delta A_{\text{DFTV}}(t) - f_1 \Delta A_{\text{VFTV}}(t) - f_2 \Delta A_{\text{BFTV}}(t)$$

(4.2)

The photoreversal absorbance signals in Figure 4.1A,B, constructed in this way, are exactly zero after completion of the photoreversal reaction and do not contain any contribution from the single, blue flash photocycle as a consequence of the definition of the correction factor $f_2$. Thus, the $f_2$ correction used here greatly simplifies the analysis of the photoreversal kinetics by removing the unrelated kinetic components from the normal photocycle prior to analysis. Comparing the DFTV and PR traces in panels A and B of Figure 4.1, the effect of corrections is twofold: the $f_1$ correction has removed the kinetic contributions from the photocycle intermediates formed by the second flash, and the $f_2$ correction has
removed the kinetic contribution from those molecules not excited by the second flash but continuing to P along the normal thermal cycle.

4.1.2 Dependence of the Photoreversal Signal on Delay

The experiment shown in Figure 4.1, at a delay of 10 ms, was carried out at 20 additional delays ranging from 1 µs to 3 s, at both 340 and 450 nm. The calculated photoreversal signals, positive at 340 nm and negative at 450 nm, were constructed as described above. Of the corresponding 21 traces, for clarity, only 7 are presented at each wavelength in Figure 4.3A.

We note that a number of these traces cross, indicating that they cannot be fitted simultaneously by a single exponential with a common time constant. For a global fit to all data sets, a sum of two exponentials with comparable amplitudes was required. In Figure 4.3B, is shown, as an example, the residuals at 450 nm for a one-exponential and a two-exponential global fit to the data of Figure 4.3A at a delay of 1 ms. Systematic deviations are apparent in the residuals for the one-exponential fit, while residuals for the two-exponential fit appear to be randomly distributed around zero. The complete set of data at the 2 wavelengths and 21 delays could be fitted with two exponentials with the common time constants $\tau_1 = 57 \pm 5$ µs and $\tau_2 = 380 \pm 40$ µs (marked by the vertical dashed lines in Figure 4.3A). The fits of individual experiments are represented by the solid lines in Figure 4.3A. The corresponding amplitudes $A_1$ and $A_2$ varied with the delay. This dependence is shown in Figure 4.4A.

The amplitudes $A_1$ and $A_2$ for the short and long photoreversal time constants show, apart from the sign and scaling factor, the same dependence on delay at 340 and 450 nm. The four discrete sets of amplitudes ($A_1$ and $A_2$ at two wavelengths) were fitted simultaneously...
with a sum of three exponentials (solid lines in Figure 4.4A). The optimal fit was obtained with time constants of 410 µs, 1.3 ms, and 500 ms identified by dotted vertical lines. The first two time constants, 410 µs and 1.3 ms, are close together and to the eye do not appear to provide a good description of the rise and decay of the $A_1$ amplitudes. This is a consequence of the partial cancellation of the positive and negative amplitudes associated with these time constants. Since the number of delay values is small, the errors in these time constants are large. The absolute value $|A_1|$ rises with a time constant of 410 µs. $|A_1|$ then decreases (1.3 ms), and in parallel, as expected for a sequential reaction, the amplitude $|A_2|$ rises. The $A_1$ amplitudes do not decay to zero, however, but reach a constant value after about 5 ms. Finally, $|A_1|$ and $|A_2|$ decay together with a time constant of 500 ms. We note that the positive (340 nm) and negative (450 nm) amplitudes are to a very good approximation scaled mirror images.

![Figure 4.4 A](image1.png)

**Figure 4.4 (A)** Dependence of the amplitudes $A_1$ and $A_2$ on the delay. $A_1$ and $A_2$ are the amplitudes of the fast (57 µs) and slow (380 µs) photoreversal components, respectively. The two positive amplitudes with filled triangles ($A_1$) and squares ($A_2$) are at 340 nm; the two negative amplitudes with open triangles ($A_1$) and squares ($A_2$) are at 450 nm. The dashed vertical lines at 410 µs, 1.3 ms, and 500 ms indicate the values of the exponential time constants for a global fit with three exponentials. (B) Single flash (430 nm) absorbance changes for the same sample at 26 wavelengths varying from 330 to 510 nm. For clarity, only the traces at the indicated wavelengths are shown. The vertical dashed lines indicate the positions of the time constants for a global fit to all of the data with a sum of three exponentials; $\tau_1 = 370$ µs is the rise time of $I_2$, $\tau_2 = 3$ ms is the rise time of the $I_2/I_2'$ equilibrium, and $\tau_3 = 400$ ms is the decay time of $I_2/I_2'$. The solid lines are the fits.

Figure 4.4B shows for comparison the single flash (430 nm) kinetics at 6 selected wavelengths out of 26 collected from 330 to 510 nm. As it will be shown below, the $I_2$ and $I_2'$ intermediates are in a thermal equilibrium after 3 ms. The model-dependent intermediate spectra for $I_1$ and the $I_2/I_2'$ mixture, shown in Figure 4.1C, were calculated from these data in the following way. It was assumed that the cycle is sequential and unidirectional with only $I_1$
present at 10 µs and only the I₂/I₂' equilibrium mixture at 10 ms. Moreover, it was assumed that the I₂ and I₂' intermediates do not absorb at 446 nm. Using these assumptions, the spectrum of the I₂/I₂' equilibrium was determined by adding, to the difference spectrum at 10 ms [spectrum labeled (*) in Figure 4.5B, calculated from Figure 4.4B], a scaled amount of the ground-state spectrum to make the absorbance around 450 nm zero. The spectrum of I₁ was then calculated from the difference spectrum at 10 µs (from Figure 4.4B) by adding this same amount of the ground-state spectrum.

The complete 26-wavelength data set of Figure 4.4B was subjected to a global fit with three exponentials (solid lines). The three vertical dotted lines indicate the values of the three exponential time constants: 370 µs, 3 ms, and 400 ms. These times correspond to the rise of I₂, the rise of I₁/I₂/I₂' equilibrium, and the return to the initial dark state, Figure 3.2C (blue curve). The presence of the 3 ms component (I₂/I₂' equilibrium) can be clearly discerned in the data in Figure 4.4B (e.g., in the 390 nm trace). The reasonable agreement between these three time constants for I₂ and I₂' (Figure 4.4B) and the corresponding three values of 410 µs, 1.3 ms, and 500 ms for A₁ and A₂ (in Figure 4.4A) together with the dependence of A₁ and A₂ on the delay suggest the following interpretation of the photoreversal kinetics data in terms of a sequential model (Figure 3.6, inner part of the photocycle model). The time constant of 57 µs is assigned to the photoreversal reaction from I₂ (I₂\textsuperscript{cis}). The very rapid photoisomerization from I₂\textsuperscript{cis} to I₂\textsuperscript{trans} is not resolved but leads to the initial positive absorbance change at 340 nm (see Figure 4.1A, difference between DFTV and BFTV at time zero and the positive spike at 10 ms in the DFTB trace). Between I₂\textsuperscript{trans} and P\textsuperscript{trans}, the chromophore deprotonates and returns to the binding pocket with a time constant of 57 µs. The time constant of 380 µs is assigned to the photoreversal reaction from I₂' (I₂\textsuperscript{cis}). After the rapid unresolved photoisomerization to I₂\textsuperscript{trans}, the chromophore has to be deprotonated and the global conformational change has to be reversed. In the sequential model, the delay dependence of A₁ and A₂ should reflect the time courses of the concentrations of I₂ and I₂', respectively. In agreement with this model, A₁ dominates at small delays, and A₂ rises concomitantly with the first decay phase of A₁. The rise time of A₁ (410 µs) agrees well with the rise time of I₂ (370 µs). The rise time of A₂ and the decay time of A₁ (1.3 ms) is only slightly ahead of the rise time of I₂' (3 ms). The common decay time of A₁ and A₂ (500 ms) agrees well with the decay time of I₂' (400 ms). In these respects, the unidirectional sequential model provides a good description for the observations and is summarized in Figure 3.6. On the other hand, if the transition between I₂ and I₂' were unidirectional, the amplitude of A₁ should go to zero in the 1.3 ms decay. This is, however, clearly not the case. The fact that A₁ remains constant after 5 ms at approximately 50% of its
maximal value and finally decays to zero in a second decay phase (500 ms) together with \( A_2 \) is direct evidence for the existence of an equilibrium between \( I_2 \) and \( I_2' \). \( I_2 \) and \( I_2' \) coexist after 5 ms and decay together. The amplitude data clearly suggest a thermal back-reaction from \( I_2' \) to \( I_2 \). Thus, protein rearrangement must be faster than decay of \( I_2' \) and \( I_2 \) (500 ms), perhaps as fast as 400 \( \mu \)s.

