Initial Events in the Photocycle of Photoactive Yellow Protein*

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The light-induced isomerization of a double bond is the key event that allows the conversion of light energy into a structural change in photoactive proteins for many light-mediated biological processes, such as vision, photosynthesis, photomorphogenesis, and photo movement. Cofactors such as retinals, linear tetrapyrroles, and 4-hydroxy-cinnamic acid have been selected by nature that provide the essential double bond to transduce the light signal into a conformational change and eventually, a physiological response. Here we report the first events after light excitation of the latter chromophore, containing a single ethylene double bond, in a low temperature crystallographic study of the photoactive yellow protein. We measured experimental phases to overcome possible model bias, corrected for minimized radiation damage, and measured absorption spectra of crystals to analyze the photoproducts formed. The data show a mechanism for the light activation of the photoactive yellow protein, where the energy to drive the remainder of the conformational changes is stored in a slightly strained but fully cis-chromophore configuration. In addition, our data indicate a role for backbone rearrangements during the very early structural events.

The 14-kDa water-soluble photosensor PYP\(^1\) (1) of the purple sulfur bacterium *Halorhodospira halophila* is among the best understood model systems for the conversion of light energy into a conformational change leading to a physiological response. The absorption spectrum of PYP matches the wavelength dependence for negative phototaxis in *H. halophila*, suggesting that it is the primary photosensor for this blue light repellent response (2). The trans-4-hydroxy cinnamic acid chromophore (HC\(_4\)) is present as a phenolate anion in a hydrophobic pocket in the protein, where it is covalently attached to the bone nitrogen atom of Cys69 by a thioester bond (3, 4). In the dark state of PYP (denoted pG; \(\lambda_{\text{max}} = 446\) nm), the phenolic oxygen (O-4) of the chromophore is stabilized by hydrogen bonds to Tyr42 and to the protonated carboxyl group of Glu46 (5–7). During the photocycle the ethylene double bond in the chromophore (C-2=C-3) isomerizes from the trans-configuration to the cis-configuration (8). This event triggers a reversible photocycle in which a number of spectrophotically distinct, short-lived intermediates have been identified, denoted I\(_0\) (\(\lambda_{\text{max}} = 510\) nm), pR (also denoted I\(_1\) or PYP\(_{\text{I1}}\); \(\lambda_{\text{max}} = 465\) nm), and pB (also denoted I\(_2\) or PYP\(_{\text{I2}}\); \(\lambda_{\text{max}} = 355\) nm) (9–12).

These intermediates have been the subject of a number of crystallographic, NMR, and UV-visible/IR spectroscopic studies, which led to the following consensus structural model for the photocycle. \(I_0\) is formed from pG in picoseconds, during which the ethylene bond C-2=C-3 in the chromophore photosomerizes, whereas the aromatic ring of the chromophore is kept in position in its hydrophobic pocket. The hydrogen bonds of O-4 to Tyr42 and Glu46 are maintained, but the hydrogen bond of the chromophoric thioester oxygen (O-1) to the backbone nitrogen atom of Cys69 is disrupted, and O-1 moves to a hydrophilic pocket formed by five aromatic residues (12–15). A second red-shifted intermediate, pR, is formed from \(I_0\) in nanoseconds, during which O-1 is repositioned from its unfavorable hydrophilic environment to an as yet unknown or possibly undefined position (15, 16). Up to this time, the hydrogen-bonding network to the aromatic head of the chromophore remains preserved (17). The blue-shifted intermediate, pB, is formed from pR in hundreds of microseconds. The residue Arg42, which shields the chromophore from the solvent, moves away from the chromophore-binding pocket disrupting the hydrogen bonds to the backbone O atoms of Tyr42 and Thr50 (18). A further repositioning of O-1, during the recovery of the ground state pG, leads to the reformation of the hydrogen bond to the backbone nitrogen atom of Cys69. The hydrogen bond between the O-4 of the phenolic oxygen to Tyr42 and Glu46 is disrupted, and O-4 becomes protonated. Furthermore, the chromophore swings out of the pocket to a strained, nonplanar conformation (18). Finally, pG recovers from pB in hundreds of milliseconds by deprotonation of O-4, resomerization, and repositioning of the chromophore into its binding pocket and reformation of the dark state hydrogen-bonding network.

