Time and Frequency Domain Investigations on Ultrafast Photoreaction Dynamics of Photoactive Yellow Protein (PYP)

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Low frequency vibrations of the native PYP and related systems in solution have been investigated in "time" (fs fluorescence up-conversion) and "frequency" (resonance Raman) domains to elucidate their role in ultrafast photoisomerization reaction dynamics of PYP. Tentative assignments of the oscillatory components to particular vibrations are proposed supported by normal mode calculations based on DFT and ab initio MO methods. It is concluded that the out-of-plane skeleton bending mode of the chromophore, γ_{16}, is responsible for the observed oscillations in native and all mutant PYPs ($f_1 \sim 135$ cm^{-1}), while in-plane ν'_{42} and ν'_{43} modes are probably responsible for oscillations observed in the PYP analogue with locked chromophore. A dynamic model called "trigger mode mediated guidance" has been proposed to explain in simple terms the ultrafast primary process initiating PYP's photocycle. Furthermore, first successful fluorescence dynamics experiments on PYP single crystals with femtosecond time resolution are presented and discussed.

Key Words: Photoactive Yellow Protein (PYP), Fs fluorescence spectroscopy, Resonance Raman spectroscopy, Coherent oscillations

1. Introduction

Recent spectacular developments in nanophotonics have once again stressed the importance of biomolecules for future nanoscience and nanotechnology applications.[1)] However, many fundamentals are still unknown and great interdisciplinary efforts and combined research of biologists, chemists, physicists and scientists of other fields are going on to understand various photo-processes in biological systems. Once understood, the processes in biomolecules could be controlled and manipulated. If controlled, they will pave the way for a wide variety of practical applications (proteins with diode functions, protein-based computers and three dimensional optical storage memories, etc.). In this article, we present our latest results on photoactive yellow protein (PYP), a small (125 kDa), water-soluble photoactive protein with excellent physico- and photochemical stability. Because several other works included in this special issue already introduce the biological function and impact of PYP, we refrain from describing PYP's basics. The reader is referred also to a recent article by Hellingwerf et al.,[2)] where review of up to date research on PYP and related systems is presented.

The initial photoisomerization event in PYP usually occurs on several hundred femtoseconds time scale.[3)] Using results of diverse experimental studies[3,6,11,14)] and quantum mechanical calculations[13)] it was demonstrated that PYP's photocycle is initiated by flipping the carbonyl group of its chromophore (p-coumaric acid) rather than its bulky aromatic ring. This result implies that the chromophore isomerization requires only minor rearrangements of the chromophore/protein structures in protein nanospace (PNS) during the initial ultrafast events. In relation to the important role of the PNS for the ultrafast reaction of the chromophore, it should be noted here that our fs fluorescence decay dynamics studies on PYP[3)] indicated an ultrafast twisting in vibrationally non-relaxed state immediately after the relaxation from the excited Franck-Condon (FC) state to the Fluorescence (Fl) state in the course of the photoisomerization process. Accordingly, we have conceived that the ultrafast fluorescence of the chromophore in PNS should exhibit coherent oscillations coupled with the twisting. With improved time resolution of the apparatus we have actually observed such oscillations recently.[6,11)] However, the origin and role of the *low frequency* intraprotein vibrations in the early ultrafast events of PYP is not very clear yet. Following simple considerations emphasize their potential importance. A flipping, twisting or complete isomerization of the chromophore in a given photoresponsive protein is presumably *unidirectional* structural deformation occurring on a characteristic time scale. Hence, specific low frequency intrachromophore vibrational modes (constituting *periodic* structural deformations) with periods on the same or comparable time scale could be effectively coupled to isomerization and play a crucial role in the later reaction. For instance, in case of PYP the fastest twisting (or flipping) reaction occurs on ~ 300 fs (~ 50 %) time scale, thus, vibrational modes (if any) with periods in 100 fs ~ 1 ps time region (corresponding frequencies

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between 33 cm^{-1} ~ 330 cm^{-1}) could be of special importance. To our best knowledge, there are no studies on PYP or related systems to identify and assign the low frequency intraprotein vibrations in the 30 cm^{-1} ~ 330 cm^{-1} spectral region.