Moreover, the delay dependence of the \( A_1 \) amplitude of Figure 4.4A is comparable to the \( I_2 \) time course of Figure 3.4B (blue curve). This shows that the 57 \( \mu \)s component is due to the photoreversal from \( I_2 \). Similarly, the delay dependence of the \( A_2 \) amplitude of Figure 4.4A is very similar to the \( I_2' \) time course of Figure 3.4C (blue curve). This shows that the 380 \( \mu \)s component is due to the photoreversal from \( I_2' \).

4.1.3 Wavelength Dependence of the Photoreversal Signal at the Fixed Delays of 1 and 10 ms

To characterize the two photoreversal components with reversal time constants of 57 \( \mu \)s and 380 \( \mu \)s spectrally, the wavelength dependence of the photoreversal signal was investigated at delays of 1 and 10 ms. At a delay of 1 ms \( A_1 \) and \( A_2 \) have comparable magnitude, whereas at a delay of 10 ms \( A_2 \) dominates (see Figure 4.4A). Data were collected at 26 wavelengths from 330 to 510 nm. The photoreversal signals were constructed as described earlier (see panels A and B of Figure 4.1 for examples at 340 and 450 nm at a 10 ms delay). Typical data for the delay of 1 ms and at seven selected wavelengths are displayed in Figure 4.5A together with their global two-exponential fits.

The two common exponential time constants for this independent data set (at 26 wavelengths), at a delay of 1 ms, were 66 \( \pm \) 5 and 410 \( \pm \) 40 \( \mu \)s. For the data set at the delay of 10 ms (data not shown), the two time constants were 51 and 390 \( \mu \)s. The rounded average values of 59 and 400 \( \mu \)s are marked by the dotted vertical lines (Figure 4.5A) and are in excellent agreement with the values of 57 and 380 \( \mu \)s determined from the delay data at the two wavelengths 340 and 450 nm (Figure 4.3A). The amplitude spectra \( A_2 \) (\( \circ \) at 10 ms delay, \( \blacksquare \) at 1 ms delay) and \( A_1 \) (\( \triangle \) at 1 ms delay) are plotted in Figure 4.5B together with the single flash difference spectrum at 10 ms (*) and the inverted ground-state spectrum (solid line).

Recall that the \( f_1 \) and \( f_2 \) corrections removed all contributions of the normal photocycle from the double flash signal. The photoreversal signal (PR) thus represents only the absorbance change due to photoreversal. In the previous section on the delay dependence, it was argued that a sequential model provides a good description of the photoreversal kinetics:
I\textsubscript{2}\textsuperscript{cis} is photoreversed with the single decay time \(\tau_1\) and I\textsubscript{2}\textsuperscript{cis} with the longer single decay time \(\tau_2\). We detected no further intermediates between I\textsubscript{2}\textsuperscript{trans} and P nor between I\textsubscript{2}\textsuperscript{trans} and P. The corresponding amplitude spectra \(A_1(\lambda)\) and \(A_2(\lambda)\) for such a two-state sequential model (I\textsubscript{2}\textsuperscript{trans} to P and I\textsubscript{2}\textsuperscript{trans} to P) are then equal to the difference spectra between the two states (I\textsubscript{2}\textsuperscript{trans}/P and I\textsubscript{2}\textsuperscript{trans}/P, respectively). The spectra of I\textsubscript{2}\textsuperscript{trans} and I\textsubscript{2}\textsuperscript{trans} may thus be constructed from the \(A_1\) and \(A_2\) amplitude spectra in a simple way. This is illustrated for the \(A_2\) amplitude spectrum at 10 ms in Figure 4.5B,C.

Figure 4.5 (A) Photoreversal signals at seven wavelengths between 330 and 510 nm at the fixed delay of 1 ms. For clarity, similar data at 19 additional wavelengths are not shown. The solid lines are the global fits with a sum of two exponentials with the common time constants \(\tau_1 = 66 \, \mu s\) and \(\tau_2 = 413 \, \mu s\). A second data set (not shown) was acquired at a delay of 10 ms with global fit parameters \(\tau_1 = 51 \, \mu s\) and \(\tau_2 = 391 \, \mu s\). The average \(\tau\)'s are marked by the vertical lines. (B) Amplitude spectra \(A_1(\lambda)\) and \(A_2(\lambda)\) associated with \(\tau_1\) and \(\tau_2\) obtained from the global fit of (A): (○) \(A_2\) for the 10 ms delay; (■) \(A_2\) for the 1 ms delay; (∆) \(A_1\) for the 1 ms delay. For comparison, the absorbance difference spectrum for the single flash experiment at 10 ms after the flash is shown (*, obtained from the data of Figure 4.4B). The \(A_2\) spectrum at 10 ms (○) and the difference spectrum (*) are scaled at 450 nm to the spectrum of the dark state P (solid line). (C) Absorption spectra of I\textsubscript{2}\textsuperscript{cis} (○), I\textsubscript{2}\textsuperscript{trans} (○) at 10 ms delay, ■ at 1 ms delay, and I\textsubscript{2}\textsuperscript{trans} (∆) calculated from (B).

