

Initial Steps of Signal Generation in Photoactive Yellow Protein Revealed with Femtosecond Mid-Infrared Spectroscopy[†]

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Received May 23, 2003; Revised Manuscript Received July 22, 2003

ABSTRACT: Photoactive yellow protein (PYP) is a bacterial blue light sensor that induces *Halorhodospira halophila* to swim away from intense blue light. Light absorption by PYP's intrinsic chromophore, *p*-coumaric acid, leads to the initiation of a photocycle that comprises several distinct intermediates. Here we describe the initial structural changes of the chromophore and its nearby amino acids, using visible pump/mid-infrared probe spectroscopy. Upon photoexcitation, the trans bands of the chromophore are bleached, and shifts of the phenol ring bands occur. The latter are ascribed to charge translocation, which probably plays an essential role in driving the trans to cis isomerization process. We conclude that breaking of the hydrogen bond of the chromophore's C=O group with amino acid Cys69 and formation of a stable cis ground state occur in ~2 ps. Dynamic changes also include rearrangements of the hydrogen-bonding network of the amino acids around the chromophore. Relaxation of the coumaryl tail of the chromophore occurs in 0.9–1 ns, which event we identify with the I₀ to I₁ transition observed in visible spectroscopy.

Activation of PYP¹ by blue light induces a negative phototactic response that enables the bacterium *Halorhodospira halophila* to swim away from harmful exposure to intense blue light (see, for a review, ref 1). Due to its rich and complex photocycle and its excellent (photo)chemical stability, PYP has become a model system for the study of biological signal generation in photoreceptor proteins. Light-induced signal generation in biology involves the amplification of an initially small configurational change generated in an active site into a conformational change of a larger scale that converts the protein into its signaling state. The initial configurational change in the photocycle of PYP (2), i.e., trans-cis isomerization of its intrinsic chromophore [*p*-coumaric acid (3, 4) depicted in Figure 1], is similar to that in other photosensors such as (bacterio)rhodopsin and (bacterio)phytochromes. A key characteristic of the PYP photocycle is a partial unfolding of the protein (4) on a

submillisecond time scale, triggered by the absorption of a photon.

The PYP photocycle has been characterized by visible transient spectroscopy: the excited state of the chromophore decays multiexponentially in a few picoseconds to a red-shifted intermediate, denoted I₀ and absorbing around 500 nm (6–11), followed by the formation of an intermediate absorbing at 480 nm in 1–3 ns (6–11), denoted I₁ (also called pR or PYP_L). A blue shift of the chromophore absorption due to protonation (13, 14) occurs after ~0.3 ms, accompanied by a partial unfolding of the protein [pB or PYP_M state (4, 14)]; this is probably the signaling state of PYP. Upon deprotonation and reisomerization of the chromophore, the ground state is recovered on a ~200 ms time scale. The structural changes of the chromophore and in the protein occurring during the photocycle have been studied with a wide range of techniques, such as time-resolved X-ray crystallography (15, 16) and time-resolved FTIR spectroscopy (17), from ~10 ns after excitation onward, and by the application of these techniques on photocycle intermediates trapped by lowering the temperature (18, 19). It was concluded that a few nanoseconds after light absorption, i.e., when PYP is in the I₁ state, important structural changes have already occurred. The chromophore has adopted the cis configuration (15–18), and changes in the hydrogen-bonding network and structural rearrangements of nearby amino acids are observed. The chromophore phenolate ring has moved only slightly in the new configuration, and its

[†] This research was supported by The Netherlands Organization for Scientific Research via the Dutch Foundation for Earth and Life Sciences (Investment Grant 834.01.002). M.L.G. is grateful to NWO-ALW for providing financial support with a long-term fellowship (Grant 831.00.004), and D.S.L. is grateful to the Human Frontiers Science Program.

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¹ Abbreviations: PYP, photoactive yellow protein; mid-IR, mid-infrared; ES, excited state; SADS, species-associated difference spectra; FTIR, Fourier transform infrared; OD, optical density; WT, wild type.

