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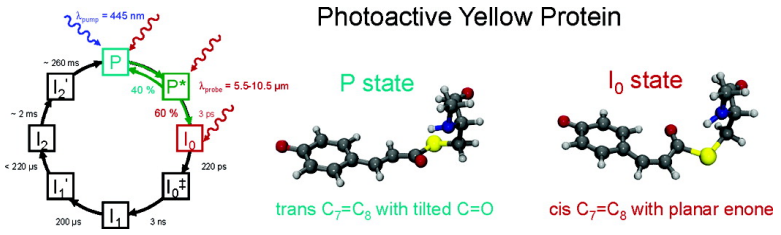

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Structural Evolution of the Chromophore in the Primary Stages of Trans/Cis Isomerization in Photoactive Yellow Protein

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Abstract: We have studied the structural changes induced by optical excitation of the chromophore in wild-type photoactive yellow protein (PYP) in liquid solution with a combined approach of polarization-sensitive ultrafast infrared spectroscopy and density functional theory calculations. We identify the νC_8-C_9 marker modes for solution phase PYP in the P and I_0 states, from which we derive that the first intermediate state I_0 that appears with a 3 ps time constant can be characterized to have a cis geometry. This is the first unequivocal demonstration that the formation of I_0 correlates with the conversion from the trans to the cis state. For the P and I_0 states we compare the experimentally measured vibrational band patterns and anisotropies with calculations and find that for both trans and cis configurations the planarity of the chromophore has a strong influence. The $C_7{=}C_8-(C_9{=}O)-S$ moiety of the chromophore in the dark P state has a trans geometry with the C=O group slightly tilted out-of-plane, in accordance with the earlier reported structure obtained in an X-ray diffraction study of PYP crystals. In the case of I_0, experiment and theory are only in agreement when the $C_7{=}C_8-(C_9{=}O)-S$ moiety has a planar configuration. We find that the carboxylic side group of Glu46 that is hydrogen-bonded to the chromophore phenolate oxygen does not alter its orientation on going from the electronic ground P state, via the electronic excited P* state to the intermediate I_0 state, providing conclusive experimental evidence that the primary stages of PYP photoisomerization involve flipping of the enone thioester linkage without significant relocation of the phenolate moiety.

1. Introduction

Biochemical reactivity is often connected to dynamically evolving protein structures and it thus becomes a task of great importance to identify intermediate protein states that occur on a broad range of time scales (femtoseconds to minutes). Photosensor proteins have a light-triggered functionality and form ideal systems for studying the structural dynamics and the underlying chromophore−protein interactions in real time.[1] Photoactive yellow protein (PYP), a photochemically stable 14 kDa photoreceptor protein, isolated from *Halorhodospira halophila*, is a structural prototype for a super family of signaling proteins containing the PAS domain structural motif.[2,3] The PYP chromophore, *p*-hydroxy-cinnamic acid covalently bonded to the apo-protein through a thioester bond to Cys69, is in the trans form in the dark or P state. The protein pocket of wild-type PYP around the chromophore is arranged in such a fashion that the anionic (deprotonated) form exists in the dark P state, and proton transfer does not occur in the early stages of the photocycle. Upon photoexcitation with blue light ($\lambda_{max} = 446$ nm) wild-type PYP transforms through a photocycle with a number of intermediate states (I_0, $I_0^‡$, I_1, and I_2), before returning to its initial dark state in a few hundred milliseconds depending on solution conditions (Figure 1, for alternative labeling schemes of PYP see refs 2−4). The structure of PYP in its dark state P has been investigated by both static and time-resolved X-ray diffraction (on PYP crystals)[5−7] and by nuclear magnetic resonance in solution.[8] Detailed structural analysis of the intermediate I_1 and I_2 states has been obtained using time-resolved X-ray diffraction[5−7] and step-scan FT-IR[9] experiments. These studies have provided insight into the geometries of the I_1 and I_2 states that appear on time scales from nano- to