Irradiation of PYP at \(<90\) K leads to the formation of a photostationary state consisting of the dark state, a bathochromic (PYP\(_{\text{BL}}\); \(\lambda_{\text{max}} = 489\) nm), and a hypsychromic intermediate (PYP\(_{\text{HR}}\); \(\lambda_{\text{max}} = 442\) nm). Upon warming, these latter intermediates thermally convert to PYP\(_{\text{BL}}\) (\(\lambda_{\text{max}} = 400\) nm) and PYP\(_{\text{HR}}\) (\(\lambda_{\text{max}} = 447\) nm), respectively, and converge at temperatures higher than 193 K to PYP\(_{\text{BL}}\) (\(\lambda_{\text{max}} = 456\) nm) (11), as depicted in the low temperature photocycle scheme shown in Fig. 1A. The same branching reaction has not been observed in the early photocycle at room temperature (12), although in a recent

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The atomic coordinates and structure factors (codes 1UWN and 1UWP) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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** The nomenclature of the chromophoric atoms is presented in Fig. 1B.

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The UV-visible absorption difference spectrum of pR-pG at 264 K involves similar conformational changes to those occurring at room temperature (10). An alternative nomenclature for these intermediates has been indicated between brackets: A490, A440, and A400, respectively (37). The room temperature intermediates are referred to as pG (λmax = 446 nm), pR (λmax = 465 nm) and pB (λmax = 355 nm) (10). An alternative nomenclature used throughout this paper. The low temperature intermediates are referred to as pG (Fig. 1A, left panel) and the light-activated PYPB state (right panel). The atoms are depicted with the nomenclature used throughout this paper.

We recently reported on the photocycle of PYP in the crystalline state (36) and showed that constant illumination of PYP crystals at cryogenic temperatures leads to a photostationary state of at least one hypsochromic and one bathochromic photoproduct that resemble PYPH and PYPB (11, 19, 37). The effect of temperature, light color, and duration on the occupancy of the low temperature photoproducts was determined. The optical properties of temperature, light color, and duration on the occupancy of the low temperature photoproducts was determined. The optical properties were used to study the effect of radicals within the crystal can be very substantial as shown in recent systematic studies (25–27). These structural changes are observable before traditional markers such as the Wilson B factor, diffraction resolution, and crystal mosaicity show visible signs of x-ray damage (27). Matsui et al. (28) showed that exposure of frozen (100 K) bacteriorhodopsin crystals to a relatively low x-ray flux converted nearly half of the protein into an orange species. They subsequently employed a data collection strategy that allowed them to study the early bacteriorhodopsin photocycle intermediate K with a reduced x-ray dose and only minor radiation damage. In contrast, in many other crystallographic studies of intermediates specific x-ray radiation damage was either overlooked or assumed irrelevant (20, 23, 29–32). The structural changes induced by radiation damage are in general very characteristic and reproducible. It is possible to correct for these differences (33) or even to profit from them (34, 35).

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Radiation damage ought to be taken into account while studying detailed structural changes using the x-ray crystallography technique. The interaction of X-rays with the crystalline sample can result in structural changes because of either sample heating or radiation chemistry. Thorough theoretical calculations seem to indicate that sample heating is in general very small (24). In contrast, the structural effects caused by the generation of radicals within the crystal can be very substantial as shown in recent systematic studies (25–27). These structural changes are observable before traditional markers such as the Wilson B factor, diffraction resolution, and crystal mosaicity show visible signs of x-ray damage (27). Matsui et al. (28) showed that exposure of frozen (100 K) bacteriorhodopsin crystals to a relatively low x-ray flux converted nearly half of the protein into an orange species. They subsequently employed a data collection strategy that allowed them to study the early bacteriorhodopsin photocycle intermediate K with a reduced x-ray dose and only minor radiation damage. In contrast, in many other crystallographic studies of intermediates specific x-ray radiation damage was either overlooked or assumed irrelevant (20, 23, 29–32). The structural changes induced by radiation damage are in general very characteristic and reproducible. It is possible to correct for these differences (33) or even to profit from them (34, 35).

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ries were collected: one of the protein in the ground state and one in the light-activated state. In addition control dark state data were used to correct the first two data series for specific radiation damage. The corrected data were used to obtain experimental phases. We show for the first time light-activated (difference) maps that have been calculated with experimental phases exclusively and have been deconvoluted from radiation damage.