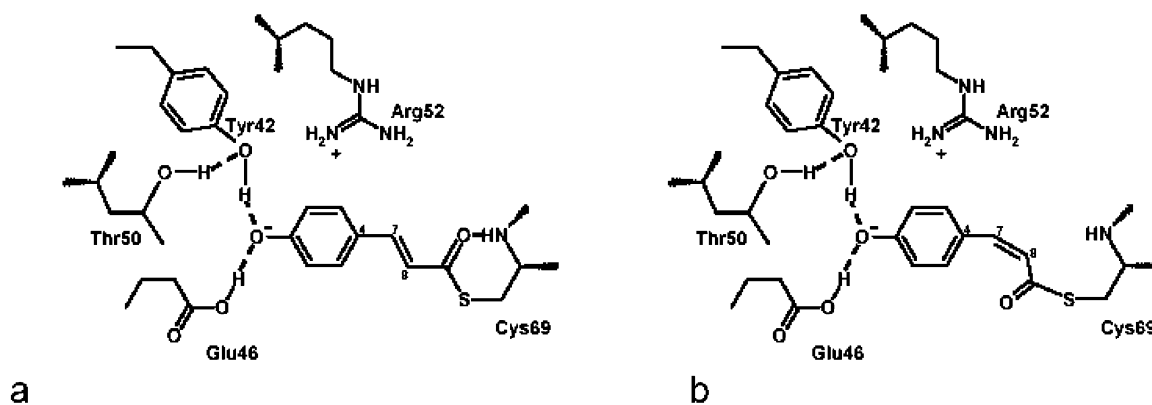


FIGURE 1: Active site of PYP, depicting the chromophore covalently linked to Cys69 and amino acids Glu46, Tyr42, Thr50, and Arg 52 near the phenolate ring of the chromophore, in the ground state (a) and in the I₁ state (b), after refs 16 and 19.

hydrogen bonds with the nearby E46 and Y42 residues remain intact (16). Together with the covalent bond with Cys69, this limits the freedom of motion of the chromophore appreciably; therefore, isomerization can take place only by simultaneous rotation around the dihedral angle of multiple bonds [e.g., $\theta_{7=8}$ and θ_{4-7} , as has been suggested previously (20, 21)]. However, the time scale of these initial events has not yet been identified.

In the present study, we provide for the first time the “missing link” between ultrafast optical information on one hand and “slower” structural information on the other by applying mid-infrared transient absorption spectroscopy with ~ 200 fs time resolution. In contrast to UV–vis spectroscopy, vibrational spectroscopy is sensitive to precise structural configurations and can even have a suboptical cycle time resolution of ~ 10 fs if a coherent emission technique is used (22). Our data for the first time yields information on the structural changes that occur in the excited state and in the first two ground-state intermediates I₀ and I₁.

MATERIALS AND METHODS

PYP was prepared as described previously (23). The sample was placed between two 2 mm thick CaF₂ plates separated by a 6 μ m Teflon spacer. The use of concentrated PYP solutions ($OD_{446} \sim 1.0$) and thin spacers diminished the presence of the intense water absorption bands in the IR absorption spectrum.

The experimental setup consists of an integrated Ti:sapphire oscillator–regenerative amplifier laser system (Hurricane, SpectraPhysics) operating at 1 kHz and 800 nm, producing 85 fs pulses of 0.8 mJ. A portion of this 800 nm light was used to pump a noncollinear optical parametric amplifier to produce the excitation pulses with center wavelength of 475 nm, i.e., at the red edge of the absorption band, to achieve excitation into the $v = 0$ level for the 1000–2000 cm^{-1} modes. The excitation pulses had a duration (uncompressed) of about 60 fs. They were attenuated to 300 nJ to reduce local heating of the sample and focused with a 20 cm lens into the sample. A second portion of the 800 nm light is used to pump an optical parametric generator and amplifier with a difference frequency generator (TOPAS, Light Conversion) to produce the mid-IR probe pulses. The probe pulses were attenuated to an intensity of about 1 nJ and were spatially overlapped with the excitation beam in the sample. The cross-correlation of the visible and IR pulses was measured in GaAs to be about 180 fs.

After overlap in the sample, the mid-IR probe pulses were dispersed in a spectrograph and imaged onto a 32-element MCT detector (Infrared Associates). The signals of the detector array were amplified (Infrared Associates) and fed into 32 home-built integrate-and-holds which were read out every shot with a National Instruments acquisition card (PCI6031E). The polarization of the excitation pulse was set to the magic angle (54.7°) with respect to the IR probe pulses. A phase-locked chopper operating at 500 Hz was used to ensure that every other shot the sample was excited and the change in transmission and hence optical density could be measured. To ensure a fresh spot for each laser shot, the sample was moved by a home-built Lissajous scanner. In a single experiment a spectral probe window of about 200 cm^{-1} was covered, so five partly overlapping regions were measured between 1850 and 1100 cm^{-1} ; experiments were repeated at least two times.