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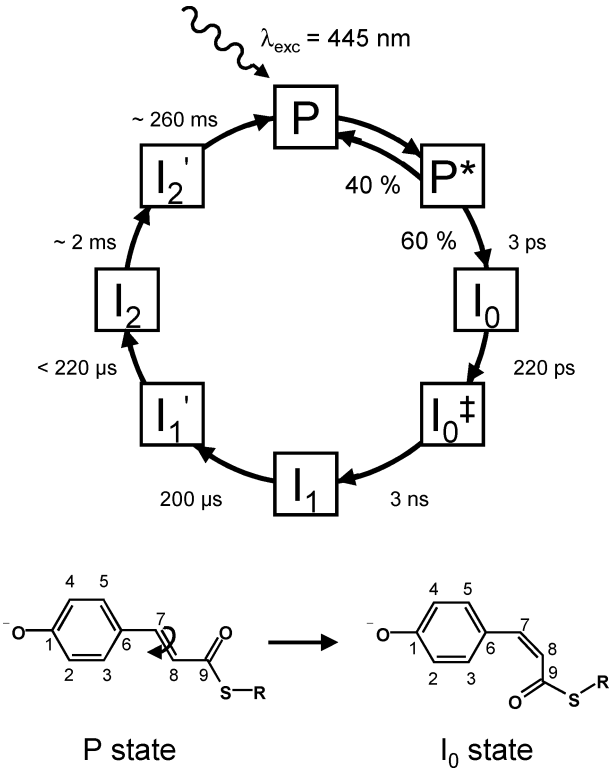

Figure 1. Level scheme of PYP showing the photocycle with the P, P*, and I_0 states and the trans and cis configurations of the PYP chromophore.

milliseconds. It has been shown that in the I_1 state the chromophore exists in a cis configuration,[9] whereas in the I_2 state the chromophore becomes protonated[9] and the protein has altered its structure significantly.[6] However, these approaches have been limited to phenomena taking place on time scales longer than 150 ps.[10−12] One approach to elucidate the nature of the early PYP intermediates is to determine the structure of intermediates trapped at low temperatures.[13−15] However, it is difficult to demonstrate how these trapped states are related to the transient pathway that occurs at room temperature in solution. Only limited structural information has been obtained with UV/vis and vis/IR pump−probe studies on the electronic excited state P* and the first intermediate state I_0 that form in the first picoseconds after photoexcitation.[16−20] In particular, no unequivocal experimental evidence has been reported to date that established that in the I_0 state the chromophore adopts a cis configuration in solution. Interestingly, a recent atomistic hybrid quantum mechanics/molecular mechanics study of PYP has indicated that on a time scale of a few picoseconds the initial dynamics of photoexcited PYP should include a chromophore isomerization to a cis geometry.[21]

To reveal the PYP chromophore structure and the orientation of the COOH group of the Glu46 amino acid side chain hydrogen-bonded to the chromophore phenolate, in the primary stages after photoexcitation, we have combined polarization-sensitive femtosecond infrared spectroscopy and quantum chemical analysis of the geometric rearrangements of the PYP chromophore in both the P and I_0 states in solution. We demonstrate the potential of the method in elucidation of structural changes in condensed phase photoinduced trans/cis isomerization reactions[22] that are often of ultrafast nature ("femtochemistry"[23,24]). We find that the chromophore of PYP in solution has a trans geometry in the P state with the C═O group tilted out-of-plane, similar to observations made on crystalline PYP with X-ray diffraction.[13,25] However, in the first intermediate (I_0) state, appearing with a 3 ps time constant, the chromophore exhibits a cis geometry with a planar enone configuration, clearly distinct from the structure derived from low-temperature trapped PYP_B that has been thought to be related to I_0.[13] Throughout the primary stages the COOH side group of Glu46 does not change its orientation.

The outline of the paper is as follows. In the Experimental Section we present details on sample preparation, time-resolved infrared spectroscopy of PYP, and quantum chemical calculations used to interpret our results. In the Results and Discussion section we first present our transient IR data and analyze the photoinduced dynamics by inspection of vibrational marker modes, providing insight into the excited state lifetime and into the nature of the trans/cis isomerization process. In the second part of the Results and Discussion section we analyze the experimentally determined polarization-sensitive vibrational mode patterns of the dark P and first intermediate I_0 states by comparison to results of quantum chemical calculations.

2. Experimental Section

2.1. Sample Preparation. We prepared PYP from *Halorhodospira halophila* as described previously.[26] We used PYP samples held in a rotation cell with two BaF_2 windows (sample thickness 50 μm) with an optical density of ∼0.8 OD at 445 nm, in water and heavy water solutions for measurements in the spectral regions of 970−1500 and 1500−1750 cm^{-1}, respectively.