Indirect evidence concerning the involvement of coherent vibrational modes in photoisomerization reactions of bacteriorhodopsin[4] and bovine rhodopsin[5] has been gained also from time resolved fluorescence studies. In the case of native PYP and its mutants, coherent oscillations have been detected first by fluorescence up-conversion technique,[6] and later by an optical Kerr-gate spectroscopy in native PYP.[7] Although observed oscillations are an interesting physical phenomenon, it still remains to be demonstrated that they are important for understanding the early events in PYP's photoreaction. The first step in this direction would be the firm assignment of observed oscillatory modes, however, "time domain" experiments alone seem not to be sufficient for unambiguous assignment of the coherence to particular vibrational mode(s). Vibrational spectroscopy, on the other hand, is better suited for this purpose. In our special case, where the emphasis is put on observation of *low frequency* vibrations, resonance Raman (RR) spectroscopy seems to be advantageous over IR methods because measurements in the low spectral region with later technique, especially on biomolecules, bear a major difficulty arising from the large absorption of water.

In this work, using RR spectroscopy, we have determined low frequency vibrational modes of the native PYP for the first time and propose a tentative assignment of observed modes. Additionally, we have found correlations between data obtained from the "time" domain experiments with those obtained by "frequency" domain experiments and discuss the role of low frequency intraprotein vibrations in ultrafast photoisomerization reaction dynamics of PYP. Finally, ultrafast fluorescence from a PYP single crystal has been successfully monitored for the first time and we discuss its possible impact on a widely circulated, but not yet unambiguously confirmed assumption, that the protein structure and its earlier ultrafast photoinduced evolution are the same in liquid as well as crystal phases.

2. Experimental Section

Native PYP, its site-directed mutants and analogues were prepared as reported previously.[8] PYP crystals (~$0.5 \times 0.05 \times 0.05$ mm^3 size, $P6_5$ space group) were obtained by vapor diffusion in which 1 μl of protein solution (30 mg/ml) was mixed with an equal volume of solution containing 45 % PEG 2000 and 100 mM MES pH 6.5. The femtosecond time-resolved fluorescence decay curves were measured using a fluorescence up-conversion apparatus.[3,6] Ten scans (with 6.67 fs steps) in alternate directions were accumulated to give a single transient with acceptable signal-to-noise ratio. As an instrument response function a cross-correlation signal between fundamental (820 nm) and its second harmonic pulses was used (FWHM ~ 110 fs). All measurements were carried out at room temperature (~ 20 ˚C). Resonance Raman (RR) spectra were obtained as described previously.[9] Analysis of fluorescence decay curves were performed using Igor Pro graphics software (WaveMetrics, Inc.). Fourier transforms of individual decays were performed using Fast Fourier Transform (FFT) algorithm to compute discrete Fourier transform spectra (DFTS). The frequency resolution in these calculations was typically ± 7 cm^{-1}. The bandwidths of the DFTS obtained by this procedure are not accurate, hence physically irrelevant. The optimized geometry and harmonic vibrational frequencies for a chromophore model, deprotonated *trans*-4-hydroxycinnamyl ethyl thiolester (HCET) and locked chromophore analogue model, deprotonated 7-hydroxy-coumarin-3-carboxyl ethyl thiolester (HCCET), were estimated using various quantum chemical calculations. Namely, Hatree-Fock (HF) and density functional theory (DFT) using the 6-31G** basis set were used for the ground state (S_0), whereas the single-excitation configuration interaction (CIS) method with the 6-31G** basis set was employed for the excited state (S_1) normal mode calculations. These calculations were done using the Gaussian98 software package (Gaussian, Inc.). The hybrid functional B3LYP was used for the DFT calculations.