RESULTS AND DISCUSSION

Figure 2 shows time traces collected over the 1100–1850 cm^{-1} region upon excitation of the PYP chromophore at 475 nm. A global analysis of the data revealed that three lifetimes are present in our data, 2 ps, 9 ps, 0.9–1 ns, and a nondecaying component (>10 ns). Before $t = 0$, a small perturbed free induction decay signal is present; therefore, no attempt was made to extract any kinetic component in the order of the instrument response, i.e., ≤ 200 fs. The state associated with the 0.9–1 ns lifetime and the nondecaying state can be identified as the I₀ and I₁ states, respectively. The spectra of the 2 and 9 ps components are due to the excited state (ES) of PYP, which is known to decay multiexponentially (6–11). We performed a target analysis (12, 13) of the data, using a specific kinetic model based on results from visible femtosecond pump–probe experiments (10, 11). This allows us to extract the species-associated difference spectra (SADS). The spectra of the two ES were virtually identical, which is why in a second round the data was fitted to a model in which the initial decay is biexponential (see Figure 3). The relative amplitudes of the 2 and 9 ps time constants were determined to be 0.7 and 0.3, respectively. The overall quantum yield of I₀ (and I₁) formation was estimated in the analysis to be 0.24 ± 0.05 , of which the major part is formed with the 2 ps time constant, in good agreement with the yield of I₁ formation determined from visible data (10, 11). The SADS of the states are shown in Figure 4. Note that in these spectra negative bands are

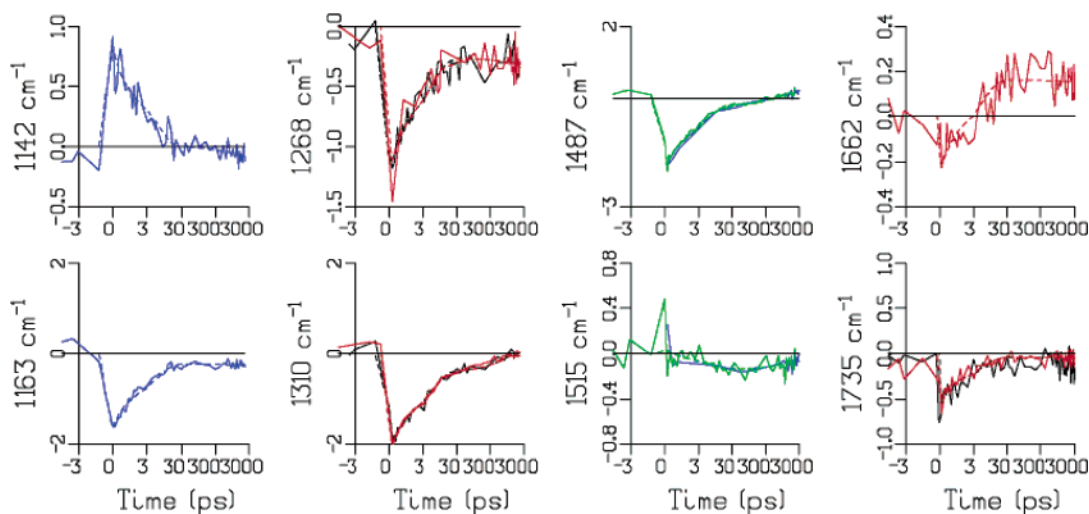


FIGURE 2: Eight of the 120 time traces collected over the 1100–1850 cm^{-1} range upon excitation of the chromophore at 475 nm with a 60 fs pulse, plotted on a linear scale up to 3 ps and on a logarithmic scale for later delay times. The y-axis is in mOD units. The dotted line represents the fit to the data; some figures show data from two different experiments, indicated by the different colors. Before time zero a small perturbed free induction decay is observed.