2.2. Transient Visible-Pump Mid-Infrared-Probe Spectroscopy. With nonlinear optical methods we generate mid-IR-probe pulses tunable from 2000 to 950 cm^{-1} with a duration of ∼230 fs (fwhm) or shorter at a repetition rate of 1 kHz.[27] This broad spectral range allows for an observation of vibrational marker modes specific for the cis

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conformation absorbing around 1000 cm^{-1}. Simultaneously, visible-pump pulses at 445 nm with 100 fs duration were generated[28] to initiate the photoreaction, resulting in a system response function of ~230 fs (fwhm) or shorter. A half-wave plate was used to change the plane of polarization of the pump pulse to be parallel or perpendicular with respect to the probe pulse. Detecting absorbance changes for both polarizations ($\Delta A_{\parallel}$ and $\Delta A_{\perp}$) enables us to derive the angle Θ^e between the excited electronic transition dipole moment and the probed infrared transition dipole moments by $0.4 \cdot P_2(\Theta^e) = (\Delta A_{\parallel} - \Delta A_{\perp})/(\Delta A_{\parallel} + 2 \cdot \Delta A_{\perp})$ (with P_2 being the second Legendre polynome), where we used $\Theta^e = a \cos([(2D - 1)/(D + 2)]^{1/2})$ with the dichroic ratio $D = A_{\parallel}/A_{\perp}$. Anisotropy free data are obtained by $\Delta A_{iso} = \Delta A_{\parallel} + 2 \cdot \Delta A_{\perp}$.

2.3. Quantum Chemical Calculations. We performed density functional theory calculations of vibrational spectra for the PYP chromophore in the electronic ground state using the B3LYP/6-31+G-(d,p) method as implemented in Gaussian98.[29] Although density functional theory is well-known for its good performance with respect to the calculation of vibrational spectra for neutral molecules,[30] it has been pointed out that it has some deficiencies in describing anionic species due to unphysical Coulomb self-interaction.[31] However, other studies found no convincing evidence to support this claim.[32] Here we find a satisfying correspondence between experiment and theory. Electronic transition dipole moments for vertically excited states were calculated with the ZINDO method applying spectroscopic parameters.[33] An active orbital space of 80 molecular orbitals (HOMO-39 to LUMO+39) was chosen.

In the ZINDO single-point excited state calculations we used the chromophore (see Figure 2) covalently bound to the amino acid Cys69, together with the amino acids Glu46 and Tyr42 that are hydrogen-bonded to the phenolate part of the chromophore, as well as Arg52, for which a strong influence has been ascribed on the energetics of the chromophore excited state.[21] We related the directions of the vibrational transition moments of the P and I_0 states to the electronic transition dipole moment, using the same Cartesian coordinate system in the P and I_0 states. This Ansatz is justified because of the observation of minor position changes for C_α and C_β of Cys69 and the negatively charged oxygen atom of the chromophore phenolate group when going from the P to the PYP_B state (up to 300 ps).[13] Moreover, our experimental results on the C=O stretching band of Glu46 also indicate negligible relocation of the oxygen atom of the phenolate group (vide infra). Therefore, the origin of the coordinate system was placed at the C_β atom of the Cys69 residue. The *z*-axis was chosen to point toward the negatively charged chromophore phenolate oxygen and the negative *x*-axis lying in the plane spanned by the *z*-axis and the Cys69 $C_\beta - C_\alpha$ bond vector. The dipole moment for the first strongly allowed $\pi \rightarrow \pi^*$ electronic transition that is excited in the experiments is directed along the chromophore molecular plane, almost parallel to the *z*-axis (1.3° and 6.1° deviation for 3PYP/1NWZ and 3PHY structures, respectively).

3. Results and Discussion

3.1. Polarization-Sensitive Ultrafast Infrared Spectroscopy. Identification of vibrational marker modes that appear at designated frequency positions for specific conformations has provided insight into, e.g., light-induced trans/cis isomerization reactions.[34−37] Moreover, polarization-sensitive spectroscopy can provide detailed structural information through the angles (Θ)