First \(A_2\) is scaled in such a way that it matches the inverted ground-state spectrum (P) at 450 nm (○). Figure 4.5B shows that the fit of the scaled \(A_2\) spectrum to P is quite good in the wavelength range from 410 to 510 nm. This supports the assumption that, at least in this wavelength range, no states other than P contribute. By adding the ground-state spectrum to the scaled \(A_2\) spectrum, the absorbance in the 410-510 nm range is now reduced to zero and
4.1.4 Photoreversal from I₂ by a Green Flash (500nm)

the I₂\textsuperscript{trans} spectrum is generated (○ in Figure 4.5C). The same procedure was applied to the $A_2(\lambda)$ amplitude spectrum at 1 ms (■ in Figure 4.5B,C). The corresponding I₂\textsuperscript{trans} spectrum is within experimental error the same as that derived from $A_2$ at 10 ms, as it should be. In the same way the I₂\textsuperscript{trans} spectrum (△) was generated from the $A_1(\lambda)$ amplitude spectrum. In agreement with the observation of an instantaneous absorbance increase around 350 nm after the second flash (arrow in Figure 4.1A), the I₂\textsuperscript{trans} and I₂\textsuperscript{cis} intermediates have considerably higher extinction coefficients than I₂\textsuperscript{cis} (Figure 4.5C). Note that the extinction coefficient is not exactly zero above 410 nm but assumes small positive or negative values. This is due to experimental error in the determination of $A_1$ and $A_2$ at individual wavelengths. These errors are of the same magnitude as the differences between the two spectra for the I₂\textsuperscript{trans} intermediate derived from $A_2$ at 10 and 1 ms in the UV region.

At first sight, the spectrum for I₂\textsuperscript{trans} (△) appears to be anomalous due to the minimum at 361 nm. We recall that $A_1$, from which this spectrum is derived, is the amplitude of the fastest photoreversal component and therefore more affected by the flash. The absorption traces at the five wavelengths 349, 353, 355, 358, and 361 nm, closest to the excitation wavelength of 355 nm, are most affected by the flash artifact which leads to an artificially lower absorbance at these five wavelengths. Leaving these five wavelength traces out of the data analysis leads to the same absorbance at the wavelengths at or below 345 nm and at wavelengths at or above 365 nm. We conclude that the apparent minimum at 361 nm is due to the flash artifact. We may therefore conclude that the spectrum of I₂\textsuperscript{trans} is blue shifted with respect to the spectrum of I₂\textsuperscript{trans} by ~10 nm but has approximately the same maximal extinction coefficient. This blue shift is also apparent from the data points above 365 nm in Figure 4.5C, which are not affected by the flash artifact. From these spectra we estimate $\lambda_{\text{max}}$ values of approximately 345 and 355 nm for I₂\textsuperscript{trans} and I₂\textsuperscript{trans}, respectively.

4.1.4 Photoreversal from I₁ by a Green Flash (500 nm)

From the spectrum of I₁ in Figure 4.1C, it is clear that selective photoreversal from I₁ should be feasible with a second flash around 500 nm. Figure 4.6 shows double flash data at measuring wavelengths of 360 and 450 nm with a first flash, at 430 nm (blue), followed by a second flash, at 500 nm (green), after a delay of 20 µs. At 20 µs, almost all molecules cycling are in I₁ as shown in Figure 3.4A-C. As expected, the green flash alone (GFTG\textsuperscript{1}, green flash data acquisition triggered on green flash) leads to very small effects due to the low absorbance of P at 500 nm. The photoreversal effect from I₁ can be most clearly discerned in the depletion signal (Figure 4.6B, 450 nm), since at this wavelength both I₁ and the initial dark
state P absorb strongly, with the extinction coefficient of P being much larger than that of I$_1$ at this wavelength (Figure 4.1C).

At 20 µs, a large positive absorbance change is observed (DFTB; the negative spike is the flash artifact), corresponding to the I$_1$ to P transition. This instantaneous positive absorbance step is not resolved when triggering on the first flash (DFTB). Triggering on the second flash (DFTG, not shown) also did not resolve this absorbance change. Thus, we can only conclude that photoreversal from I$_1$ is faster than 1 µs. The reduction in the number of cycling molecules at 20 µs by the 500 nm flash leads to a corresponding reduction in the amplitude of the depletion signal at 10 ms, since fewer I$_2$ molecules are formed.

![Figure 4.6](image)

Figure 4.6 Photoreversal from I$_1$. The first flash (at 430 nm) was followed after 20 µs by a second flash (at 500 nm). Conditions: pH 6, 20 °C, 20 mM Tris, and 50 mM KCl. (A) Absorbance change at 360 nm. The trace labeled BFTB is the response after a blue single flash. DFTB labels the double flash response triggered on the first, blue flash. The trace labeled GFTG is the response after a single green flash. (B) Absorbance change at 450 nm. The meaning of the labels is as in (A). The positive unresolved absorbance change at 20 µs is due to about 18% photoreversal from I$_1$ to the initial dark state P.

In Figure 4.6A (360 nm), no absorbance change is observed at 20 µs (apart from the negative spike), since the extinction coefficients of I$_1$ and P are quite small and similar at this wavelength (see Figure 4.1C). At 10 ms after the flash, the double flash amplitude (DFTB) is reduced by ~18% with respect to the single flash amplitude (BFTB). This reduction factor is the same as for the depletion signal at 10 ms and is due to the reduction in the population of I$_2$ caused by the photoreversal of the preceding intermediate I$_1$. Additional experiments were carried out at delays of 300 ns, 10 µs, and 10 ms (data not shown). At 300 ns and 10 µs, the amplitude of the photoreversal signal from I$_1$ was about the same as at 20 µs. This is as expected since I$_1$ has a rise time of about 3 ns and decays to I$_2$ in 300 µs, Figure 3.4A. At a delay of 10 ms, photoreversal from I$_1$ could not be detected, since, at this delay, I$_1$ contribution is below detection limit in photoreversal signal. Photoreversal from I$_1^{cis}$ is represented in Figure 3.6.
4.2 pH Dependence of the Photoreversal Kinetics at Acid and Neutral pH

The I$_2$ and I$_2'$ intermediates are in a pH dependent equilibrium from about $\sim$ 20 ms until the end of the cycle as shown by Figure 3.4 B,C. This pH dependence was further investigated in double flash experiments with a violet flash (355 nm, selective for I$_2$/I$_2'$; I$_1$ absorbs to a much smaller extent at this wavelength (see Figure 3.2A)) as the second flash at a delay of 20 ms (maximum of I$_2$/I$_2'$ population during cycle, Figure 3.4B,C). The photoreversal kinetics was measured at only two wavelengths (340 and 450 nm) and at the following fifteen-pH values: 4.6, 4.8, 5.1, 5.4, 5.7, 6.0, 6.3, 6.6, 6.75, 6.9, 7.35, 7.7, 7.9, 8.1, and 8.4.

Figure 4.7: (A) Photoreversal signals, at 340 nm (positive) and 450 nm (negative) at pH 5.1 (black) and 8.1 (red), calculated using eq 4.2. For clarity, the data obtained at 13 other pH values ranging from 4.6 to 8.4 are not shown. The solid curves represent a simultaneous exponential fit to the 340 nm and 450 nm traces. Conditions: 20°C, 50 mM KCl and 50 mM MES, PYP concentration: 53 $\mu$M. The delay between first (blue, 430nm) and second (violet, 355nm) flashes is 20 ms. (B), (C) pH dependence of the photoreversal amplitudes A$_1$ and A$_2$ at 340nm (B) and 450nm (C). A$_1$(•) and A$_2$(○) are the amplitudes of the fast (48 to 60 $\mu$s) and slow (330 to 770 $\mu$s) components, respectively, obtained from the simultaneous fit of the 340 nm and 450 nm traces of panel A. The solid curves of panels B and C represent a common fit to the pH dependence of all four amplitudes with the Henderson-Hasselbalch equation, with a pK$_a$ of 6.1 (dash vertical lines) and a Hill coefficient of 1.9.