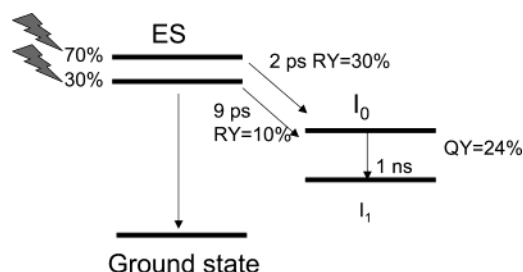


FIGURE 3: Schematic depiction of the first part of the PYP photocycle. Indicated are the rate constants, the relative yield for I_0 formation from each of the excited states, and the quantum yield for I_1 formation obtained from fitting this model to the data.

due to the disappearance of trans absorption, and positive (going) bands originate from excited- or product-state absorption.

The assignment of the dynamic band shifts for each of the ES, I_0 , and I_1 states is based on literature reports (17, 18, 20, 21, 25–31) and on experiments performed on a mutant in which residue Glu46 (glutamate) has been replaced by a glutamine (data not shown) and is given in Table 1. The I_1 spectrum shows a very good resemblance to the ~ 50 ns–1 μ s FTIR difference spectrum reported by Brudler et al. (17) and to the FTIR difference spectrum reported for I_1 in a cryotrapped intermediate (18). A difference with the spectrum reported by Brudler et al. (17) is the amide I signal at $-1643/+1626$ cm^{-1} in their spectrum due to the response of the protein to the isomerization. On the time scale of our investigation such signals are absent; the spectra correspond quite well with Raman spectra (25, 28, 29) and those of model chromophores in solution (26, 27) and therefore can be assigned to structural changes in the chromophore itself, with the exception of bands 9 and possibly 10. Apparently the protein starts to respond on the 10–50 ns time scale. FTIR difference spectra have also been reported for the cryotrapped intermediates PYP_B and PYP_H, which, after forming PYP_{BL} and PYP_{HL}, respectively, convert to I_1 . Note that there is a difference in visible absorption between I_0 , PYP_B, and PYP_H, (495, 490, and 440 nm, respectively). Nevertheless, our I_0 spectrum resembles both cryotrapped intermediates quite well but differs in the downshift of mode

3 while only PYP_B has a mode at 1485 cm^{-1} , close to our mode 4. Interestingly, these modes are both due to the phenol ring of the chromophore. We suggest that these differences are due to processes involving the chromophore or surrounding residues that are hampered at low temperature (see discussion below).

From the strong similarity of our transient spectra with published trans-cis difference spectra of model chromophores, the PYP_B, PYP_H, and the I_1 state, we conclude that the pCA chromophore in both the I_0 and the I_1 transient states has isomerized.

The trans features in region 2 [ascribed to normal modes containing the C–C single stretches, including the C–C(–S–)=O stretch at 1302 cm^{-1} (18, 29)] have disappeared already in the initial ES spectrum and partly also those of the C=C stretch vibrations [reported at $-1663/+1621/-1607$ cm^{-1} for pCA methyl ester (26)]. This may for a large part be explained by a decrease of pure single and double bond character, due to displacement of electronic charge from the C₇–C₈ isomerizable double bond into the adjacent single bonds upon excitation (36). Other changes observed in the ES spectrum are shifts of phenolate bands 3, 4 [ring–O[–] symmetric stretch (28, 29)], and 5. At first sight these shifts seem to indicate that protonation changes of the phenolate ring occur: The upshift of the ~ 1560 cm^{-1} bands, the upshift of the 1168 cm^{-1} band in 850–950 ps, and the disappearance of the 1444 and 1495 cm^{-1} bands (29) are quite similar to the FTIR spectrum reported for PYP_M (37), in which the chromophore is protonated. It is, however, unlikely that the chromophore actually gets protonated, since the 1755/1740/1732 feature (“9”) shows that the hydrogen bond with Glu46 is weakened, but not lost, in the ES and strengthened in I_0 and I_1 ; see below. Furthermore, protonation leads to a strong blue shift of the electronic absorption of the chromophore, and this occurs on the submillisecond time scale. Recently, on the basis of time-dependent DFT calculation it was suggested (38) that in the I_0 state [i.e., the cryotrapped structure reported by Genick et al. (19) was used in these calculations] a subpopulation of the chromophore is protonated, which led to a much smaller electronic blue shift than that of the PYP_M state in line with visible pump–