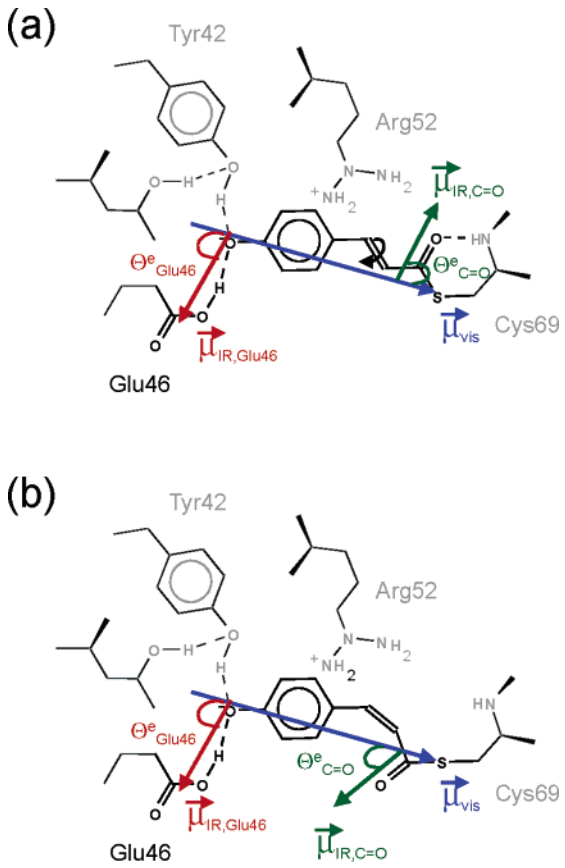

Figure 2. Polarization-sensitive vis/IR spectroscopy on the trans/cis isomerization of the chromophore of PYP. When the marker modes involve local vibrational motions, the experimentally measured anisotropies (with relative angle Θ^e between the electronic ($\vec{\mu}_{vis}$) and infrared ($\vec{\mu}_{IR}$) transition dipole moments) can directly be correlated to geometric configurations of the (transient) states. For delocalized normal modes experimental findings have to be compared to results of quantum chemical calculations. Our results show that for the dark P state, with the chromophore in the trans configuration (a), a local mode picture only holds for the νC=O mode of Glu46 whereas the stretching motion of the chromophore carbonyl moiety strongly mixed with other chromophore modes. For the cis conformation in the I_0 state (b) on the other hand both vibrational modes can be considered as local modes.

between the transition dipole moments of the electronic transition (excited by an ultraviolet- or visible-pump pulse) and the infrared active vibrational mode (measured by an IR-probe pulse). This approach has been used to determine the relative orientations of CO or NO ligands bound to and photodissociated from the heme iron in myoglobin and hemoglobin,[38−41] and for the excited state twisting of the chromophore of green fluorescent protein.[42] For PYP, direct insight into the trans/cis isomerization process could be provided by determination of the direction of the C=O stretching motion (see Figure 2).

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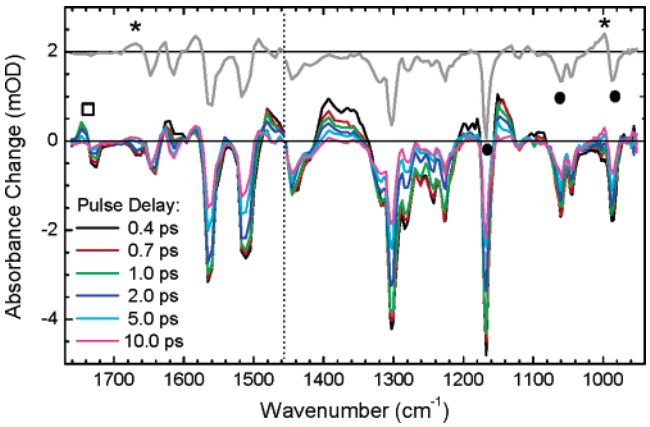

Figure 3. Transient absorbance difference spectra of PYP measured at different pulse delays under polarization anisotropy free conditions, showing initial bleaching of vibrational bands of the dark P state and the overlapping transient absorbance bands of the excited P*, the "hot" recovered P state, and the product I_0 state at early delays. The gray curve, indicating the averaged transient absorbance measured from 20 to 300 ps, consists of (negative) bleach signals in the ground P state and positive absorbance signals of the I_0 product state. This curve has been displaced 2 mOD units for clarity purposes. The vibrational marker modes are depicted as solid dots (P state) and as asterisks (I_0 state), while the location of νC=O of Glu46 is shown as an open square.