Crystallography is a powerful technique that can elucidate small, structural changes that occur in early photocycle intermediates. PYP has for long been a model compound for these studies, because of the superior diffraction behavior of its crystals. Nevertheless, dispute remains among crystallographers, quantum chemists, and spectroscopists about the very fine details of the first events upon photoabsorption. The aim of this study is to contribute toward a more unified picture of these early events by employing off-line spectroscopy, radiation damage correction, and experimental phasing.

EXPERIMENTAL PROCEDURES

Overproduction and Crystallization of Selenomethionine-labeled PYP—Selenomethione (SeMet)-labeled PYP was overproduced and purified as described for the native protein (42). Blue light was selected from a 150-W halogen light source with a mW, as determined through a pinhole of 25-μm diameter optical fiber. The radius of the focused laser spot was 90 μm.

Initial Events in the Photocycle of PYP

The combination of excellent experimental phases (Fig. 2) and the use of the MAD technique on crystals of PYPH, and PYPB, as presented in Fig. 1, is a critical tool for the identification of the photointermediates. The primary photointermediate in the light-activated state was confirmed using a thermocouple, which measured a temperature between 4 and 9 K higher than that set at the Oxford cryoloop and protected from dehydration by soaking in paraffin oil.

Data Collection and Radiation Damage Correction—Experimental phases were obtained by the use of the MAD technique on crystals of SeMet-labeled PYP. Because of the low solvent content (31%) of the P63 crystals, a two-wavelength approach was chosen (inflection point and remote wavelength) to aid density modification procedures in solving the phase ambiguity that would have resulted from a single-wavelength anomalou dispersion experiment. A total of four data sets were collected for the low temperature intermediate study, two for the dark state and two for the photoactivated state (Table I). A low resolution data set was collected at the inflection point (12,685 k), whereas the high energy remote (13,200 k) data set consisted of a low and high resolution sweep. The crystals of dimensions 160 × 80 × 80 μm3 diffracted to atomic resolution (Table I). All of the data were collected on the MAD beamline ID14-4 at the European Synchrotron Radiation Facility (Grenoble, France), whereas the synchrotron was operated in 16-bunch mode (~50 m of normal intensity). Severe attenuation (between 14- and 70-fold) was used to reduce radiation damage. The exposure time consisted of a few seconds/frame, and the data were collected to 1.2 Å. The total absorbed dose for all four data sets, augmented by the increased absorption of the crystal caused by the presence of selenium atoms, was estimated with the aid of the program RADDose (45) to be about 2 × 106 Gy.

A control experiment was carried out on a second crystal of SeMet-labeled PYP kept in the dark throughout the experiment. A series of four data sets was collected, in the order inflection point (ip1), remote (rm1), inflection point (ip2), and remote (rm2). Inspection of the Fcalc − Fobs and Fobs difference maps showed minor but significant radiation damage. The small corresponding changes in intensities were used to correct the four data sets collected on the first crystal, after linear scaling to compensate for differences in absorbed dose and diffraction power between the two crystals. The correction scheme has similarities to the one recently published by Diederichs et al. (33) but is less dependent on redundancy and uses an external data series to allow optimal deconvolution of photocytivation and radiation damage. The scaling of the data sets was critically checked by inspection of difference maps around the x-ray susceptible C terminus of the protein. Upon correction, no significant differences remained around this residue in the Flight − Fdark difference map. The merging R factor (on intensities) between the remote data sets of the first crystal was 5.3% before and 4.4% after correction, indicating that (i) only one correction for radiation damage was needed and (ii) the light-induced differences are very small.

Three data sets were collected on the SeMet late pB state crystal, low and high resolution data sets at the inflection point and a low resolution data set at the high energy remote (Table I). No correction for radiation damage was made for this semi-quantitative study.

Phasing and Refinement—Experimental phases were obtained using the programs SHARP (46) and SOLOMON (47) as directed by SHARP. The structures of the dark and late pB state were refined using maximum likelihood refinement in REFMAC (47), incorporating the experimental phases as restraints. 5% of the data were used in an Rmerge set. An overall Rmerge of 13% was achieved. The combination of experimental phases (Fig. 2) and the use of these phases in refinement allowed the identification of several multiple conformations in the ground state (Ser8, Asp10, Ala16–Met18, Leu23, Gln32, Asp53, Gln60, Lys60, Asp65, Gla61, Ser65, Met61, Gln69, Met100, Met109, and Arg124). The final R factors (Table I) were comparable with those for the 0.82 Å resolution PYP ground state structure of Getzoff et al. (25).