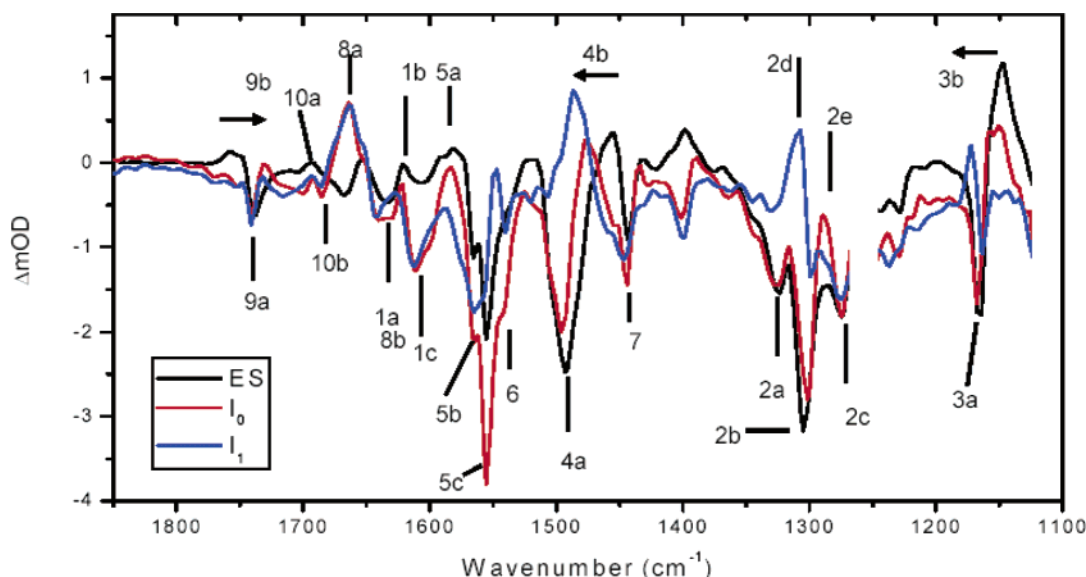


FIGURE 4: Species-associated difference spectra of WT PYP induced by excitation of the chromophore with a short 475 nm laser pulse. The ES decay was fitted with a biexponential rate of 2 and 9 ps⁻¹; the I₀ to I₁ transition rate was 0.9–1 ns⁻¹. Since the quantum yield of the I₀ and I₁ states was only 24%, their presence in the raw data was ~4 times smaller relative to the ES spectrum.

probe experiments. These authors found that in the I₁ state the chromophore was deprotonated again. Our dynamic shifts of the phenol bands might therefore be explained by a partial localization of the proton on the chromophore in the ES and I₀ states. Alternatively, or in addition, these modes may be sensitive to the chromophore becoming more neutral, due to the instantaneous change in dipole moment of 26 D upon light absorption, recently measured for PYP. This dipole moment change was interpreted as a charge translocation from the phenolic oxygen toward the ethylene chain, probably leading to a molecule with almost zero permanent dipole moment (39). Such a charge translocation to the ethylene chain may lead to a further decrease in pure C=C double bond and C–C single bond character (39) and, therefore, be important in initiating the trans-cis isomerization. For bacteriorhodopsin the presence of substantial light-induced charge redistribution in the retinal chromophore was shown to be a prerequisite for the occurrence of a photocycle (40 and references cited therein); therefore, this motive seems to be a common trait in biological signal generation.

The C=O stretch of Glu46 at 1740 cm⁻¹ upshifts to 1755 cm⁻¹ in the ES spectrum, reflecting a weakening of the H-bond of Glu46 with the phenolic oxygen. This could be caused by a small increase in distance between Glu46 and the chromophore. However, in light of the observations discussed above, either the disappearance of the electron from the phenolic oxygen or the partial localization of the proton closer to the chromophore may be a more likely cause. Indeed, the upshift disappears with the ES to I₀ transition, when, as argued above, a more negative phenolic oxygen is re-formed. In the I₀ and I₁ spectra subsequently, a downshift of the Glu46 C=O is observed, indicating a *strengthening* of the Glu46–chromophore H-bond relative to the ground-state structure, probably due to a small decrease in the Glu46–chromophore distance. Likely this initiates the proton transfer from Glu46 to the chromophore on the submillisecond time scale, leading to the swinging of the phenol ring out of the protein pocket.