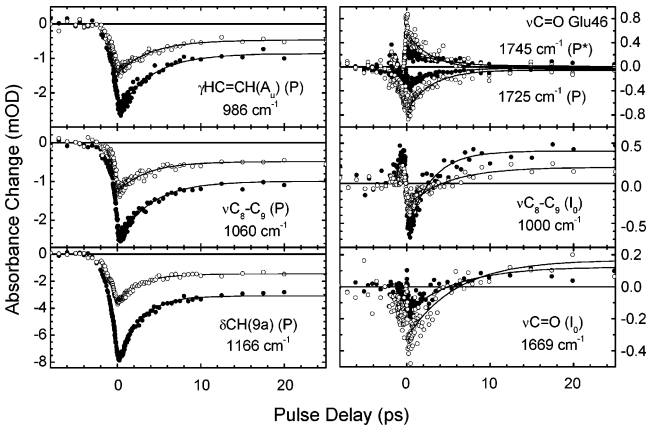

Figure 4. Transient dynamics of vibrational marker mode bands of the dark P, the excited P*, and the first intermediate I_0 states recorded with parallel (solid dots) and perpendicular (open dots) polarizations of vis-pump and IR-probe pulses.

However, as our quantum chemical calculations on the PYP chromophore indicate (vide infra), local vibrational motions of the chromophore atoms are often strongly mixed and as a result no distinct vibrational transition due to a local C=O stretching mode can be identified in the P state. Our approach instead is to identify first vibrational transitions that may be used alternatively as structural marker bands for the dark P, the excited P*, and the first intermediate I_0 states, as described in section 3.2. Following that, we compare in section 3.3 the frequency positions and anisotropies of all strong fingerprint vibrational transitions measured in transient mid-IR pump−probe spectra with quantum chemical calculations using specific chromophore configurations, for which reported PYP chromophore geometries[8,13,25] have been used as input.

3.2. Transient Polarization-Sensitive Infrared Spectra of PYP. We have monitored the early time structural dynamics of PYP after excitation at the absorption maximum at 445 nm through the IR-active vibrations in the fingerprint region (950−2000 cm^{-1}).

In addition, we have measured transient difference absorbance spectra for parallel and perpendicular polarizations of the visible-pump and IR-probe pulses with 230 fs temporal resolution. For pulse delays up to 300 ps these transient spectra show contributions of the early stages of the PYP photocycle, that is, the relaxation of P* back to the P state and the transformation to the I_0 state (Figure 1). In Figure 3 anisotropy free absorption changes upon photoexcitation are presented for different pulse delays. Negative signals result from bleaching of the IR-active fingerprint pattern of the P state at thermal equilibrium. On the other hand, positive IR-active absorption signals can be due to several factors: (i) vibrations in the electronic excited state P*, (ii) vibrations of the ground state P with high internal vibrational energy ("hot" P state, generated after internal conversion from the excited P* state), and/or (iii) vibrations in the first intermediate state I_0. The transient spectra show a partial refilling of the bleached transitions, consistent with 40 ± 5% of the excited PYP molecules returning to the P state.

Due to the branching pathways from P* toward P and I_0, the observed dynamics are rather complex, as contributions from different states may overlap, in particular in the spectral range between 1100 and 1700 cm^{-1}. This dynamic behavior described by multiple time constants does not necessarily imply a multiexponential decay of the excited state P*, but can also be due to intramolecular vibrational redistribution and vibrational cooling[43] of the initially "hot" P and I_0 states. Since these latter processes lead to a complex time-dependent behavior of frequency positions, shapes, and magnitudes of transient bands, a numerical treatment using singular value decomposition or decay associated spectra[19,20] for extraction of the contributions from the different states is not warranted.

Fortunately, two spectral regions with isolated bands enable us to assign structural marker modes for the P, P*, and I_0 states. In Figure 4 we show the transient dynamics at selected frequencies of the vibrational marker modes. The vibrational transition of the C=O stretching mode of the COOD side group of Glu46 is found between 1720 and 1760 cm^{-1}.[9,44] Electronic excitation to P* causes a frequency upshift from 1725 to 1747 cm^{-1}, indicating a weakening of the hydrogen bond of the COOD group with the phenolate moiety of the PYP chromophore in the P* state.[19] This is consistent with a net charge relocation upon electronic excitation from the phenolate to the (C_9=O)−S side of the chromophore[45] that may be quantitatively addressed by inspection of frequency shifts of C=O stretching bands caused by changing hydrogen bond interactions.[46] When P* decays to P or to I_0, the hydrogen bond strength recovers to about its original strength, as is evidenced by the disappearance of the response in the 1720−1760 cm^{-1} spectral region. More importantly, when going from P via P* to I_0, the anisotropy of the C=O stretching mode of Glu46 remains the same, indicating no effective reorientation of this amino acid side group. As a result, we can conclude that the initial stages of photoisomerization of PYP involve structural rearrangements of the chromophore without a relocation of the oxygen end of the phenolate group.