3. "Time Domain" Investigations

Fluorescence decay dynamics of native PYP and related systems have been measured at equidistant (10 nm step) wavelengths in a broad spectral region (460 nm ~ 620 nm) covering whole steady state fluorescence spectra. Additional details of these observations have been summarized in recent articles by Mataga et al..[10,11] Briefly, for intact proteins, the fluorescence decay curves with coupled oscillations can be well reproduced by double exponential decays superimposed with exponentially damping two oscillatory modes. On the other hand, for denatured PYP no oscillations in fluorescence decays have been observed. The dynamics is dominated by *solvation effect*.[11] Three exponential model function with a rise component was sufficient to reproduce accurately the dynamics in this case. Our analysis shows that the initial phases of the oscillatory modes in proteins depend on observation wavelength and a phase shift of ~ π from "blue" to "red" is clearly observed for both modes.[11] Hence, the observed oscillations could be explained by the transition frequency modulation mechanism. Further evidence supporting this mechanism is the fact that the amplitudes of vibrations are rather small at the maximum compared with that at the blue and red edges of the fluorescence spectrum. Theoretically, at the wavelengths near the fluorescence maximum, a twice-higher frequency is expected to be involved as a wave packet passes the maximum of the spectrum twice in one period. Practically, however, the amplitude of the "double" frequency is expected to be rather small due to a broad fluorescence spectrum and finite time/frequency resolution. Furthermore, in our experiments the bandwidth we can excite impulsively is estimated to be ~ 175 cm^{-1} (~ 90 fs at 410 nm), i.e., not enough to capture the expected double mode at around ~ 270 cm^{-1}. The described pattern with suppressed oscillating component near the fluorescence maximum is clearly observed for ~ 135 cm^{-1} mode in Fourier transformations† of decays presented in Fig. 1.

The general features in other mutants examined in this work are qualitatively rather similar to the above described picture and similar oscillatory modes (130 ~ 140 cm^{-1} and 40 ~ 70 cm^{-1}) have been observed as well. Although an accurate quantitative analysis of damping times for each coupled mode in correlation with specific mutation site would be most desirable, such an analysis at this stage is hampered by the time resolution of present

† We note that it would be deceptive to Fourier transform numerically extracted oscillatory components alone, because important details such as peak intensities in the transformed spectra depend sensitively on the method used to subtract off the underlying *non-exponential* excited state population decay. Hence, we present in Fig. 1 and Fig. 3 the DFTS of whole fluorescence transients.

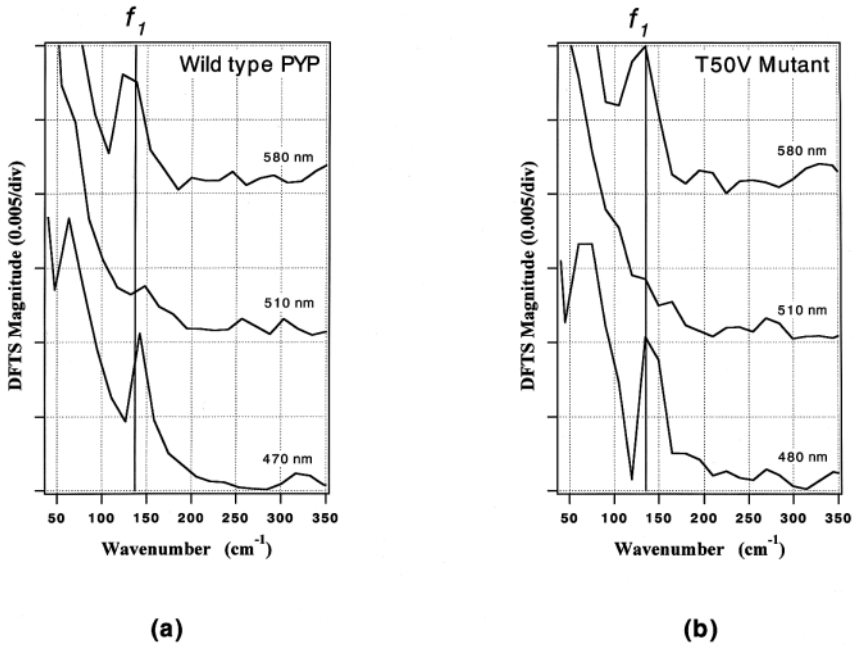

Fig. 1 Low-frequency components in the fluorescence decays of native PYP (a) and T50V mutant (b) at three characteristic wavelengths on blue, top and red sides of the fluorescence spectra obtained by Fourier transformations. The spectra were shifted by 0.01 step on the Ordinate axis for clarity.

experiments and methods/models used to subtract off the underlying non-exponential decays.