The frequency upshift of the chromophore's C=O stretch from ~1640 to 1663 cm⁻¹ (feature 8a,b) in the ES to I₀ transition clearly demonstrates the breaking of the hydrogen bond with Cys69 (25), probably due to the flipping of the C=O around the ethylene chain of the chromophore. This flipping of the C=O group has been observed during the first few nanoseconds of the I₁ intermediate in time-resolved X-ray data (16) and in an early cryotrapped (PYP_{BL}) intermediate (19). Furthermore, an increase of bleaching of trans C=C modes at 1607 cm⁻¹ is observed and the appearance of an upgoing C–C product band at 1289 cm⁻¹ typical for the formation of the cis isomer (18). The complete disappearance of the C=C (and C–C) trans modes and the appearance of C–C cis mode, together with the decrease in distance between Glu46 and the chromophore and the breaking of the H-bond with Cys69, show that the cis ground state is formed on the picosecond time scale. Probably the chromophore assumes in I₀ a stretched cis configuration, similar to that observed in the cryotrapped PYP_B structure.

The changes in the region of the C–C(–S–)O vibrational modes ("feature 2"), in particular, the rise of the positive band at ~1310 cm⁻¹, show that a further cis relaxation around these bonds takes place on the time scale of the I₀ to I₁ transition. The quantum yield for the I₀ to I₁ transition is estimated to be 90–100%, indicating that I₀ is a stable cis intermediate from which no return to the trans ground state occurs. Time-resolved X-ray studies show that in the I₁ intermediate the chromophore is in a slightly stretched cis conformation. In the first few nanoseconds the carbonyl oxygen is in a flipped position on the opposite side of the chromophore (16; see structure depicted in Figure 1b).

On no time scale is breaking of the hydrogen bond with Glu46 observed in our spectra, excluding the possibility of a large movement of the phenol ring of the chromophore during isomerization. Therefore, isomerization does seem to occur via a double isomerization mechanism about the vinyl bond and the thioester linkage in which the connections of the chromophore with the protein remain intact (20, 21).

Table 1: Assignment of Features Observed in the Mid-IR Difference Spectra for States ES, I₀, and I₁^a

Frequency/Assignment	ES	I ₀	I ₁
-1633/+1621/-1607 1a-c C=C pCA <i>trans</i> → <i>cis</i> markers ^{17,18,26,27-29}	~	+	+
-1326/-1302/-1274/+1308/+1289 2a-e C-C(-S)=O pCA <i>trans</i> → <i>cis</i> markers ^{17,18,27,29}	+	+	+
-1168 3a,b Phenol ring Y9a pCA <i>trans</i> → <i>cis</i> and ionic markers ^{18,27-29}	+ 1149 b	+ 1155 b	+ 1173 b
-1495 4a,b Phenol ring Y19a, ring-O ⁻ symmetric stretch ionic marker ^{28,29}	+ 1457 b	+ 1476 b	+ 1486 b
+(1580-1585)/-1565/-1555 5a-c Phenol ring Y8b ionic marker ^{25,27-30}	+	+	-
-1540 6 Phenol ring Y8a ^{28,30}	+	+	+
-1444 7 Phenol ring Y19b ^{28,29}	+	+	+
+1665/-(1635-1640) 8a,b C=O of pCA ²⁵	-	+	+
-1740 9a,b COOH stretch of Glu46 ^{18,20}	+ 1755	+ 1732	+ 1732
+1693/-1685 10a,b Arg52 C=N ³¹	~+	+	+

^a Frequencies are in cm⁻¹. In the left column +/- denotes a positive and negative band, respectively, and in the right three columns +/-/- indicates the presence/absence of these bands in the different states. If present and different for each of the states, the frequency of the product band is indicated in the right three columns.

The small +1693/-1685 cm⁻¹ band shift ("10") observed in all spectra we tentatively assign to Arg52, of which the C=N stretch lies in this frequency region (31). Groenhof et al. (32) found in molecular dynamics simulations that upon excitation partial charge transfer from the chromophore to Arg52 takes place. These changes of Arg52 are in line with the time-resolved X-ray data of Ren et al., who observe, within a few nanoseconds, significant changes in residues not in direct contact with the chromophore (16). Here we show that these changes, probably due to the changed electron distribution/polarization of the chromophore, are sensed within ~200 fs and remain present during the nanosecond time scale of the experiment.