Unfortunately, REFMAC neither allows the refinement of occupancies nor the calculation of atomic coordinate uncertainties via matrix-inversion, as can be done using SHELXL. This limitation was overcome by using a modified version of Cruickshank’s diffraction precision indicator (48, 49); the coordinate error of an atom in the ground state structure, with a mean B value of 11 Å2, is calculated to be 0.632 Å.

The low temperature cis-model was built based on the Fextra – Fcalc difference and light-activated state extrapolated maps (Fig. 3). The extrapolated maps (18, 29) were calculated for a range of possible populations α using amplitudes Flight − Fdark and on intensities (1 − α) and phases based on a model that excluded residues Tyr12, Gla36, Arg52, Phe52, Val60, Thr70, Phe96, and Tyr96 and the chromophore HIC. The latter residues were left against Fcalc, while keeping the rest of the ground state model fixed, using cis-chromophore stereochemical terms that were based on density functional studies (50). The weights on critical terms such as bond angles (Sy, C1, O1, C1, C1, C2, C3, C2, C3, C1), and torsion angles (C1, C2, C3, C1) were minimized. A combination of Fextra, Rmerge, Rfree, electron density maps and the correlation between Fobs, Fcalc, Fobs − Fcalc, and Fcalc − Fdark difference maps gave an optimum population of the cis-chromophore of around 21%, consistent with population estimates from off-line microspectrophotometry measurements (36).

RESULTS AND DISCUSSION

Full trans-to-cis Isomerization of the Chromophore—To study the first structural events associated with the PYP photocycle, we populated low temperature intermediates by continuous illumination of a PYP crystal with blue light at 85 K leading to a photostationary state of the ground state pG, PYPH, and PYPB, as presented in Fig. 1A. We applied continuous illumination during x-ray diffraction data collection, because our spectroscopic studies on PYP crystals indicated that…
**Table I**  
Diffraction data and refinement statistics

All data were collected on ID14-4 of the ESRF, Grenoble, France. Low- and high-resolution sweeps of data collected at the same wavelength were combined. Friedel pairs were not merged given the strong anomalous signal in each data set. Data were collected for minor radiation damage after merging. Phasing and model refinement was done using the corrected data. iso, isomorphous; ano, anomalous; ip, inflection point; rm, high-energy remote; acen, acentric reflections; cen, centric reflections.

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* $R_{\text{free}}$ is defined as $\Sigma ||\langle h,i \rangle - \langle k\rangle||/\Sigma ||\langle h,i \rangle||$.  
* Phasing power is defined as $||(F_{\text{calc}}/\text{phase-integrated lack of closure})$.  
* $R_{\text{free}}$ as defined in Ref. 46.  
* $R_{\text{free}}$ as defined in Ref. 46.  
* $R_{\text{free}}$ (as defined in Ref. 46) is defined as $[\Sigma ||F_{\text{calc}}|| - k||F_{\text{obs}}||]/\Sigma ||F_{\text{calc}}|| \times 100$, where 5% of randomly selected reflections were used for $R_{\text{free}}$.  
* Incomplete refinement.
PYP\textsubscript{B} is not stable at low temperatures. The latter intermediate thermally decays in the dark with a decay time of 100 s at 100 K (38). We verified experimentally that the obtained photostationary state of PYP, PYP\textsubscript{H}, and PYP\textsubscript{B} contained a relatively high occupancy of PYP\textsubscript{B}. These spectroscopic studies on crystals did not give any evidence for the formation of unwanted, secondary photoproducts under the conditions used (38). We collected data from selenomethionine labeled PYP, which has identical kinetics of the recovery reaction of the dark state as native PYP, to obtain experimental phases by the MAD method. A control series was collected where the crystal was not photoactivated. X-ray damage studies on native P6\textsubscript{3} and P6\textsubscript{3} PYP crystals (51) showed that the most susceptible residues include Asp\textsubscript{34}, Glu\textsubscript{46}, Asp\textsubscript{48}, Cys\textsubscript{69}, Ser\textsubscript{72}, and Val\textsubscript{125} and the chromophore HC4. The control series showed some of these changes, and these differences where used to correct the data collected on the photoactivated crystal using Val\textsubscript{125} as a marker because it is not believed to play any role in the photocycle.

