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 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 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 response. The absorption spectrum of PYP matches the wave-length dependence for negative phototaxis in *H. halophila*, suggesting that it is the primary photosensor for this blue light response (2). The trans-4-hydroxy cinnamic acid chromophore (HC4) is present as a phenolate anion in a hydrophobic pocket in the protein, where it is covalently attached to the 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 spectroscopically distinct, short-lived intermediates have been identified, denoted I0 ($\lambda_{\text{max}} = 510$ nm), pR (also denoted I1 or PYPL; $\lambda_{\text{max}} = 465$ nm), and pB (also denoted I2 or PYPH; $\lambda_{\text{max}} = 355$ 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. In the first red-shifted intermediate, I0 is formed from pG in picoseconds, during which the ethylene bond C-2=C-3 in the chromophore photoisomerizes, whereas the aromatic ring of the chromophore is kept in position in its hydrophobic pocket. The hydrogen bonds of O-4 of 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 hydrophobic pocket formed by five aromatic residues (12–15). A second red-shifted intermediate, pR, is formed from I0 in nanoseconds, during which O-1 is repositioned from its unfavorable hydrophobic 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 Arg52, 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, reisomerization, and repositioning of the chromophore into its binding pocket and reformation of the dark state hydrogen-bonding network.

Irradiation of PYP at $\sim 90$ K leads to the formation of a photostationary state consisting of the dark state, a bathochromic (PYPα; $\lambda_{\text{max}} = 489$ nm), and a hypochromic intermediate (PYPβ; $\lambda_{\text{max}} = 442$ nm). Upon warming, these latter intermediates thermally convert to PYPBL ($\lambda_{\text{max}} = 400$ nm) and PYPH ($\lambda_{\text{max}} = 447$ nm), respectively, and converge at temperatures higher than 193 K to PYPα ($\lambda_{\text{max}} = 456$ 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
Fig. 1. A, the low temperature photocycle as observed by UV-visible spectroscopy. The photocycle intermediates are depicted with the nomenclature used throughout this paper. The low temperature intermediates are referred to as PYP$_B$, PYP$_H$, PYP$_BL$, and PYP$_HL$. An alternative nomenclature for these intermediates has been indicated between brackets: A$_{440}$, A$_{447}$, and A$_{467}$ respectively (37). The room temperature intermediates are referred to as pG ($\lambda_{max} = 446$ nm), pR (248 nm) and pB (355 nm) (10). An alternative nomenclature for these intermediates has been indicated between brackets: $I_0$, $I_1$, and $I_2$. B, the chemical structures of the chromophore in the pG state (left panel) and the light-activated PYP$_B$ state (right panel). The atoms are depicted with the nomenclature used throughout this paper.

report the presence of a second early intermediate with lower extinction coefficient and spectral overlap with the ground state pG has been postulated (13). The low temperature intermediates PYP$_B$ and PYP$_L$ were proposed (12, 19) to be analogous to the room temperature intermediates $I_1$ and pR, although the absorption maxima of the latter intermediates are slightly more red-shifted. No large protein conformational changes appear to take place in the room temperature transition from the dark state to pR as indicated by Fourier transform infrared in solution (17) and x-ray crystallography (16). The UV-visible absorption difference spectrum of pR-pG at room temperature appears virtually identical to that of PYP$_L$-PYP at 193 K (11). The difference Fourier transform infrared spectra of PYP$_B$, PYP$_H$, and PYP$_L$ minus PYP indicate that the major conformational changes in the early photocycle are restricted to the region around the C-2=C-3 bond (19). It is likely that the low temperature branch from pG to PYP$_B$, PYP$_BL$, and PYP$_L$ involves similar conformational changes to those occurring during the conversions from pG to $I_0$ and pR, although this has not been unambiguously proven (12, 15, 19).

A crystallographic analysis of a photocycle intermediate trapped at 150 K led to a model that showed a 80$^\circ$ double bond torsion angle between C-2=C-3, indicating that the isomerization reaction around this double bond is incomplete (20). This intermediate was tentatively assigned to PYP$_{BI}$ (Fig. 1A) on the basis of a 400-nm shoulder in the absorption spectrum of the frozen PYP crystal. In a later theoretical study by the same group, the cryogenically trapped intermediate was reassigned to PYP$_{BI}$ (21). Their current model for the photocycle holds that the C-2=C-3 bond in the chromophore isomerizes to a highly unfavorable orthogonal position (20). It has been proposed that this energetically unfavorable chromophore conformation is essential for the signaling activity of PYP (20, 22, 23). Does the chromophore-binding pocket of PYP have a unique configuration that has allowed the trapping of a double bond in an orthogonal position? To address this question we applied novel techniques that are of general importance for the structural elucidation of low occupancy reaction intermediates in x-ray crystallography.

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 the early bacteriorhopsin 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).

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 PYP$_B$ and PYP$_H$ (11, 19, 37). The effect of temperature, light color, and duration on the occupancy of the low temperature photoproducts was determined. The optimized conditions were subsequently used on the beamline to determine the structure of PYP$_B$.