The photoreversal signals at pH 5.1 and 8.1 are shown in Figure 4.7A. For clarity, only the traces at two of the fifteen pH values are shown together with their simultaneous fits (solid lines). As at pH 6 (Figure 4.5A), the kinetics required two exponentials over the pH range from 4.6 to 6.9. These two phases can be clearly discerneed in the data at pH 5.1. From pH 7.3 on, only the slow component was required. The fast time constant was virtually pH-independent, varying between 48 and 63 $\mu$s. The second time constant varied between 330
and 770 µs in the pH range investigated. Comparing the time traces in Figure 4.7A, it is clear that there are significant differences between pH 5.1 and 8.1. The total initial photoreversal signal is somewhat larger at pH 5.1 than at 8.1, suggesting that more I$_2$/I$_2'$ can be photoreversed.

The amplitude data provide further insight. The pH dependence of the amplitudes A$_1$ and A$_2$ (for the corresponding time constants $\tau_1$ and $\tau_2$) are plotted in Figure 4.7B (340 nm) and C (450 nm). These figures confirm that, with increasing pH, A$_1$ becomes smaller approaching zero around pH 7 at both wavelengths, whereas A$_2$ shows a corresponding increase. The solid curves are simultaneous fits of the amplitudes at 340 and 450 nm with the Henderson-Hasselbalch equation, with $pK_a = 6.1$ and $n = 1.9$. The amplitudes A$_1$ and A$_2$ were assigned to photoreversal from I$_2$ and I$_2'$, respectively (Figure 4.4A). The results of Figure 4.7B and C thus suggest that, with increasing pH, the I$_2$/I$_2'$ equilibrium shifts from I$_2$ at pH 4.6 to I$_2'$ at pH 8.4. These results, from the pH dependence of the photoreversal amplitudes, thus support the observations from the photocycle as described in Figure 3.5C, where a pK$_a$ of 6.4 was obtained.

### 4.3 Photoreversal from I$_2'$ at pH 10, dependence on delay and wavelength

I$_1$, I$_1'$ and I$_2'$ contribute to the photocycle at pH 10 and are in equilibrium beyond about ~3 ms during photocycle, Figure 3.8C. The delay dependence of the photoreversal kinetics was investigated at this pH with a violet flash (355 nm, selective for I$_2'$; I$_1'$ and I$_1$ absorb to a much smaller extent at this wavelength (see Figure 3.8A)) after a variable time delay. The pure photoreversal signal was calculated from the DFTV, VFTV and BFTV and the $f_1$ and $f_2$ factors using eq 4.2.

The photoreversal signals were measured at 365 and 450 nm at 19 delays ranging from 500 ns to 5 s. A selection of 6 of these traces at each wavelength is shown in Figure 4.8A. The solid lines are from a two-exponential global fit to the data at all 19 delays. The data clearly show the presence of two phases with exponential decay constants of $\tau_1 = 60$ µs and $\tau_2 = 3.5$ ms, identified by the dashed vertical lines.

The corresponding amplitudes A$_1$ (60 µs component) and A$_2$ (3.5 ms) are plotted as a function of the delay in Figure 4.8B. The positive amplitudes refer to the photoreversal signal at 365 nm, the negative amplitudes to the signal at 450 nm. The solid lines in Figure 4.8B are simultaneous fits to the delay dependence of the amplitudes with a sum of two exponentials. The optimal fit was obtained with exponential time constants of 1.1 ms (rise) and 680 ms (decay). The 1.1 ms rise time corresponds closely with the 1 ms rise time of I$_2'$ (Figure 3.8C).
4.3 Photoreversal from I\textsuperscript{2}′ at pH 10: Dependence on Delay and Wavelength

In view of the small number of delay times, the 680 ms decay time agrees well with the 830 ms decay time of I\textsuperscript{2}′ (Figure 3.8C).

**Figure 4.8:** (A) Photoreversal signals, at 365 nm and 450 nm at six delays ranging from 500 μs to 1 s (first flash at 430 nm, second flash at 355 nm). For clarity, the data at 13 other delays are not shown. The solid lines represent a two-exponential global fit to all of the data with \( \tau_1 = 60 \) μs and \( \tau_2 = 3.5 \) ms marked by the dashed vertical lines. Conditions: pH 10, 20 °C, 50 mM KCl and 20 mM Tris. PYP concentration: 58 μM. (B) Dependence of the photoreversal amplitudes \( A_1 \) and \( A_2 \) on the delay. \( A_1 \) and \( A_2 \) are the amplitudes of the fast (60 μs) and slow (3.5 ms) components, respectively, obtained from the global fit of the delay data of panel A. The two positive amplitudes with open squares (\( A_1 \)) and circles (\( A_2 \)) are for the data at 365 nm; the two negative amplitudes with filled squares (\( A_1 \)) and circles (\( A_2 \)) are for the data at 450 nm. The dashed lines at 1.1 ms and 680 ms indicate the values of the time constants for a global fit with two exponentials. The solid curves represent the fit. (C) Photoreversal signals at seven wavelengths between 330 and 510 nm at the fixed delay of 10 ms. For clarity, similar data at 23 additional wavelengths are not shown. The solid lines are the global fits with a sum of two exponentials with the common time constants \( \tau_1 = 60 \) μs and \( \tau_2 = 2.3 \) ms, marked by the vertical dashed lines. (D) Amplitude spectra \( B_1(\lambda) \) and \( B_2(\lambda) \) associated with \( \tau_1 \) and \( \tau_2 \) obtained from the global fit of (B): \( B_1 \) (close red circles), \( B_2 \) ( ), sum of \( B_1 \) and \( B_2 \) (open red squares) and down scaled \( I_2^{\text{exc}} - P \) difference spectrum (blue open triangles). The solid curve is a scaled P spectrum.

The delay dependence of \( A_1 \) and \( A_2 \) in Figure 4.8B thus closely matches the time course of the \( I_2^1 \) intermediate (Figure 3.8C). The slow ms component \( A_2 \) has a much smaller amplitude than \( A_1 \) (about 40%).
To characterize the spectral intermediates involved in photoreversal, its kinetics were measured at 30 wavelengths from 330 to 510 nm at the fixed delay of 10 ms (where the photoreversal amplitude is maximal, Figure 4.8B). The time traces at seven selected wavelengths are shown in Figure 4.8C. The solid lines are from a simultaneous fit to all 30 traces with two exponentials. The two exponential time constants are $\tau_1 = 60 \mu s$ and $\tau_2 = 2.3$ ms and are identified by vertical dashed lines in Figure 4.8C. The agreement with the delay data (panel A) is excellent. The corresponding amplitude spectra $B_1(\lambda)$ (red balls) and $B_2(\lambda)$ (■) associated with these two exponentials are plotted in Figure 4.8D. The sum of $B_1(\lambda)$ and $B_2(\lambda)$ (open red squares) is also plotted. This sum equals the initial absorbance change after the second flash with respect to the initial dark state P.