Another way to gain further insight into the primary dynamics of PYP is to study PYP analogues with modified chromophores. Particularly, fluorescence dynamics of the PYP analogue with locked chromophore is of primary interest. Its absorption at ~ 443 nm and rather small Stokes shift (~ 1100 cm^{-1}) indicate that the chromophore is not exposed to water environment fitting well into the PNS, and no solvation effect in its primary dynamics was observed.[11] In this analogue, the vinyl double bond of the chromophore is locked by the presence of a covalent bridge. As expected, the fast fs- and ps- components totally disappear and the fluorescence decay becomes very slow (~ 60 ps), indicating that the ultrafast photoisomerization does not occur at all. This observation nicely shows the ability of transient fluorescence spectroscopy in "visualizing" the correlation between the chromophore structure and its photoisomerization rate. Furthermore, weak oscillatory features on both short and long wavelength regions of locked chromophore analogue spectrum could be observed.[11] A model function with three oscillatory components could nicely fit the experimental decays. A decay example together with the dynamics of the native PYP is shown in Fig. 2. The fits predict the frequencies of coupled oscillatory modes for the locked chromophore analogue to be at around ~ 60 cm^{-1}, ~ 115 cm^{-1} and ~ 165 cm^{-1}. For convenience, further analysis and comparative discussions concerning the frequencies of coupled modes in different systems are hereafter curried out in the "frequency" domain representation presented below.

In Fig. 3 DFTS of "time" domain experiments for native PYP, denatured PYP, its several mutants and locked chromophore analogue are summarized. In denatured PYP there are no detectable vibrational modes. The heavy background at low frequency region is due to the fast decay (solvation!) in the time space. In native PYP and all mutants there are two dominant modes involved (~ 60 cm^{-1} and ~ 140 cm^{-1}). However, in locked chromophore analogue the picture is rather different and intriguing. One can clearly see two modes at around ~ 115 cm^{-1} and ~ 165 cm^{-1} instead of a single mode at ~ 135 cm^{-1} observed for the native protein. While in the locked chromophore analogue a fast photoisomerization reaction does not occur at all, it is reasonable to speculate that the observed difference in DFTS in one or another way is related to it.

4. "Frequency Domain" Investigations and Comparative Discussion

Low frequency RR spectra of the calibration standard (neat fenchone), PYP_{dark}, PYP_L and denatured PYP are shown in Fig. 4. Denatured PYP did not show any significant low frequency Raman bands. This is in full agreement with non-oscillating fluorescence dynamics in "time" domain experiments described above. In the 100 ~ 210 cm^{-1} spectral region there are at least four Raman bands for PYP_{dark} at 104, 153, 183 and 202 cm^{-1}. The Raman band at 153 cm^{-1} seems to be a singlet while bands at 183 cm^{-1} and 202 cm^{-1} are observed as not-well-resolved doublet. For PYP_L only one band at 191 cm^{-1} can be clearly distinguished in the same spectral region within present S/N ratio.

We note that comparison of results from "time" and "frequency" domain experiments should be carried out with some important precautions. In "time" domain experiments, we are observing the oscillations of the *spontaneous* fluorescence reflecting *only* the excited state properties of the protein, hence, there is no question that the oscillations also originate from the excited electronic state of the protein (a wave packet motion on the excited state potential surface). Contrary, resonance Raman spectroscopy probes the vibrational modes in the ground state coupled to *excitation* of the protein. Hence, the time resolved experiments described here *are not* "time" domain analogues of RR and Fourier transforms can not be directly compared with it. This problem could be theoretically overcome by applying time resolved excited state Raman spectroscopy technique.[12] How-