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For the second spectral region we refer to low-temperature FT-IR studies of trapped intermediate states of wild-type PYP and mutants with isotopically altered chromophores, in which it has been established that structural marker bands for the chromophore are located in the range between 970 and 1200 cm^{-1}.[14,47,48] In particular, our results show that the bleaching band at 1060 cm^{-1} and the appearance of the I_0 band at 1000 cm^{-1} provide direct insight into the first steps of geometrical rearrangement in PYP that occurs upon excitation of the chromophore. The PYP difference spectrum measured at room temperature is not fully identical, but has a strong resemblance to the FT-IR spectrum of low-temperature trapped PYP_B (and not PYP_H).[14] The structure of the PYP_B state has been determined by X-ray diffraction to be in a cis geometry.[13,15] Thus, our results with room-temperature PYP allow us to conclude that the I_0 state is formed with a cis chromophore geometry. We note that earlier CARS measurements on wild-type PYP and the E46Q mutant have focused on the 1100−1700 cm^{-1} frequency range, where the phenolate bands are found, without probing the (weaker) νC_8-C_9 trans/cis marker bands. Based on this, no decisive conclusion on trans/cis isomerization could be drawn on the early stages of I_0 and $I_0^{\ddagger}$ intermediates.

Both the appearance of the I_0 product band at 1000 cm^{-1} and the disappearance of the Glu46 C═O stretching band at 1747 cm^{-1} can be fit using a single-exponential decay function with a 3 ± 1 ps time constant, consistent with a single-step conversion from P* to I_0. This result is contrary to the assumption of a two-step isomerization process reported previously.[19] Based on arguments given above, however, the two-step isomerization process derived from a global analysis of transient IR spectra is likely resulting from the convoluted chromophore band contributions of P*, "hot" P, and I_0 states. The Glu46 C═O stretching vibration on the other hand is an ideal marker mode for the decay of the P* state, as it is not a chromophore band and will not be affected significantly by IVR and vibrational cooling dynamics of the chromophore.

In accordance with the earlier reported transient CARS[37] and transient IR[19] studies, we do not observe any long tail components on a time scale of 20−50 ps. In contrast, several time-resolved fluorescence and pump−probe measurements have indicated signal contributions with time constants of 10 ps[49] or even 46 ps.[50,51] Whether these discrepancies in time scales are due to sample inhomogeneities remains as a subject of further research. We do not observe significant absorbance changes for pulse delays from 20 ps up to 300 ps (maximum delay in the current experiments). This could mean that the $I_0^{\ddagger}$ state does not appear with the reported time constant of 220 ps.[17] Recent analysis of pump−dump−probe experiments has resulted in the suggestion that the $I_0^{\ddagger}$ state does not exist.[51] We suggest an alternative interpretation. Visible pump−probe experiments probing electronic transitions are known to be strongly sensitive to solvent rearrangements ("solvation dynamics").[52−54] In contrast, vibrational transitions are less sensitive to solvation dynamics, except for the special case of hydrogen bond interactions.[43] In the case of PYP the transient IR spectra monitor changes on the chromophore as well as specific vibrational marker modes of amino acid side groups involved in hydrogen bonding, i.e., those of Tyr42, Glu46, and Cys69. If on the other hand other amino acid groups of the protein pocket rearrange (e.g., Arg52), negligible changes occur in the transient IR spectra, except perhaps for amide I and amide II vibrations, but substantial changes are expected in transient visible pump−probe spectra. Further experiments are necessary to elucidate whether the I_0 and $I_0^{\ddagger}$ have similar chromophore structures, and changes are caused by an altering protein pocket, responding at these later times to the structural changes of the chromophore at early pulse delays.