The fact that both $A_1$ and $A_2$ have the same delay dependence and rise and decay in parallel with $I_2^c$, suggests that at alkaline pH photoreversal proceeds via $I_2^{cis}$ under our experimental conditions. From Figure 3.8A we note that the extinction coefficient of $I_1^c$ at 355 nm is only about a fourth of that of $I_2^c$. According to Figure 3.8C, the relative concentration of $I_1^c$ is only about half that of $I_2^c$ at the delay of 10 ms. Together these factors lead to an expected photoreversal signal from $I_1^c$ below the detection limit. Similar arguments hold for $I_1$. As shown in Figure 4.8D, the initial absorbance change (open red squares) fits reasonably well with the scaled ($I_2^{cis} - P$) difference spectrum (blue open triangles, Figure 4.5B) for wavelengths larger than 440 nm. Below 440 nm however, significant systematic deviations are apparent. Around 345 nm, this difference is presumably due to the higher extinction coefficient of $I_2^{trans}$ with respect to $I_2^{cis}$, Figure 4.5C. The positive discrepancy in the range 390 to 440 nm is most likely due to a contribution from the $I_1^c$ intermediate. This suggests that the second flash initially produces $I_2^{trans}$ from which some $I_1^{trans}$ is generated before the start of the data acquisition. This $I_1^c$ contribution could arise from rapid equilibrium with $I_2^{trans}$ after reisomerization. Comparison of $B_1(\lambda)$ with the scaled spectrum of P (Figure 4.8D) indicates that, in the fast component, an $I_2$-like intermediate ($I_2^{trans}$) decays and an $I_1$-like intermediate rises (negative mismatch near 490 nm). This transition is probably associated with proton release from the chromophore. The fact that at pH 10 it is accelerated (60 $\mu s$) with respect to pH 6 (380 $\mu s$) is consistent with faster deprotonation at the higher pH. The $B_2(\lambda)$ spectrum shows that in the subsequent slow transition the $I_1^{trans}/I_1^{trans}$ equilibrium decays to P. This is supported by the positive contributions to $B_2$ around 390 nm ($I_1^c$) and 480 nm ($I_2^c$). The photoreversal transition from $I_2^{cis}$ to P consists of three sequential steps: rapid unresolved isomerization of $I_2^{cis}$ and formation of an $I_2^{trans}/I_1^{trans}$ equilibrium, the 60 $\mu s$ transition from
Figure 3.13. Comparing these results at pH 10 (Figure 4.8A) with previous results at pH 6 (Figure 4.5A), striking differences are apparent. At pH 10, the two time constants are more widely separated and $\tau_2$ is increased by a factor of about ten (58 and 380 µs at pH 6; 60 µs and 3.5 ms at pH 10). The dependence of both amplitudes $A_1$ and $A_2$ on the delay is the same at pH 10 and requires only two exponential time constants 1.1 ms and 680 ms, which closely match the rise and decay times of $I_2'$, Figure 3.8C. At pH 6, the delay dependencies of $A_1$ and $A_2$ were very different, requiring three exponentials and suggesting photoreversal from sequential $I_2$ and $I_2'$ intermediates, Figure 4.4A. Moreover they showed that $I_2$ and $I_2'$ are in equilibrium. In contrast to the case at pH 6, the slow ms component $A_2$ has a smaller amplitude than $A_1$ (about 40%). Whereas both amplitude spectra at pH 6 had only contributions in the 330 – 380 nm region from $I_2$ and $I_2'$ intermediates, at pH 10 $B_1$ has a spectrum corresponding to $I_2$ or $I_2'$, but $B_2$ has contributions from $I_1'$ and $I_1$. The data at pH 10 provide no evidence for a sequential $I_2$ to $I_2'$ step nor for an $I_2/I_2'$ equilibrium. It is concluded that at pH 10 very little or no $I_2$ remains. At this pH there is only one intermediate with a protonated chromophore, $I_2'$, and $I_1'$ and $I_2'$ are in equilibrium.

Using a second green flash (495 nm) and a delay of 20 µs, efficient photoreversal from $I_1$ at pH 10 is detected, with a time constant for the return to P of less than 1 µs (data not shown). This result is the same as that obtained at pH 6 (Figure 4.6). The photoreversal from $I_1$ is represented in Figure 3.13.

### 4.4 Discussion

From measurements of the photoreversal kinetics of PYP, the following results were obtained: 1) $I_2$ and $I_2'$ can be distinguished from their corresponding photoreversal time constants, 2) the delay dependence of the photoreversal amplitudes indicate that $I_2$ and $I_2'$ are in equilibrium beyond ~10 ms, 3) this equilibrium is pH dependent with $pK_a \sim 6.1$, as determined from the pH dependence of the photoreversal amplitudes, 4) $I_2^{\text{trans}}$ and $I_2'^{\text{trans}}$ have higher extinction coefficients than $I_2^{\text{cis}}$ and $I_2'^{\text{cis}}$ respectively, and cis to trans photoisomerization is faster than 50 ns, 5) efficient photoback reaction from $I_1$ is feasible with a green flash, 6) no $I_2$ is detected at pH 10.
Using double flash excitation with a variable time delay between the blue (430 nm) and violet (355 nm) flashes, the kinetics of the photoreversal from the I$_2^{cis}$ and I$_2^{cis}'$ intermediates to the initial dark state P were investigated. Figure 3.6 summarizes key aspects of the PYP photocycle and the integration of the photoreversal processes into the overall photocycle in the acid/neutral pH range. Photoisomerization to the I$_2^{trans}$ and I$_2^{trans}'$ intermediates is the first step in photoreversal and is expected to be very rapid (on the order of a few picoseconds, [Heyne, 2005]). We could not resolve these isomerization reactions, but we detected them by the instantaneous positive absorbance change in the 350 nm range immediately after the second flash (positive spike in trace DFTB and positive difference between traces DFTV and BFTV at time zero in Figure 4.1A). This initial cis-trans isomerization has been observed in previous single flash studies with a photo-steady-state mixture of I$_2$/I$_2'$ and P but could not be resolved either [Hendriks, 1999]. We note that our experiments do not provide definitive evidence that the initial unresolved absorbance is due to isomerization. This is the most likely explanation, however. In all photoreceptors studied to date, photoisomerization occurred in the excited state and was the first event after excitation. Indeed, light-induced isomerization is time-resolved with femtosecond IR spectroscopy [Heyne, 2005], and is found to occur in 3 ps, Figure 1.6.

Using a logarithmic time base and triggering on the second flash, we could time-resolve the slower dark phases of the photoreversal kinetics of I$_2$ and I$_2'$. From the data analysis of photoreversal signals, we obtained two exponential kinetic processes with time constants $\tau_1 = 57 \mu$s and $\tau_2 = 380 \mu$s (from the delay dependence) and $\tau_1 = 59 \mu$s and $\tau_2 = 400 \mu$s (from the wavelength dependence), which can be attributed to the dark phases of the photoreversal reactions of the I$_2$ and I$_2'$ intermediates, respectively. In a sequential model, the delay dependence of the amplitudes $A_1$ and $A_2$, for the dark phases of the photoreversal reactions, should then reflect the time course of the I$_2$ and I$_2'$ populations. Comparison of the corresponding time constants showed good agreement, except possibly for the rise of $A_2$ (1.3 versus 3 ms for the rise of I$_2'$). An exact agreement is not to be expected in view of the large errors in the time constants for the delay dependence of $A_1$ and $A_2$. This is due to the limited number of delay times (21 delay times between 1 $\mu$s and 3 s; see Figure 4.4A). Fitting these few data points over six decades of time with three exponentials necessarily leads to considerable errors. The fact that $A_2$ rises simultaneously with the decay of $A_1$ provides strong evidence for the sequential model.