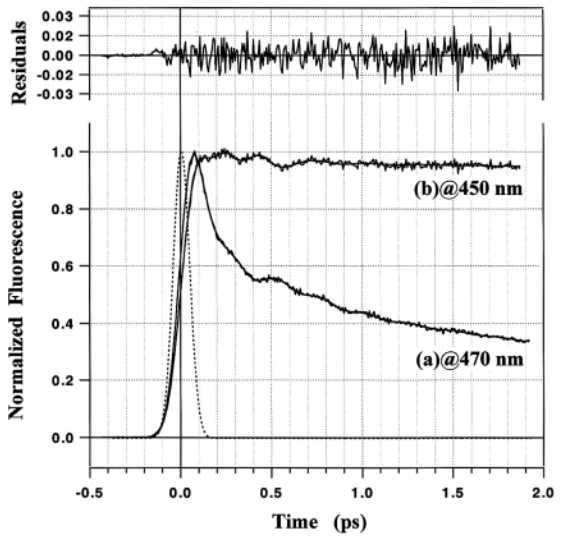

Fig. 2 Examples of fluorescence decays for the native PYP (a) and locked chromophore analogue (b) at the blue edge of the steady state spectrum. The solid lines show the best fits and the dashed line shows the instrumental response. Upper panel shows the fitting residuals for (b).

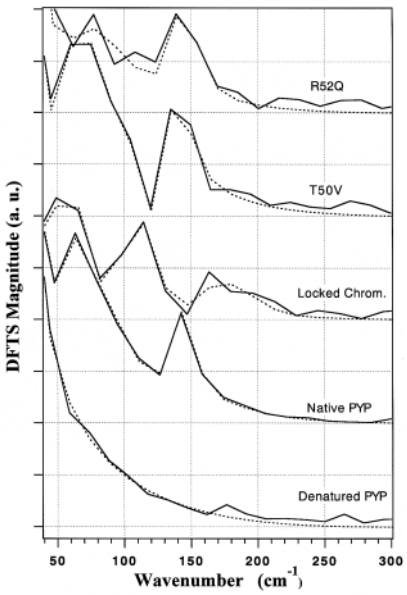

Fig. 3 Frequency components in the fluorescence decays at the blue edge of the steady state fluorescence spectra of native PYP, denatured PYP, its several mutants and locked chromophore analogue obtained by Fourier transformation. The solid lines are Fourier transforms of experimental data and dashed lines are Fourier transforms of the fits in "time" space. The spectral resolution depends on the scan step (6.67 fs) and the number of points in the time domain data set, and is ± 7 cm^{-1} in this case. The spectra were shifted by a 0.01 step on the Ordinate axis for clarity.

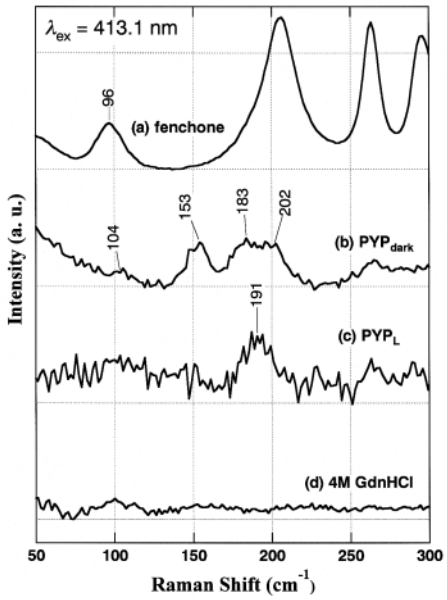

Fig. 4 Raman spectrum of the calibration standard fenchone (a) and RR spectra of native PYP_{dark} (b), PYP_L (c) and denatured PYP (d).

ever, our attempts to measure RR spectra of PYP's excited state or its transient photointermediate states were unsuccessful, because of a large fluorescence from the sample. On the other hand, model quantum mechanical calculations have proven to be a good way for normal mode estimation. In present calculations, HCET for native- and HCCET for analogue PYP chromophores were used as models.† This choice of model chromophores is based on a precaution that truncation of a covalent bond between the chromophore and protein could potentially affect low frequency modes.

Optimized structures and calculated atomic displacements for selected normal modes of HCET and HCCET are presented in Fig. 5, and observed and calculated frequencies for native PYP and its model (*trans*-HCET), and locked chromophore analogue and its model (HCCET) are summarized in Table 1 (a) and (b), respectively. The Raman bands in native PYP's spectrum at 183 cm^{-1} and 202 cm^{-1} are assigned to in-plane skeleton bending modes ν_{42} and ν_{41}, while the bands at 104 cm^{-1} and 153 cm^{-1} are assigned to out-of-plane skeleton bending modes γ_{17} and γ_{16}, respectively. Furthermore, a close examination of the computed frequencies in the ground S_0 (HF, B3LYP) and excited S_1 (CIS) states reveals that calculations predict a slight downshift tendency for the out-of-plane (γ) modes in the (excited) S_1 state whereas in-plane (ν) modes are almost unchanged in both (ground) S_0 and (excited) S_1 states. This result qualitatively supports the assignment of the f_1 (135 cm^{-1}) mode observed in "time" domain fluorescence experiments to γ_{16} (130 cm^{-1} for CIS) because the RR spectrum of the native PYP shows a band at 153 cm^{-1} (146 cm^{-1} for DFT). Concerning the f_2 (~ 50 cm^{-1}) mode observed in "time" domain experiments, the calculations predict two possibilities, i.e., it could be the ν_{43} or γ_{18} mode. How-