It has been demonstrated by Brodersen et al. (38) that the modeling of multiple conformers could be strongly biased by the phases from the refined model. An example was shown using a 1.05 Å data set, where a second, 23% conformation could not be located in $\sigma$-weighted $2mF_o - DF_c$ or even $mF_o - DF_c$ maps while using refined model phases. Experimental single-wavelength anomalous dispersion data, however, revealed the true identity of the second side chain conformation. Global indicators such as $R_{free}$ were not sensitive enough to catch errors caused by such low occupancy conformations (38). This conclusion is likely to hold equally well while refining low occupancy photointermediates.

To avoid any possible model errors, we have used the multi-wavelength anomalous dispersion (MAD) method on selenomethionine substituted crystals of PYP. Two MAD data se-
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 diffusion 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—Selenomethionine (SeMet)-labeled PYP was overproduced and purified using similar procedures as previously reported methods (39, 40). The substitution of methionine by SeMet was verified by matrix-assisted laser desorption ionization-time of flight mass spectrometry to be 100% for all five methionines in the protein. We checked for effects on the kinetics of the recovery reaction of the dark state of purified SeMet-labeled PYP in solution, because it was postulated that the sulfur atom of Met100 catalyzes the reisomerization process in the photocycle (41). We found a rate constant of 4.5 s<sup>−1</sup>, very similar to that observed for the recovery reaction of the unlabeled, recombinant, and native proteins (9, 39). The SeMet protein crystallized in space group P6<sub>3</sub> under conditions nearly identical to those described for the native protein (42).

Cryo-trapping and Single-crystal Microspectrophotometry—An off-line microspectrophotometer (43) was used to study the kinetics of PYP in the crystalline state. The primary photointermediate in the light cycle of PYP was trapped at 85 K by continuous exposure of crystals to light during x-ray diffraction data collection. The optimization of experimental conditions and the spectroscopic identification and estimation of the occupancy of photointermediates are reported elsewhere (36). Blue light was selected from a 150-W halogen light source with a 80-nm band filter centered at 440 nm and guided to the crystal by two glass fibers. The temperature was regulated by an N<sub>2</sub> gas stream from a 600 series Oxford Cryostream. The temperature at the position of the crystal was confirmed using a thermocouple, which measured a temperature between 4 and 9 K higher than that set at the Oxford controller (44). Throughout the paper, the controller-set temperature is denoted.

The pB state was populated at -12 °C to slow down the rate of recovery of the ground state of PYP. Excitation of the P6<sub>3</sub> crystal was carried out with a shutter-controlled 488-nm laser pulse of 1-s duration from a tunable Argon ion laser (Melles Griot). Light was guided to the reflective objective by a 600-μm-diameter optical fiber. The radius of the focused laser spot was 90 μm. The crystal was mounted in a cryoloop and protected from dehydration by soaking in paraffin oil (Hampton Research). The laser power at the sample position was 0.55 mW, as determined through a pinhole of 25-μm radius. Cryo-trapping of a late pB state was carried out by freeze-trapping the crystal. The presence and occupation of the late pB state in the crystal was confirmed by recording a spectrum after trapping (data not shown).

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 P6<sub>3</sub> 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 anomalous 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 (12,885 keV), whereas the high energy remote (13,200 keV) data set consisted of a low and high resolution sweep. The crystals of dimensions 160 × 80 × 80 μm<sup>3</sup> 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 (40% of normal intensity). Severe attenuation (between 14- and 70-fold) was used to reduce radiation damage. The exposure time consisted of several seconds/frame, and the data were collected to 1.2Å. 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 RADDOS (45) to be about 2 × 10<sup>16</sup> 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 (r1), inflection point (ip2), and remote (r2). Inspection of the F<sub>ip1</sub> - F<sub>r1</sub> and F<sub>ip2</sub> - F<sub>r2</sub> 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 diffractive 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 photoactivation 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 F<sub>light</sub> - F<sub>dark</sub> difference map. The merging R factor (on intensities) between the remote data sets of the first crystal was significantly improved 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 R<sub>free</sub> set. The combination of excellent experimental phases (Fig. 2F) and of these phases in refinement allowed the identification of several multiple conformations in the ground state (Ser<sup>S</sup>, Asp<sup>10</sup>, Ala<sup>16</sup> - Met<sup>18</sup>, Leu<sup>25</sup>, Gly<sup>32</sup>, Asp<sup>53</sup>, Gly<sup>60</sup>, Lys<sup>60</sup>, Asp<sup>65</sup>, Glu<sup>61</sup>, Ser<sup>65</sup>, Met<sup>61</sup>, Glu<sup>69</sup>, Met<sup>100</sup>, and Arg<sup>124</sup>). The final R factors (Table I) were comparable with those for the 0.82 Å resolution PYP ground state structure of Getzoff et al. (23).