In a unidirectional sequential model, the $A_1$ amplitude should decay to zero in parallel with the rise of $A_2$. Instead, $A_1$ drops to only about 50% of its maximal value around 5 ms,
remains constant, and finally decays together with $A_2$ to zero in 500 ms. This suggests the presence of a thermal back-reaction from $I'_2$ to $I_2$ and the corresponding existence of an $I_2/I'_2$ equilibrium (Figure 3.6). Since chemical reactions are generally reversible, a strictly unidirectional photocycle is unlikely to be correct. In fact, evidence for a pH-dependent equilibrium between the $I_1$ and $I_2$ intermediates in wild type and in the mutant E46Q was recently presented [Borucki, 2003], [Imamoto, 2004].

The photoreversal method provides a powerful tool to prove directly the existence of thermal back-reactions. Suppose the intermediate $I_n$ is photoreversed and the delay is set such that both the $I_n$ and $I_{n+1}$ states are populated. The second flash, which selectively excites $I_n$, leads to an instantaneous decrease in the concentration of $I_n$. If a back-reaction with $I_{n+1}$ exists, the concentration of $I_n$ will increase again by chemical relaxation of the $I_n/I_{n+1}$ equilibrium. This allows a determination of the rate of the back-reaction. This idea was successfully applied to the equilibrium between the M and N intermediates of bacteriorhodopsin using the photoreversal from M [Druckmann, 1993] and should find application to PYP as well. Since the absorption spectra of $I_2$ and $I'_2$ are very similar, the 355 nm flash used here cannot perturb the postulated $I_2/I'_2$ equilibrium. Application to the $I_1/I_2$ equilibrium is more promising since these intermediates have very different absorption spectra, allowing selective excitation.

Comparing the spectra of $I_2^{\text{trans}}$ and $I'_2^{\text{trans}}$ in Figure 4.5C with those of $I_2^{\text{cis}}$ and $I'_2^{\text{cis}}$, we conclude that the extinction coefficient of $I'_2$ is much higher in the $\text{trans}$ than in the $\text{cis}$ form. This result confirms the experimental observation of Figure 4.1A that, at 340 nm (and neighboring UV wavelengths), there is a large instantaneous and unresolved increase in absorbance due to the $\text{cis}$-$\text{trans}$ isomerization (difference between DFTV and BFTV traces at 1 µs). The origin of this difference in oscillator strength between the $\text{trans}$ and $\text{cis}$ forms of the protonated chromophore is not well understood, but the effect is large and seems to be common. For example, in the mutant E46Q, the dark $\text{trans}$ form of the chromophore also has a much larger extinction coefficient around 350 nm than the $I_2^{\text{cis}}$ form in the normal photocycle [Borucki, 2003].

The absorption maxima of $I_2^{\text{cis}}$ ($\sim 370$ nm) and $I'_2^{\text{cis}}$ ($\sim 350$ nm) differ by about 20 nm as shown in Figure 3.2A. Our analysis of the $A_1$ and $A_2$ amplitude spectra, resulting in intermediate spectra of $I_2^{\text{trans}}$ ($\lambda_{\text{max}} \sim 345$ nm) and $I'_2^{\text{trans}}$ ($\lambda_{\text{max}} \sim 355$ nm) of Figure 4.5C, shows that these spectra also differ. The spectrum of $I_2^{\text{trans}}$ is blue shifted with respect to that of $I'_2^{\text{trans}}$ by roughly 10 nm. These observations are consistent with the view that the chromophore environment differs in the $I_2$ and $I'_2$ states. Apart from the value of the
extinction coefficient at corresponding $\lambda_{\text{max}}$ values, the $I_2^{\text{trans}}$ and $I_2^{\text{cis}}$ spectra are comparable in terms of the spectral shape and the value of the absorbance maxima. However, the $I_2^{\text{trans}}$ and $I_2^{\text{cis}}$ spectra are quite different in the value of the absorbance maxima, in addition to the value of the extinction coefficient at their $\lambda_{\text{max}}$.

The dark phase of photoreversal, involving chromophore deprotonation, movement of chromophore back into the binding pocket, and reversal of global conformational change, is quite rapid: $\sim$58 µs for $I_2$ and $\sim$400 µs for $I_2'$. Moreover, these reversals occur in a single concerted step. The difference in the photoreversal kinetics of these two $I_2$ intermediates is a consequence of the different structure of the two states. Reversal from $I_2$ involves chromophore deprotonation and a coupled movement of the chromophore back into the binding pocket and is thus rapid (58 µs). In the reversal from $I_2'$, the global conformational change has to be reversed as well, leading to slower kinetics. Our data thus provide a first estimate of about 400 µs for the time constant of the protein refolding reaction. In the thermal decay of $I_2$ which occurs in a few hundred milliseconds, the isomerization is rate-limiting, and the reaction appears to occur with a single time constant.

It has been argued that $I_2$ and $I_2'$ are difficult to distinguish with electronic absorption spectroscopy since their spectra are similar. The most convincing evidence for the existence of different $I_2$ intermediates (protonated chromophore) comes from time-resolved FTIR measurements [Xie, 2001], [Brudler, 2001]. $I_2$ and $I_2'$ can also be distinguished by the ability of $I_2'$ to bind dyes [Borucki, 2002], [Hendriks, 2002] when a hydrophobic surface patch is exposed as a result of the conformational change [Meyer, 1989]. Here we showed that $I_2$ and $I_2'$ can be differentiated kinetically using the double flash method with variable time delay, which was applied here for the first time to PYP.

Using a second flash at 500 nm, we directly showed photoreversal from $I_1$. This reaction is fast (<1 µs) and possibly faster than our time resolution of about 50 ns. It is not surprising that the photoreversal kinetics from $I_1$ are much faster than from $I_2$. In the earlier $I_1$ intermediate, the chromophore remains deprotonated with the hydroxyl of the chromophore hydrogen-bonded to E46 and Y42. Thus, only the isomerization around the $C_7=C_8$ double bond has to be photoreversed. In $I_2$ and $I_2'$, the cycle has advanced much further: chromophore protonation, loss of hydrogen bonds, surface exposure of the chromophore, and global conformational change. All of this has to be reversed in addition.

In recent work on the $M$ intermediate of the light-driven proton pump bacteriorhodopsin, we were able to demonstrate a similar kinetic heterogeneity of the $M$ substrates by photoreversal kinetics [Dickopf, 1997]. In the photoreversal from $M$,
reisomerization and chromophore protonation are involved. As was the case for the I$_2$ intermediate of PYP, two photoreversal time constants were observed in BR (100 and 600 ns), which could be assigned to two sequential M intermediates that differed in the protonation state of the proton release group [Dickopf, 1997], [Druckmann, 1992].