† The chromophore of PYP has the low symmetry *C*s. Thus, there are only two A' and A" configurations and the symmetry notations are not so useful in this case. Briefly, in common nomenclature the notations $\gamma$and ν correspond to out-of-plane and in-plane vibrations, respectively, and the subscript numbers (1, 2, 3, ...) are numberings from higher- to lower-frequency. However, because the number of atoms (also the number of normal modes) is different for the native and analogue chromophores, one should be careful when comparing modes with similar notations such as γ_{16} or ν_{43}. Thus, in our special case the same notation was used for the similar mode regardless of its order, i. e., we used the notation of the HCMT[15] for the similar modes for both native chromophore model (HCET) as well as locked chromophore analogue model (HCCET) and the later modes are marked with a dash.

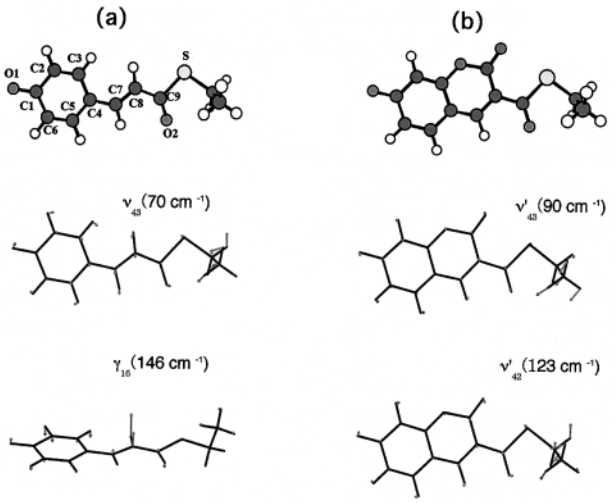

Fig. 5 A sketch of the optimized structures for *trans*-HCET (a) and HCCET (b), and some selected low frequency vibrational modes in the ground state. Arrows in each sketch show corresponding atomic displacement vectors. The frequency values obtained from DFT (B3LYP) calculations for presented modes are also given.

Table 1 Observed and calculated frequencies (normal modes in ground (S_0) and first excited (S_1) states) for (a) native PYP and its model *trans*-HCET, and (b) locked chromophore analogue and its model (HCCET).

(a)

Fluorescence **(S_1)** ν_{obs}/cm^{-1}	Raman **(S_0)** ν_{obs}/cm^{-1}	HF **(S_0)** ν_{cal}/cm^{-1}	B3LYP **(S_0)** ν_{cal}/cm^{-1}	CIS **(S_1)** ν_{cal}/cm^{-1}	Assign.[14]
		35	31	19	γ_{20}
		47	40	31	γ_{19}
		67	70	69	ν_{43}
50		81	55	63	γ_{18}
	104	106	81	101	γ_{17}
135	153	154	146	130	γ_{16}
	183	186	177	183	ν_{42}
	202	237	219	238	ν_{41}
		241	228	195	γ_{15}
		259	245	257	ν_{40}

(b)

Fluorescence **(S_1)** ν_{obs}/cm^{-1}	Raman **(S_0)** ν_{obs}/cm^{-1}	HF **(S_0)** ν_{cal}/cm^{-1}	B3LYP **(S_0)** ν_{cal}/cm^{-1}	CIS **(S_1)** ν_{cal}/cm^{-1}	Assign.[14]
		32	29	25	γ'_{20}
60		61	58	59	γ'_{19}
115		102	90	106	ν'_{43}
		81	79	61	γ'_{18}
		118	115	95	γ'_{17}
165		185	177	149	γ'_{16}
		139	123	141	ν'_{42}
		216	207	215	ν'_{41}
		255	238	237	γ'_{15}
		240	225	241	ν'_{40}

ever, because the probability of out-of-plane modes involvement in the photoisomerization process is higher, we speculate that its assignment to γ_{18} mode is more plausible.