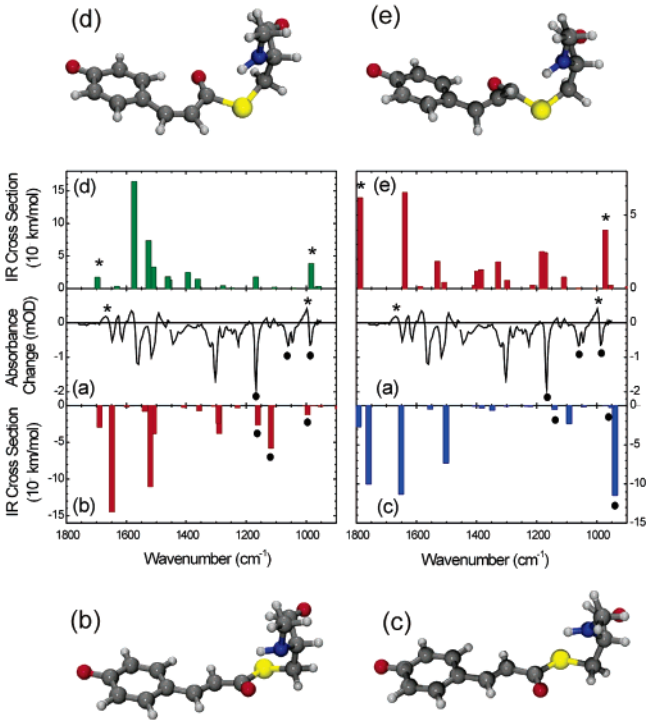

Figure 5. Comparison of the vibrational mode patterns in experiment (a) and theory (b−e). The mode patterns (b−e) have been calculated using structures previously reported. Trans chromophore in the dark P state as derived from crystalline PYP X-ray diffraction (b) and solution phase PYP NMR (c). Cis chromophore in the optimized I_0 product state (d) and in the PYP_B product state as derived from crystalline PYP X-ray diffraction (e). The vibrational marker modes are depicted as solid dots (P state) and as asterisks (I_0 state).

3.3. Infrared Active Vibrational Band Assignments and Relation to Chromophore Geometry. For determination of the chromophore structure we used the transient mid-IR spectra averaged over pulse delays from 20 to 300 ps after excitation (Figure 5a), that is, when P* has fully relaxed into either the ground state P or the intermediate state I_0 and vibrational cooling is complete. For these long pulse delays the difference spectrum is the result of negative absorption (bleach) signals caused by vibrational transitions of the P state and positive absorption signals due to vibrational bands of the I_0 state. The observed

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vibrational patterns with derived angles are summarized in Table S1. For analysis we have focused on the 1800−1660 and the 1400−970 cm^{-1} spectral ranges, where several marker modes for the geometry and the protonation status of the chromophore are found, and the vibrational response is not superimposed by amide I and amide II contributions.

Vibrational spectra of chromophores with conjugated π-orbital moieties are strongly sensitive to small structural changes. The degree of planarity of the chromophore defines the degree of conjugation as well as the degree of coupling between local vibrational motions, leading to delocalized normal modes. For PYP the degree of planarity of the enone moiety strongly determines the vibrational band patterns. Moreover, as the chromophore in PYP is anionic, but linked to nearby hydrogen donors Tyr42, Glu46 on the oxygen of the phenolate moiety, and the Cys69 on the chromophore C═O group, these hydrogen-bonding interactions also influence the chromophore geometries. Determination of the (transient) vibrational spectra by quantum chemical calculation methods should provide insight into the three-dimensional structure of the PYP chromophore, albeit a formidable task to optimize all possible coordinates.

To calculate chromophore properties of PYP in the natural environment in a reasonable amount of time, we thus chose to apply experimentally determined molecular structures available in the Brookhaven protein database. It turns out that the available molecular structures provide insight into the key role that the planarity of the chromophore enone moiety plays in dictating vibrational mode patterns. We based our calculations on the following data sets (as taken from the RCSB Protein Data Bank: (i) 3PYP geometries derived from an X-ray diffraction study on PYP crystals.[13] This data set includes atomic positions with 0.85 Å resolution for the PYP dark P state and for the trapped intermediate PYP_B. The more recently reported 1NWZ structure has an improved resolution of 0.82 Å for the coordinates of the P state.[25] The chromophore coordinates 3PYP and 1NWZ have similar values, whereas the positions of the COOH group of Glu46 differ for the two structures. (ii) 3PHY geometry derived from a nuclear magnetic resonance study on PYP in solution.[8] This data set contains 26 possible structures. All of these chromophores exhibit a planar geometry, with the C═O group tilted less than 0.6° out of the chromophore plane.