In previous work [Hendriks, 1999] the photoreversal kinetics from I$_2$ were investigated with single flash (355 nm) experiments on a photostationary mixture of I$_2$, I$_2$', and P at pH 5.6. This low pH was required to accumulate a sufficiently high population of I$_2$/I$_2'$ under steady illumination, thus limiting this method to the low pH range. The observed exponential time constant was 147 µs at 20 °C and represents the average value over the I$_2$ and I$_2'$ populations in the initial photo-steady-state mixture under these conditions. Likewise, the observed spectrum of I$_2^{\text{trans}}$ was a superposition of the spectra of I$_2'^{\text{trans}}$ and I$_2'^{\text{trans}}$. The efficient excitation of the remaining 30% in the ground state by the 355 nm flash was ignored (see trace VFTV in Figure 4.1A,B). The value of 147 µs (measured at pH 5.6) lies between our value of 57 µs for I$_2$ and 380 µs for I$_2'$ (measured at pH 6), as expected for an average value. For the mutant M100A, which has a lifetime for I$_2'$ of ~18 min at pH 6, nearly 100% of the PYP molecules are accumulated in the I$_2$/I$_2'$ equilibrium under steady illumination [Devanathan, 1998]. Using single flash excitation, an exponential time constant for photoreversal of 231 µs was obtained, consistent with the results presented here. The fact that the wild-type and M100A photoreversal kinetics are similar is not surprising, since current thinking is that M100 catalyzes the reisomerization but does not affect other steps in the photocycle [Devanathan, 1998]. Thus, photoreversal kinetics should be similar to wild type and is consistent with the above interpretation.

Measurements of the pH dependence of the photoreversal kinetics of I$_2$ and I$_2'$ at a delay of 20 ms (Figure 4.7) provide further support for the pH$_a$ of the I$_2$ / I$_2'$ equilibrium. The kinetics is characterized by a fast (50-60 µs) and a slow (300-800 µs) component, which are due to photoreversal from I$_2$ and I$_2'$ respectively, Figure 4.5A. The corresponding amplitudes are pH dependent (Figure 4.7B,C) with a pH$_a$ of 6.1 in good agreement with the value of 6.4 obtained from the photocycle kinetics, Figure 3.5C. The amplitude for I$_2$ goes to zero beyond pH 7.3. The photoreversal data thus suggest that no I$_2$ remains at alkaline pH, whereas the photocycle data indicate that some I$_2$ remains since [I$_2$] /([I$_2$] + [I$_2'$]) is about 0.25 at pH 8.6 (Figure 3.5C). We note that the end value of the I$_2$ population at high pH in Figure 3.5C is quite sensitive to the exact choice of the I$_1$ spectrum. We found that only three intermediates I$_1$, I$_1'$ and I$_2'$ are present alkaline pH above pH 8, i.e. no I$_2$ as shown Figure 3.11.
Our results for photoreversal with the second flash at 355 nm are very different at pH 10 and pH 6. At pH 6, we observed photoreversal from two sequential intermediates $I_2$ and $I_2'$ that were in equilibrium, and had their own photoreversal time (58 µs and 380 µs for $I_2$ and $I_2'$ respectively). At pH 10, there is only one intermediate with protonated chromophore, $I_2'$. The photoreversal kinetics has again two phases (60 µs and 3.5/2.3 ms). However, their interpretation is very different. The first unresolved step is again the rapid isomerization from $I_2^{cis}$ to $I_2^{trans}$. Some $I_1^{trans}$ is also already present around 50 ns after the second flash, possibly from rapid equilibration with $I_2^{trans}$. In the 60 µs phase, $I_2^{trans}$ decays to $I_1^{trans}$. This transition is associated with chromophore deprotonation. In the next, slower phase (3.5 ms), $I_1^{trans}$ and $I_1'$ decay to P. The associated amplitude spectrum $B_2$ provides clear evidence for the participation of $I_1^{-}$ and $I_1'$-like intermediates in the photoreversal. The photoreversal results thus provide further support for the existence of the $I_1'$ intermediate at alkaline pH.

In future work with the double flash method, it will be of particular interest to investigate the kinetics of the associated proton release and to study the perturbation of equilibrium with selective excitation of one of the equilibrium partners.

### 4.5 Conclusions

The kinetics of photoreversal from $I_1$, $I_2$ and $I_2'$ at pH 6 was measured and analyzed in detail with a violet (at 355 nm) or a green (at 500 nm) flash as the second flash. $I_2$ and $I_2'$ can be distinguished on the basis of their photoreversal time constants of 57 µs and 380 µs respectively. The time delay between the two flashes was varied from 1 µs to 3 s with a violet flash as the second flash. Temporal variation of the $I_2$ and $I_2'$ intermediates determined from the delay dependent photoreversal amplitudes is in agreement with their time dependent populations measured in the normal cycle with a single flash excitation experiment. From the delay dependence of the photoreversal amplitudes it was concluded that $I_2$ and $I_2'$ are in equilibrium beyond ~10 ms.

Moreover, the $I_2$ and $I_2'$ intermediates are in pH dependent equilibrium in about ~ 20 ms with $pK_a \sim 6.1$, as determined from the pH dependent photoreversal amplitudes with a violet flash as the second flash. This observation supports the measured $pK_a$ with the single flash excitation experiment from the titration curves of the normal cycle populations at ~ 20 ms time scale.

The initial event just after absorption of the second flash is probably the reisomerization of the chromophore from cis to trans. This is indicated by the instantaneous
absorbance increase around ~ 350 nm after second flash, in the wavelength dependence of
the photoreversal amplitudes at the fixed delays of 1 and 10 ms, when a violet flash is used as
the second flash. This might be due to the formation of $I_2^{trans}$ and $I_2'^{trans}$ from $I_2^{cis}$ and $I_2'^{cis}$
respectively. The chromophore in any intermediate with $trans$ configuration is observed to
have a higher extinction coefficient than its $cis$ counterpart. This $cis$ to $trans$ isomerization
event could not be resolved in this study and is assumed to be faster than our time resolution
of 10 ns. The wavelength dependence of the photoreversal amplitudes further indicates that,
$I_2^{trans}$ and $I_2'^{trans}$ formed just after the second flash, decay to the initial state P, with two
separate time constants.

Selective photoreversal from $I_1$ with a green flash indicated that the photo-
backreaction from $I_1$ to the initial state is faster than 1 µs.

Moreover, the delay and the wavelength dependence of the photoreversal kinetics at
pH 10 were measured with a violet flash as the second flash. The delay dependence of the
photoreversal amplitudes confirm the rise and decay of the $I_2'$ intermediate, indicating the
photoreversal from $I_2'$. The photoreversal kinetics differ significantly in the acid/neutral and
alkaline pH range indicating that different intermediates contribute to the photocycle in the
two pH ranges.

These results have established the double flash experiment as a significant technique
to elucidate the mechanism of the normal photocycle.
Summary and Outlook

The pH dependence of the photocycle kinetics of photoactive yellow protein was measured and interpreted in terms of a specific reaction mechanism. Transient absorption spectroscopy with single and double flash excitation is used to investigate the photocycle of wild type PYP. The measured transient absorbance changes with a single blue flash excitation in the whole pH range from pH 4.6 to 11, and the time window of 50 ns to 50 s, indicate that four intermediates $I_1$, $I_1'$, $I_2$ and $I_2'$ contribute to the photocycle, during three transitions. These intermediates are characterized spectrally from the measured transient absorbance changes using extrapolated difference method and scaled subtraction method. This is the key information to acquire the mechanism of the photocycle kinetics. The measured absorbance maxima of the corresponding intermediates $I_1$, $I_1'$, $I_2$ and $I_2'$ are at 460 nm, 430 nm, 370 nm and 350 nm respectively. The intermediates, $I_1$ and $I_2'$ contribute in the whole pH range. However, $I_1'$ and $I_2$ contribute minimally in the acid (pH 4.6 to 8.4) and alkaline pH (pH 8 to 11) range respectively. The temporal variation of each intermediate is determined from the measured transient absorbance changes and the intermediate spectra. This provides the information about the connectivity among the intermediates. It is observed that these intermediates are in equilibrium, and some intermediate populations are pH dependent during the photocycle.