Both the data and the experimental phases are of excellent quality (Table I). Iterative refinement located several conformations of the selenium atoms. The resulting anomalous residual maps (46) showed a positive peak for the Cys\textsubscript{69} S\textsuperscript{-} ground state position, whereas f\textsuperscript{a} of sulfur is only 0.2 e\textsuperscript{-} at the wave-
lengths used. This indicates the high quality of the data as well as the power of experimental phasing procedures with modern software packages.

The resolution of the data was limited to 1.2 Å because of the high attenuation and low exposure times used to minimize radiation damage; however, the final experimental maps showed the complete structure at full atomic detail. Multiple conformations of several residues were found, including a previously unreported double conformation of the Ala16-Met18 backbone. Experimental electron density maps of the chromophore in the ground state and upon photocexcitation are shown in Fig. 2. Spectroscopic studies (36) have shown that a total photoactivation of about 20% was expected. The low temperature photointermediates seem to overlap largely with the ground state, because no major differences are observed between the two experimental maps. The only clear and significant difference (feature B) is the additional electron density attached to the C-2 atom of the chromophore. This position corresponds to the new carbonyl oxygen O-1 position of the chromophore, as determined by Genick et al. (20). Whereas the position of this oxygen atom became visible in the experimental map despite its low occupancy, no new position (feature G) for the heavier Cys69 S-atom could be found. Whereas positive and negative peaks higher than ±6 σ were located in or close to the chromophore-binding pocket. The strongest peaks were found around the thioester link between the chromophore and Cys69; the carbonyl oxygen disappears at −15.9 σ (feature A) and reappears at 10.3 σ (feature B), the ethylene bond disappears at −9.3 σ (feature C) and reappears at 8.7 σ (feature D), whereas the Cys69 Sy atom disappears at −14.4 σ (feature E) and reappears in two positions, at 7.2 (feature F) and 7.1 σ (feature G), respectively.

Inspection of the Flight – Fdark difference maps showed additional subtle changes, e.g., around the chromophore phenyl ring. These difference maps were used in combination with a Fourier map calculated using extrapolated Fextramap amplitudes (Fig. 3C) for building and refinement of a low temperature intermediate of PYP. This intermediate model shows a completed cis-configuration without major stereochemical distortions. We have assigned this intermediate to PYPB based on the occupancy of this intermediate under the same experimental conditions in our spectroscopic analysis (36). Upon trans-to cis-isomerization of the C-2=C-3 ethylene bond, the chromophore carbonyl group rotates 180°. The movement of Cys69 Sy, however, is small and therefore not visible in the experimental electron density map (Fig. 2B). The absence of significant features next to the heavy Cys69 Sy atom excludes an intermediate model that shows a large movement of this atom. Previous studies have shown that Cys69 Sy is susceptible to x-ray damage (51). To minimize radiation damage, a minimal dose was used for data collection, thus compromising the resolution of the data. Nevertheless, data collected on a reference crystal still showed some specific radiation damage, which could be used to correct the Flight and Fdark data. The effects of the radiation damage correction on the features present in the Flight – Fdark difference maps are presented in Fig. 3 (A and B). It is obvious that the minor movement of Cys69 Sy is better defined in the Flight – Fdark difference map after correction (Fig. 3B) than in the difference map prior to the radiation damage correction (Fig. 3A). Another subtle difference can be observed on the x-ray damage susceptible carbonyl group of Glu46. Whereas only negative density is seen before the correction (Fig. 3B), small correlated positive and negative features are visible in the corrected map (Fig. 3A). These results indicate that care should be taken with the interpretation of features associated with x-ray susceptible atoms in electron density difference maps. We note that several crystallographic PYP intermediate studies show exclusively negative features on Glu46 (15, 16, 20). In addition, radiation damage may have affected the positive density around Cys69 Sy (position F) in a previous study on a low temperature PYP intermediate, where this feature did not appear very prominent and remained unexplained (20). However, the density around Cys69 Sy is much better defined in our radiation damage corrected maps (Fig. 3B) and led to the assignment of the Cys69 Sy position in our model of PYPB that included the cis-configuration of the C-2=C-3 bond.