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 is calculated with a mean B value of 11 Å<sup>2</sup> calculated to be 0.632 Å<sup>2</sup>. The low temperature cis-mode was built based on the F<sub>light</sub> - F<sub>dark</sub> 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 F<sub>light</sub> = F<sub>light</sub> - α F<sub>ground</sub> and phases based on a model that excluded residues Tyr<sup>22</sup>, Glu<sup>46</sup>, Arg<sup>52</sup>, Phe<sup>54</sup>, Val<sup>56</sup>-Thr<sup>57</sup>, Phe<sup>68</sup>, and Tyr<sup>69</sup> and the chromophore HIC. The latter residues were refined against F<sub>calc</sub>, 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 (5y, C-1, O-1, C-1, C-2, C-3, C-2, C-3, C-1'), and torsion angles (C-1, C-2, C-3, C-1') were minimized. A combination of F<sub>ray</sub> = 0 (Table I), the weight on F<sub>light</sub> - F<sub>dark</sub> 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, PYP<sub>H</sub>, and PYP<sub>1H</sub>, 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.

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PYP$_{B}$ 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$_{B}$, and PYP$_{B}$ contained a relatively high occupancy of PYP$_{B}$. 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$_{3}$ and P6$_{3}$ PYP crystals (51) showed that the most susceptible residues include Asp$^{34}$, Glu$^{46}$, Asp$^{48}$, Cys$^{69}$, Ser$^{72}$, and Val$^{125}$ 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$^{125}$ 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$^{69}$ S$_{Y}$ ground state position, whereas $f$ of sulfur is only 0.2 $e^{-}$ 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 Ala106–Met108 backbone. Experimental electron density maps of the chromophore in the ground state and upon photoexcitation are shown in Fig. 2. Spectroscopic studies (36) have shown that a total photoinitiation 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.

Fig. 2C shows the residual map (46), calculated between the dark and the photoactivated data using a model of the selenium atoms only. All positive and negative peaks higher than 2·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), whereas the ethylene bond disappears at −9.3 σ (feature C) and reappears at 8.7 σ (feature D), whereas the Cys69 S 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 $F_{\text{light}} - F_{\text{dark}}$ 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 $F_{\text{extr}}$ 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 S atom, 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 S atom excludes an intermediate model that shows a large movement of this atom. Previous studies have shown that Cys69 S atom 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 $F_{\text{light}}$ and $F_{\text{dark}}$ data. The effects of the radiation damage correction on the features present in the $F_{\text{light}} - F_{\text{dark}}$ difference maps are presented in Fig. 3 (A and B). It is obvious that the minor movement of Cys69 S atom is better defined in the $F_{\text{light}} - F_{\text{dark}}$ 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 carboxyl 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 S (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 S is much better defined in our radiation damage corrected maps (Fig. 3B) and led to the assignment of the Cys69 S 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 PYPH intermediate, although the formation of PYPHL 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 PYPHL at temperatures above 93 K (Fig. 1A). Interestingly, the position for the S 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 PYPHL and PYPBL (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 S 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 $I_1$ 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 Phe66, Phe75, Tyr94, 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 Tyr98 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 PYP<sub>B</sub> (Fig. 1A) to be the first fully isomerized structure of the photocycle. The PYP<sub>B</sub> structure presented here is fully cis-and cannot be mistaken for PYP<sub>B</sub> 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 Cys69ββ 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 light−dark difference map, which matches a small translation of the backbone. These changes stop at residue Asp65, 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 Val<sup>66</sup> to the NH-2 atom of Arg<sup>52</sup> (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 Arg<sup>52</sup> does not move significantly in the PYP<sub>B</sub> structure presented here, it is known to move long before the chromophore moves out of its pocket (15, 18). However, it is unknown what triggers this movement. In the ground state, Arg<sup>52</sup> makes several hydrogen bonds: NH-1 to Thr<sup>50</sup> O and NH-2 to both Val<sup>66</sup> O and Tyr<sup>94</sup> O (Fig. 4). All of the hydrogen bonds are indirectly connected to the chromophore and might therefore communicate structural changes within the chromophore on to Arg<sup>52</sup>. Thr<sup>50</sup> could transmit changes through a tight hydrogen-bonding network that includes H-bonds from Tyr<sup>94</sup> and Glu<sup>46</sup> to the phenolate oxygen of the chromophore. Isomerization around the C-1–C-2–C-3–C-1 torsion angle in the direction, surmounting the energy barrier formed by the steric hindrance between CH4 O-1 and Tyr<sup>94</sup>. Isomerization in the +θ direction would involve steric clashes between HC4 O-1 and Ala<sup>67</sup>. The energy landscape for passing the HC4 O-1 Tyr<sup>94</sup> and HC4 O-1 Ala<sup>67</sup> gates will change upon movement of the Val<sup>66</sup>–Thr<sup>50</sup> 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 PYP<sub>α</sub>. The experimental phases were crucial for the calculation of Fig. 2B, which excludes large movements of Cys<sup>69</sup> 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 photo-induced changes. The chromophore in PYP<sub>α</sub> 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 PYP<sub>B</sub>.

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...
the repositioning of the thioester carbonyl oxygen in a hydrophobic pocket and the widening of the bond angles Cβ–Sγ–C1 and C–C–C–C–1’, necessary to stretch the chromophore so that its hydrogen bonds with Tyr36 and Glu66 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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REFERENCES