We have tried to measure also the low frequency RR spectrum of the PYP analogue with locked chromophore at two excitation wavelengths (413.1 nm and 406.7 nm) but it revealed that in both experiments weak low frequency Raman bands were obscured by a strong fluorescence from the sample (data not shown). Unfortunately, the non-resonance Raman experiments failed, too, because the Rayleigh scattering from the sample was too big to see small Raman bands. At present stage we have to content ourselves with the discussion and assignments of the oscillatory modes observed for locked chromophore analogue in "time" domain based only on the DFT calculations. The ~ 60 cm^{-1} mode of locked chromophore analogue (Table 1 (b)) could be either γ'_{18} or γ'_{19} mode (61 cm^{-1} and 59 cm^{-1} in CIS calculations for HCCET, respectively). Similarly, the ~ 115 cm^{-1} mode could be correlated with γ'_{17} or ν'_{43}, and ~ 165 cm^{-1} mode could be either out-of-plane γ'_{16} or in-plane ν'_{42} mode, respectively. However, because in locked chromophore analogue the fast photoisomerization does not occur at all, one could speculate that both modes at 115 cm^{-1} and 165 cm^{-1} are of in-plane origin, i.e. they correlate with ν'_{43} and ν'_{42} modes, respectively. In this analogue system, where the chromophore's large scale out-of-plane movements, flipping or twisting are unlikely due to its rigid structure, only totally symmetric A' (in-plane) modes with no role in photoisomerization reaction, couple to the transient fluorescence and are responsible for the observed oscillations. Nevertheless, the lowest mode (~ 60 cm^{-1}) observed in "time" domain experiments, is more sensitive to the PNS structure (i.e. chromophore-apoprotein interactions). Hence, there is a possibility that this mode could have an out-of-plane origin also in locked chromophore analogue (γ'_{18} or γ'_{19}), because the stronger coupling to amino acid residues in PNS could activate corresponding A" out-of-plane modes. We note that these assignments are based on the vibrational analysis of the chromohore alone and in this regard present assignments are *tentative*. Further experimental studies are required for firm assignments. In calculations, one needs also to consider the effects of the PNS on the low frequency modes. Hence, further *ab initio* DFT calculations mimicking apoprotein-chromophore interactions by introducing intermolecular hydrogen bonds around HCET, steric and/or electrostatic effects of the protein moiety etc. may shed more light on the RR spectra and help to obtain detailed structural information that can not be easily derived from the experiments alone.

Based on above presented results and analysis, here we suggest a possible photoisomerization model for PYP and call it "trigger mode mediated guidance". After a blue light absorption, the system is elevated into its Franck-Condon excited electronic state with excess vibrational energy due to a number of intrachromophore vibrational modes. FC→Fl conversion takes place rapidly accompanied with ultrafast IVR (intramolecular vibrational redistribution) leading to Fl state with selectively excited coherent mode.[11] This mode, identified as γ_{16}, manifests itself as oscillations in the fluorescence decay, flips the C8 carbon and corresponding hydrogen atoms of the chromophore (see corresponding atomic displacements of γ_{16} mode in Fig. 5) and effectively triggers the isomerization. Because the mutation effect almost does not affect this mode, we suppose that it only triggers the reaction without controlling its rate. On the other hand, for the lowest mode (~ 50 cm^{-1}) the relative spread

of frequencies depending on mutation was remarkably larger and its damping time considerably decreases as PNS becomes more disordered by mutation. Hence, we speculate that this mode could "guide" the twisting and effectively control the reaction rate. Finally, the non-uniform protein environment secures the unidirectionality of the photoisomerization, i.e., it explains why the isomerizable bond, once it reaches the transition state, does not return immediately to the *trans* conformation that perfectly matches the protein environment but instead forms the *cis* conformation, causing the mismatch with the protein environment that drives the rest of the light cycle. The essential difference between the proposed model and the model presented previously[14] is the inclusion of the concept of the low frequency coherent "trigger" and "guidance" modes into the reaction scheme. Further dynamics studies using ~ 30 fs time resolution as well as complete vibrational assignment of PYP's low frequency vibrational modes are currently in progress.