The reported C−H and N−H distances are too short[55] and thus result in unrealistically high C−H and N−H vibrational frequencies as well as wrong combinations of local C−H and N−H bending motions in fingerprint normal modes. Thus, we performed a relaxation of all hydrogen atom positions by partial geometry optimization. In the B3LYP results for vibrational analysis of the different chromophore geometries, imaginary frequencies have been found, albeit only for low-frequency modes, and the conclusions drawn on the fingerprint region remain essentially unaffected.

For the calculations of the infrared vibrational transitions we constrained the atomic data set to the chromophore including the covalently bound Cys69. For the PYP trans chromophore in the electronic ground dark P state we used the 3PYP geometry (after optimization of C−H and N−H distances) (Figure 5b) and the 3PHY geometry (Figure 5c). For the PYP cis chromophore in the first intermediate I_0 state we used the atomic positions given in the 3PYP data set (after optimization of C−H and N−H distances) (Figure 5e), as well as a structure obtained by further partial geometry optimization of the chromophore while keeping the Cys69 amino acid and the phenolate oxygen fixed (Figure 5d).

In the Supporting Information we describe the details of our analysis of the transient spectra, where we refer to previous vibrational spectroscopic studies on PYP,[9,37,44,56,57] including isotope substitution (H/D exchange at C_2 and C_4 positions in the phenolate moiety, H/D exchange at C_8, and $^{12}C-^{13}C$ exchange at C_9 of the ethylenic group) to facilitate the vibrational band assignments.[14,58] The results from the numerical analysis are summarized for the dark P state in Table S2 using the 3PYP/1NWZ structure (Figure 5b) and in Table S3 for the 3PHY geometry (Figure 5c). Results obtained for the I_0 state using the 3PYP geometry (Figure 5e) and the structure based on partial chromophore geometry optimization (Figure 5d) are collected in Table S4.

For the assignment of the experimentally observed chromophore vibrational bands of the P and I_0 states we compare the experimental values for frequencies ν^e and angles Θ^e with the calculated frequencies ν^c and angles Θ^c for the four different input structures (see Figure 5). Based on this comparison we conclude that the 3PYP/1NWZ trans chromophore geometry with the C═O tilted out-of-plane, as derived from X-ray diffraction on crystalline PYP,[13,25] describes our observations of the dark P state in a more appropriate way than the 3PHY geometry with a fully planar trans chromophore as deduced from NMR measurements on solution phase PYP.[8] On the other hand, the 3PYP geometry with a cis chromophore, as derived from X-ray diffraction on PYP_B,[13] does not have a satisfying correspondence with our observations of the first intermediate I_0 state. In contrast, a cis chromophore with a planar enone geometry is a better representation of the I_0 state. Based on this, we added the resulting assignment for structures (b) and (d) in Table S1. When comparing the calculated IR cross sections for the vibrational marker modes for structures (b) and (d) with the experimental band intensities for the dark P and intermediate I_0 states, we derive that besides the 40% quantum yield for P state recovery, the remaining 60% fraction reaches I_0 (with a 10% error).

4. Conclusions

Summarizing, we have determined the geometries of the chromophore and the hydrogen-bonded COOD side group of Glu46 in the dark ground P and the first intermediate I_0 states by comparison of experimental transient infrared absorbance spectra and ab initio calculations of vibrational spectra. This is the first demonstration of application of polarization-resolved mid-infrared spectroscopy for the elucidation of structural changes of a medium-sized chromophore subject to a photoinduced trans/cis isomerization. This work shows the potential of the method as a tool for time-resolved vibrational spectroscopic[43] investigations of condensed phase ultrafast chemistry ("femtochemistry"[23,24]). We derive that the chromophore of PYP

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in the P state has a trans geometry with the C═O group tilted out of the chromophore plane. In the I_0 state, however, the chromophore has a cis geometry with a planar enone moiety. Thus, we can conclude that isomerization occurs with a 3 ps time constant and that subsequent events leading to the signaling state are the result of protein structural changes to accommodate the isomerized chromophore. Moreover, the COOD group of Glu46 does not change its orientation going from the P to the electronically excited P* and the I_0 states. From this we conclude that in the primary stages of trans/cis isomerization substantial structural rearrangements only occur on the enone moiety of the chromophore, whereas the phenolate does not significantly change its position.

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Supporting Information Available: Full listing of ref 29, details on vibrational band assignments, and results on calculations summarized in Tables S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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