In conclusion, using time-resolved mid-IR spectroscopy, we have resolved the structural changes in the very early

part of the PYP photocycle. Our measurements are the first to characterize the vibrational features of the excited state from which isomerization occurs with a 20–30% overall quantum yield. We have found additional evidence for a charge translocation upon excitation from the phenolic oxygen toward the ethylene chain, which may be important for the weakening of the isomerizable C₇–C₈ double bond. Isomerization occurs on the 2 ps time scale and is accompanied by breaking of the hydrogen bond of the carbonyl oxygen with the protein. The transition from I₀ to I₁ occurs with a yield of 90–100%, and we therefore conclude that I₀ is a stable long-living *cis* ground-state configuration, which structurally relaxes on the nanosecond time scale of the I₀ to I₁ transition. We have further shown that the H-bond of the chromophore with Glu46 remains intact on all time scales relevant for this investigation. The combination of charge translocation and isomerization upon light absorption seems to be a common theme in photosensors. The protein appears to play an active role in combining the two and, thereby, in directing the photocycle, since it stabilizes the negative charge on the phenolic oxygen in the ground state by an extensive hydrogen bond network.

ACKNOWLEDGMENT

Assistance and technical support from Han Voet, Jos Thieme, and the mechanical and electronic VU workshops is gratefully acknowledged.

REFERENCES

- Hellingwerf, K. J., Hendriks, J., and Gensch, T. (2003) *J. Phys. Chem. A* 107, 1082–1094.
- Meyer, T. E., Yakali, E., Cusanovich, M. A., and Tollin, G. (1987) *Biochemistry* 26, 418–423.
- Hoff, W. D., Dux, P., Hard, K., Devreese, B., Nugteren-Roodzant, I. M., Crielgaard, W., Boelens, R., Kaptein, R., Van Beeumen, J., and Hellingwerf, K. J. (1994) *Biochemistry* 33, 13959–13965.
- Baca, M., Borgstahl, G. E. O., Boissinot, M., Burke, P. M., Williams, D. R., Slater, K. A., and Getzoff, E. D. (1994) *Biochemistry* 33, 14369–14377.
- Van Brederode, M. E., Hoff, W. D., van Stokkum, I. H. M., Groot, M. L., and Hellingwerf, K. J. (1996) *Biophys. J.* 71, 365–380.
- Baltuška, A., van Stokkum, I. H. M., Kroon, A., Monshouwe, R., Hellingwerf, K. J., and van Grondelle, R. (1997) *Chem. Phys. Lett.* 270, 263–266.
- Ujj, L., Devanathan, S., Meyer, T. E., Cusanovich, M. A., Tollin, G., and Atkinson, G. H. (1998) *Biophys. J.* 75, 406–412.
- Devanathan, S., Pacheco, A., Ujj, L., Cusanovich, M. A., Tollin, G., Lin, S., and Woodbury, N. (1999) *Biophys. J.* 77, 1017–1023.
- Imamoto, Y., Kataoka, M., Tokunaga, F., Asahi, T., and Masuhara, H. (2001) *Biochemistry* 40, 6047–6052.
- Gensch, T., Gradinaru, C. C., van Stokkum, I. H. M., Hendriks, J., Hellingwerf, K. J., and van Grondelle, R. (2002) *Chem. Phys. Lett.* 356, 347–354.
- Larsen, D. S., Vengris, M., van Stokkum, I. H. M., van der Horst, M. A., de Weerd, F. L., Hellingwerf, K. J., and van Grondelle, R. (2003) (manuscript in preparation).
- Holzwarth, A. R. (1996) Data analysis of time-resolved measurements, in *Biophysical Techniques in Photosynthesis* (Amesz, J., and Hoff, A. J., Eds.) pp 75–92, Kluwer, Dordrecht.
- Hoff, W. D., van Stokkum, I. H. M., van Ramesdonk, H. J., van Brederode, M. E., Brouwer, A. M., Fitch, J. C., Meyer, T. E., van Grondelle, R., and Hellingwerf, K. J. (1994) *Biophys. J.* 67, 1691–1705.
- Hendriks, J., van Stokkum, I. H. M., and Hellingwerf, K. J. (2003) *Biophys. J.* 84, 1180–1191.
- Perman, B., Srajer, V., Ren, Z., Teng, T., Pradervand, C., Ursby, T., Bourgeois, D., Schotte, F., Wulff, M., Kort, R., Hellingwerf, K. J., and Moffat, K. (1998) *Science* 279, 1946–1950.