5. Fluorescence Dynamics of PYP Single Crystal

In the final part of this article we present first results of femtosecond fluorescence dynamics of PYP single crystals. We would like to mention that these measurements were not easy to perform and required accurate verifications and several important precautions. However, in this article we refrain from detailed experimental descriptions (will be presented elsewhere) and present only the observed results. The study on protein single crystals was motivated by the fact, that up to date, only experiments with longer time resolution (ns - ms) have been performed.[15,16] Because of changes in protein tertiary structure occur much faster than the applied experimental time resolution in previous experiments,[15] obtained dynamics mirrored an averaged structural change over a broad distribution of possible intermediate conformations. Indeed, different dynamics behaviors of a protein system in solution and in single crystals have been observed. However, these differences were simply explained by an argument that in crystals, where the proteins are densely packed, the structural restraints imposed by the interprotein contacts in the crystal lattice alter the kinetics behavior. In femtosecond time resolved experiments, however, where proteins are "frozen" in time, this explanation is no more valid and differences in dynamics (if any) can be directly tested. Are the intraprotein structural changes and their temporal evolution similar in both solution and crystal phases or not? If yes, then the fluorescence dynamics should be similar in both cases as well and vise versa. On Fig. 6 two transients, taken with ~ 250 fs instrumental response near the fluorescence maximum (~ 500 nm), one from PYP in aqueous solution and the other from a PYP single crystal are presented. Evidently, the dynamics are different. While in PYP solution a fast component (~ 670 fs (43 %)) is present, it disappeared from a PYP crystal dynamics. This experiment demonstrates that the crystal condition considerably modifies PYP's properties, most probably due to differences in the protein nanospace structure, low content of water in the crystals, etc. Our first results are somewhat skeptical about the applicability of PYP's molecular structure derived from X-ray crystallography in single crystals, on PYP in solution. It is of primary importance to characterize the differences in molecular events and dynamics between PYP solution, crystals as well as amorphous aggregated states, etc. precisely, and experiments towards this goal are now going on in our laboratory.

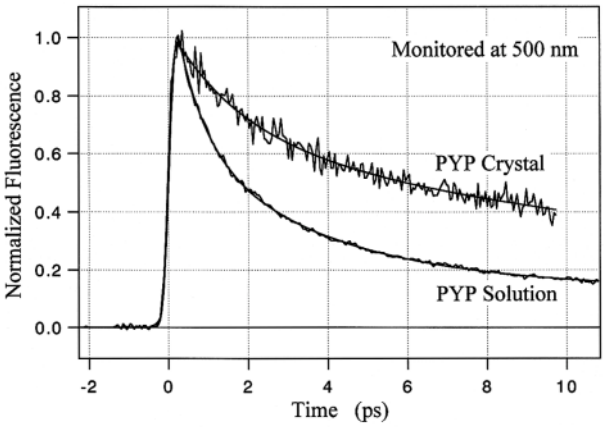

Fig. 6 Comparison of fluorescence decay dynamics of PYP in solution and as a single crystal near the maximum of the steady state fluorescence spectrum (~500 nm). Best fits for PYP in solution (670 fs (43 %), 3.6 ps (45 %) and ~100 ps (12 %)) and as a single crystal (3.6 ps (60 %) and ~400 ps (40 %)) are shown with solid lines. Note that because of the time resolution (~250 fs) and the monitoring wavelength of the fluorescence transient in solution, the coupled oscillations could not be resolved in present experiments.

6. Conclusion

Though in present investigations some properties of the "bulk" material have been addressed, it was demonstrated that detailed knowledge of protein nanospace structure (PNS) and dynamics of PYP are essential for understanding its fast photobiology. Moreover, we have shown that PYP single crystals can be investigated with fs time resolution, and if combined with SNOM or space resolved fluorescence up-conversion techniques, investigations on PYP crystals and various aggregates could shed more light on unique properties of this family of photoactive proteins.

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