A second sulfur position in the photoactivated state (feature G) can be observed both in the difference maps and in the extrapolated map (Fig. 3), indicating the presence of a second photoactivated chromophore configuration. The distribution of the sulfur atom over its two new positions is not equal, although the features F and G in Fig. 2C have similar heights. The negative feature E for the sulfur atom is smaller than feature A for the lighter oxygen atom. This indicates that most of the photoactivated sulfur atoms only show a very small movement, which is confirmed by the absence of any new sulfur position in the experimental map (Fig. 2B). Refinement against the extrapolated data (Fig. 3C) indicates a ratio of 3:1 in favor of the sulfur position corresponding to our PYPB model. This ratio is in good agreement with spectroscopic data (36). The second photoactivated sulfur position (feature G) could belong to a PYPB intermediate, although the formation of PYPBL or other species cannot be excluded. The second sulfur position is similar to that reported for the sulfur position of the low temperature intermediate of Genick et al. (20), trapped at 150 K. This intermediate has previously been assigned to PYPB (21), which seems unlikely, because this intermediate thermally relaxes to PYPBL at temperatures above 93 K (Fig. 1A). Interestingly, the position for the Sy atom in the full cis-chromophore configuration (feature F) can also be observed in the electron density difference map presented in Fig. 1 of the latter report (20), indicating that also under these experimental conditions a mixture of photoproducts is formed. The ratio of those photoproducts is likely to be different as a result of the higher temperature and different excitation wavelength used by Genick et al. (20).

The presence of very subtle structural differences among the first low temperature intermediates is confirmed by Fourier transform infrared spectroscopic studies that indicate that all changes between PYPB and PYPH and between PYPBL and PYPHL (Fig. 1A) are restricted to the region directly around the C-2=C-3 bond and that the hydrogen network surrounding the chromophore is not affected in any of these intermediates (19). Additional studies at different temperatures and using different illumination conditions are needed to deconvolute the atomic structures of the PYPH, PYPHL, and PYPBL intermediates. One of these intermediates could have its Cys69 sulfur atom at position G, which is incompatible (20) with an undistorted excited state if the rest of this intermediate would largely overlap with our PYPB structure. However, the formation of a cis-chromophore at 85 K is in contrast with the interpretation that an energetically unfavorable "transition state" (22) was frozen out at 150 K (20).

Several lines of evidence indicate that the low temperature intermediate PYPB is equivalent to the earliest room temperature intermediate Ic formed within a few picoseconds (12, 13, 21, 52). The assignment of our 85 K structure to PYPB implies that the first events in the photocycle include the complete isomerization of the C-2=C-3 bond in the chromophore and the
flipping of the thioester linkage. This argues for a new photocycle model where the light energy that drives the photocycle is not stored by the formation of distorted torsion angles around C–2–C–3 in the chromophore (20, 22) but in the potential energy of a cis-conformation (21). This energy could originate from (i) the repositioning of the thioester carbonyl oxygen in a hydrophobic pocket, formed by the five aromatic moieties of Phe62, Phe75, Tyr48, Phe96, and the chromophore itself, (ii) the breakage of a hydrogen bond between this oxygen atom and the backbone nitrogen atom of Cys69, and (iii) the widening of the bond angles Cβ–Sγ–C–1 and C–2–C–3–C–1, necessary to stretch the chromophore to maintain its hydrogen bonds with Tyr48 and Glu46 upon isomerization. The atoms O-1, C-1, and C-2 are in fact the only atoms that move more than 0.5 Å upon isomerization. It was shown that O-1 stays in this position only for a few nanoseconds at room temperature and reforms its hydrogen bond with the backbone nitrogen atom of Cys69 after ~1 ms (15). Our model strengthens the conclusion from room temperature Laue studies (15) that the most important trigger for the entry into the photocycle is the flipping of the carbonyl group of the chromophore.