$I_1$ decays partially to $I_2$ during the first transition (~ 400 µs) in the acid/neutral pH range, whereas this intermediate decays in part to $I_1'$ in the alkaline pH range in the same transition. After the second transition (~ ms), the $I_1/I_2/I_2'$-equilibrium is formed in the acid/neutral pH range, in which the $I_1/I_2$-equilibrium decays further in part to $I_2'$. However, in the alkaline pH range the $I_1/I_1'$ equilibrium decays forming the $I_1/I_1'/I_2'$ equilibrium during this transition. It is observed that $I_2$ and $I_2'$ are in a pH dependent equilibrium in the ~ 20 ms time range with a $pK_a ~ 6.3$ in the acid/neutral pH region. The signaling state $I_2'$ is the main species above this $pK_a$. Similarly, $I_1'$ and $I_2'$ are in a pH dependent equilibrium with a $pK_a ~ 10$, in the alkaline pH region during the same time range (~ 20 ms), with the $I_1'$ the main species above this $pK_a$. Moreover, $I_1'$ is the alkaline form of $I_2'$ with the surface exposed chromophore. The
former pKₐ might be assigned to the carboxylate side chain of E46 and the latter to the phenolate oxygen of the chromophore.

The third rate constant k₃ is due to the recovery to P of the I₁/I₂/I₂' equilibrium in the acid/neutral pH range and of the I₁/I₁'/I₂' equilibrium in the alkaline pH range, respectively. This rate is strongly pH dependent with a well known bell-shaped curve with pKₐ’s of ~ 6.3 and ~ 9.7. These pKₐ’s are very close to those determined from the intermediate equilibrium populations. In both cases, the recovery rate is proportional to the [I₂'] concentration, indicating that the dark state recovery is controlled by the I₂' population of the cycle. Thus the transient accumulation of I₂', the active state of PYP, is controlled by the proton concentration. This is analogous to the case of the photoreceptor rhodopsin, where the equilibrium between the signaling state MII and its precursor MI is also strongly pH dependent. The habitat of *Hr. Halophila* is alkaline, and this study provides the working pH range of this photoreceptor. The largest accumulation of signaling state is at ~ pH 8.

Moreover, for the first time the well-known pKₐ’s of the recovery rates are explained in terms of the corresponding pH dependence of the equilibrium intermediate populations. This investigation provides the most extensive data set to date (pH region from pH 4.6 to 11, the time window of 50 ns to 50 s and wavelength range of 320 nm to 510 nm) and analyses this data field in terms of the number of the intermediates, their spectra, connectivity and equilibria. The mechanism of converting photon energy to conformational alteration in PYP presented in this study contributes to understand the signal transduction mechanism of PAS domain superfamily as PYP is proposed as the PAS structural prototype.

The mechanism of the photocycle kinetics acquired with single flash excitation experiments was further supported by double flash excitation experiments, where the kinetics of photoreversal from the I₁, I₂ and I₂' intermediates was investigated. A first flash, at 430 nm, initiated the photocycle. After a variable time delay, the I₁ intermediate was photoreversed by a second flash, at 500 nm, or a mixture of I₂ and I₂' intermediates was photoreversed by a second flash, at 355 nm. The corresponding photoreversal times measured at pH 6 from I₁, I₂, and I₂' are <1 µs, 59 µs and 400 µs respectively. The first step in photoreversal is rapid *cis-trans* isomerization of the chromophore. By varying the delay from 1 µs to 3 s, it was possible to excite selectively the intermediates I₁, I₂, and I₂'. The good agreement of the delay dependence of the two amplitudes, A₁ and A₂ (respective photoreversal times of 59 µs and 400 µs), with the time dependence of the I₂ and I₂' populations provided strong evidence for the sequential model. The persistence of A₁ beyond delay times of 5 ms and its decay, together with A₂ around 500 ms, suggest moreover that I₂ and I₂' are in thermal equilibrium. This was
the first evidence for this equilibrium, which was later supported by measurements of the normal cycle. Depending on the progression of the photocycle, reversal becomes slower with the time delay, thus mirroring the individual steps of the forward photocycle. In this way, the kinetics and equilibria of the single flash cycle may be probed by photoreversal.

Moreover, measurements of the pH dependence of the photoreversal kinetics in the pH range of 4.8 to 8.5 with a second flash of 355 nm applied at a delay of 20 ms confirmed the pKₐ value (~6.1) of the I₂/I₂' equilibrium obtained from measurements of the single flash cycle. Photoreversal experiments at pH 10 with the second flash at 355 nm indicate the presence of only one I₂-like intermediate, which is assigned on the basis of its λₘₐₙ value to I₂'. The amplitude spectra of the photoreversal signal support that I₂'ₐₜ₅, I₁'ₐₜ₅ and I₁'ₐₜ₅ intermediates participate in this reversal.

Photoreversal from I₂' is slower than from I₂, since, in addition to chromophore protonation, the global conformational change has to be reversed. These data thus provide a first estimate of about 59 µs for deprotonation and 400 µs for the structural change, which also occurs in the thermal decay of the signaling state but is obscured there since reisomerization is rate-limiting.

In future work, these investigations may be extended to key mutants (e.g. E46Q, Y42F, K110A, E12A) and PYP/phytochrome hybrids under various conditions e.g. azide, heavy water, temperature to probe their effect on the photocycle. The extrapolated difference method and the scaled subtraction method might be used to determine the spectra of the intermediates and the associated time courses. Alternatively, the double flash excitation experiment might assist to verify the photocycle kinetics acquired from the single flash excitation experiment.

In particular, photocycle study of K110A and E12A mutants may explain the role of the K110 and E12 salt bridge, during the formation of the signaling state I₂' as proposed in [Borucki, 2005]. Still unexplained curved Arrhenius plots for the measured recovery rate as a function of temperature [Meyer, 1989] may be explained in terms of the intermediate equilibrium populations. Preliminary experiments for some of these cases have shown interesting observations. For example, there is a large azide effect on the photocycle at pH 6.3, where the recovery is slowed down by a factor of ~10 upon addition of 850 mM azide. Whether this effect is just like the salt effect reported in [Borucki, 2005], [Harigai, 2003] or whether azide acts as a proton donor/acceptor as observed in bacteriorhodopsin [Otto, 1989], remains to be seen.
Interesting isotope effects were observed during the course of this Ph. D. work. The dark spectrum is red-shifted by 2 nm, in D₂O and recovery is slowed down by a factor of ~ 2 at pH 6. These observations are similar to those reported in [Hendriks, 2003]. Moreover, by measuring the proton inventory plots of various transitions as acquired for bacteriorhodopsin [Brown, 2000], it may be possible to distinguish, whether a particular transition involves one or more protons.

Finally, these investigations may be extended to other photoreceptors especially the hybrid proteins containing PYP and phytochrome domains [Kyndt, 2004]. A major question here is why these photoreceptors have two chromophore domains. How do these domains interact? Does the blue light absorbed by PYP control or reset the red light sensitive phytochrome domain? The interest here is on the selective excitation of the particular domain (e.g. phytochrome) and observe its effect on the other domain.
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Publications


Presentations

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