16. Ren, Z., Perman, B., Srajer, V., Teng, T.-Y., Pradervand, C., Bourgeois, D., Schotte, F., Ursby, Th., Kort, R., Wulff, M., and Moffat, K. (2001) *Biochemistry* 40, 13788–13801.
17. Brudler, R., Rammelsberg, R., Woo, T. T., Getzoff, E. D., and Gerwert, K. (2001) *Nat. Struct. Biol.* 8, 265–270.
18. Imamoto, Y., Shirahige, Y., Tokunaga, F., Kinoshita, T., Yoshihara, K., and Kataoka, M. (2001) *Biochemistry* 40, 8997–9004.
19. Genick, U. K., Soltis, S. M., Kuhn, P., Canestrelli, I. L., and Getzoff, E. D. (1998) *Nature* 392, 206–209.
20. Xie, A., Hoff, W. D., Kroon, A. R., and Hellingwerf, K. J. (1996) *Biochemistry* 35, 14671–14678.
21. Imamoto, Y., Kataoka, M., and Liu, R. S. H. (2002) *Photochem. Photobiol.* 76, 584–589.
22. Groot, M. L., Vos, M. H., Schlichting, I., van Mourik, F., Joffre, M., Lambry, J.-C., and Martin, J.-L. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99, 1323–1328.
23. Hendriks, J., Gensch, T., Hviid, L., van der Horst, M. A., Hellingwerf, K. J., and van Thor, J. J. (2002) *Biophys. J.* 82, 1632–1643.
24. Genick, U. K., Borgstahl, G. E. O., Ng, K., Ren, Z., Pradervand, C., Burke, P. M., Srajer, V., Teng, T.-Y., Schildkamp, W., McRee, D. E., Moffat, K., and Getzoff, E. D. (1997) *Science* 275, 1471–1475.
25. Unno, M., Kumauchi, M., Sasaki, J., Tokunaga, F., and Yamauchi, S. (2002) *Biochemistry* 41, 5668–5674.
26. Xie, A., Kelemen, L., Hendriks, J., White, B. J., Hellingwerf, K. J., and Hoff, W. D. (2001) *Biochemistry* 40, 1510–1517.
27. Van Thor, J. J., Pierik, A. J., Nugteren-Roodzant, I., Xie, A., and Hellingwerf, K. J. (1998) *Biochemistry* 37, 16915–16921.
28. Zhou, Y., Ujj, L., Meyer, T. E., Cusanovich, M. A., and Atkinson, G. H. (2001) *J. Phys. Chem. A* 105, 5719–5726.
29. Kim, M., Mathies, R. A., Hoff, W. D., and Hellingwerf, K. J. (1995) *Biochemistry* 34, 12669–12672.
30. Brudler, R., Meyer, T. E., Genick, U. K., Devanathan, S., Woo, T. T., Millar, D. P., Gerwert, K., Cusanovich, M. A., Tollin, G., and Getzoff, E. D. (2000) *Biochemistry* 39, 13478–13486.
31. Tamm, L. K., and Tatulian, S. A. (1997) *Q. Rev. Biophys.* 30, 365–429.
32. Groenhof, G., Lansink, M. F., Berendsen, H. J. C., Snijders, J. G., and Mark, A. E. (2002) *Proteins: Struct., Funct., Genet.* 48, 202–211.
33. Gensch, T., Hellingwerf, K. J., Braslavsky, S. E., and Schaffner, K. (1998) *J. Phys. Chem. A* 102, 5398–5405.
34. Herbst, J., Heyne, K., and Diller, R. (2002) *Science* 297, 822–825.
35. Yamada, A., Yamamoto, S., Yamato, T., and Kakitani, T. (2001) *J. Mol. Struct. (THEOCHEM)* 536, 195–201.
36. Sergi, A., Gruning, M., Ferrario, M., and Buda, F. (2001) *J. Phys. Chem. B* 105, 4386–4391.
37. Imamoto, Y., Mihara, K., Hisatomi, O., Kataoka, M., Tokunaga, F., Bojkova, N., and Yoshihara, K. (1997) *J. Biol. Chem.* 272, 12905–129808.
38. Thompson, M. J., Bashford, D., Noddleman, L., and Getzoff, E. D. (2003) *J. Am. Chem. Soc.* (in press).
39. Premvardhan, L. L., van der Horst, M. A., Hellingwerf, K. J., and van Grondelle, R. (2003) *Biophys. J.* 84, 3226–3239.
40. Zadok, U., Khatchatourians, A., Lewis, A., Ottolenghi, M., and Sheves, M. (2002) *J. Am. Chem. Soc.* 124, 11844–11845.

BI034878P