Early Backbone Rearrangements from Val66 to Thr70—A recent theoretical study (21) predicted PYPB, (Fig. 1A) to be the first fully isomerized structure of the photocycle. The PYPB structure presented here is fully cis-and cannot be mistaken for PYPa because the low temperature does not permit formation of the latter intermediate. One possible origin of this disagreement lies in the assumption made in the theoretical study that Cys69Cβ remains in a fixed position. As shown in Fig. 3, all of the atoms of Cys69 have moved slightly, together with the highly conserved stretch of amino acids in the backbone of PYP from Val66 to Thr70. A chain of small but correlated features can be observed in the $F_{\text{light}} - F_{\text{dark}}$ difference map, which matches a small translation of the backbone. These changes stop at residue Asp45, the residue from which main chain atoms are disordered in the ground state.

Our structure shows a clear hydrogen bond between the carbonyl oxygen of Val66 to the NH-2 atom of Arg52 (O–N distance 3.1 Å, C=O–N angle 160°). This hydrogen bond is rarely mentioned, probably because of its increased length in some structures (the maximal O–N distance is 3.77 Å in Protein Data Bank entry 2phy (7)). However, the C=O–N angles are always around 165°, indicating a (weak) electrostatic hydrogen bond (53). Could this hydrogen bond be of importance in the PYP photocycle?

The conversion of light into a structural signal is believed to occur through an opening of the arginine gateway. Whereas Arg52 does not move significantly in the PYPB structure presented here, it is known to move long before the chromophore in PYPa (22). The chromophore to maintain its hydrogen bonds with Tyr48 and Thr50 broken, whereas the hydrogen bond to Val66 is the only one left intact (Fig. 4).

It was shown by Thompson et al. (21) that the ground state structure of PYP anticipates an isomerization around the θ(C–1–C–2–C–3–C–1′) torsion angle in the θ direction, surmounting the energy barrier formed by the steric hindrance between HC4 O-1 and Tyr48. Isomerization in the +θ direction would involve steric clashes between HC4 O-1 and Ala67. The energy landscape for passing the HC4 O-1 Tyr48 and HC4 O-1 Ala67 gates will change upon movement of the Val66–Thr70 backbone. This might play a role in providing the unidirectionality of the photoisomerization.

Conclusions—Experimental phasing has allowed us to identify a new low temperature photointermediate of PYP. Off-line absorption spectroscopy on crystals showed that this intermediate, formed at 85 K, was PYPc. The experimental phases were crucial for the calculation of Fig. 2B, which excludes large movements of Cys69 Sγ. Systematic studies showed that this atom is highly susceptible to x-ray radiation damage. We have used a minimum dose to collect atomic resolution data and corrected the data for minor specific damage using control data sets allowing the deconvolution of specific damage from photoinduced changes. The chromophore in PYPc was found fully isomerized from the trans- to cis-configuration. We feel that the ultra high resolution difference maps of Genick et al. (20) that were obtained upon data collection at a temperature 65 K higher than the temperature used in our study should be reinterpreted using a mixture of photoinduced and possibly radiation damage-induced intermediates succeeding or occurring in parallel to PYPB.

Our data support a new photocycle model, in which the light energy that drives the photocycle is stored by the potential energy of a cis-conformation (21). This energy originates from

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**Fig. 4.** Experimental map of the cryo-trapped late pB intermediate superimposed with a model of the Arg52 gateway in the ground state (dark gray) and pB state (light gray/blue). The map is contoured at 1.2 $\sigma$. The carbonyl oxygen of Val66 to the NH-2 atom of Arg52 and might play a role in triggering the opening of the arginine gateway. The potential importance of the weak hydrogen bond of the carbonyl oxygen of Val66 to the NH-2 atom of Arg52 is further supported by the structure of a cryo-trapped late pB state of PYP obtained by flash-freezing a crystal exposed to 488-nm laser light. In the observed structure, most of the chromophore is back in position, whereas the Arg52 is still partly present outside the binding pocket with the hydrogen bonds to Tyr48 and Thr50 broken, whereas the hydrogen bond to Val66 is the only one left intact (Fig. 4).
the repositioning of the thioester carbonyl oxygen in a hydrophobic pocket and the widening of the bond angles $\mathrm{C\beta-S-Y-C-1}$ and $\mathrm{C-2-C-3-C-1'}$, necessary to stretch the chromophore so that its hydrogen bonds with $\mathrm{Tyr}^{46}$ and $\mathrm{Glu}^{66}$ are maintained upon isomerization. The flipping of the carbonyl group, combined with minor movements of a part of the backbone that has nonideal secondary structure in pG (16), forms the trigger for the entry into the photocycle.

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