

EFemtosecond Structural Dynamics Drives the *Trans/Cis* Isomerization in Photoactive Yellow Protein

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Abstract.

A variety of organisms have evolved mechanisms to detect and respond to light, in which the response is mediated by protein structural changes following photon absorption. The initial step is often the photo-isomerization of a conjugated chromophore. Isomerization occurs on ultrafast timescales, and is substantially influenced by the chromophore environment. Here we identify structural changes associated with the earliest steps in the *trans* to *cis* isomerization of the chromophore in photoactive yellow protein. Femtosecond, hard X-ray pulses emitted by the Linac Coherent Light Source were used to conduct time-resolved serial femtosecond crystallography on PYP microcrystals over the time range from 100 femtoseconds to 3 picoseconds to determine the structural dynamics of this important reaction.

One Sentence Summary.

The *trans* to *cis* isomerization of the central chromophore in a protein is structurally characterized on ultrafast time scales with TR-SFX.

Trans-cis isomerization constitutes a major class of chemical reactions of critical importance to biology, where for example light-dependent isomerization of a retinal chromophore underlies vision (1). Since isomerization occurs on the femtosecond (fs) to picosecond (ps) time scale, time-resolved methods are necessary to follow the reaction in real time. The spectral response after photon absorption reveals the dynamics of the molecules involved (2-5) but does not directly observe the associated structural changes, which have to be inferred by computational approaches (6). Until recently it has been impossible to directly determine the structure of molecules on ultrafast time scales. With the recently availability of hard X-ray pulses on the fs time scale emitted by free electron laser (X-ray FEL) sources such as the Linac Coherent Light Source (LCLS), the ultrafast fs to ps time scale has become experimentally accessible (7-11). Photochemical reactions (12) are initiated by photon absorption, which promotes electrons into the excited state. Thereafter, the nuclei experience - and the structure evolves on - the excited state potential energy surface (PES) (13, 14). The shape of the surface controls the subsequent nuclear dynamics. After returning to the ground state PES, the reaction continues and is driven thermally. Although structures of longer-lived excited state intermediates have been characterized with ~100 ps time resolution at synchrotrons (15-19), the fs structural dynamics of ultrafast photochemical reactions can only be investigated at an X-ray FEL (11).

The photoactive yellow protein (PYP) is an ideal macromolecular system which can be used to investigate ultrafast *trans* to *cis* isomerization. Its chromophore, p-coumaric acid (pCA), can be photoexcited by absorbing a photon in the blue region of the spectrum. Upon photon absorption PYP enters a photocycle involving numerous intermediates (Fig. 1). The primary photochemical event that controls entry into the photocycle is isomerization of pCA about its C2=C3 double bond (see Fig. 1B for the pCA geometry). The pCA chromophore remains electronically excited for a few hundred fs (3, 5, 20). Excited state dynamics is thought to drive the configurational change from *trans* to *cis* (3, 21). The chromophore pocket within the PYP protein is sufficiently flexible to allow certain relatively large atomic displacements, but at the same time imposes structural constraints that may affect the pathway and dynamics of isomerization (22, 23). In particular, the pCA chromophore is constrained by a covalent bond to the Cys69 side chain of PYP (Fig. 1B), by unusually short hydrogen bonds between its phenolate oxygen and nearby glutamate and tyrosine side chains (24), and by a hydrogen bond between the carbonyl oxygen of its tail and the main chain amide of Cys69.

Previously, we showed that time-resolved pump-probe serial femtosecond crystallography (TR-SFX) could be successfully carried out on PYP on the ns to microsecond (μ s) time scales. Difference electron density (DED) maps of very high quality, which compare the structures before (dark) and after (light) absorption of a photon (25), were obtained to near-atomic (1.6 Å) resolution. These experiments used a nanosecond (ns) laser pulse to initiate isomerization and subsequent structural changes. If enough photons are supplied, a ns pulse is sufficiently long that each molecule has many opportunities to absorb a photon. This enhances the overall reaction yield to as high as 40% (25). However, achieving fs time resolution requires that a fs pump laser pulse be used, which restricts the overall reaction yield to the much lower value of the primary quantum yield (around 10%) and correspondingly reduces the structural signal. The energy of fs pulses i.e. the number of photons per pulse must also be limited to avoid damaging effects from their significantly higher peak power. Here, we present results of TR-SFX experiments covering the time range from 100 fs to 3 ps. We directly follow the *trans-cis* isomerization of the pCA chromophore and the concomitant structural changes in its protein environment in real time.

Full details of the experiment and data analysis are provided in the Supplementary Materials (SM). Light-initiated structural changes in PYP were investigated on the 100 fs to ps time scale at the Coherent X-ray Imaging (CXI) instrument of the LCLS (26). Electronic excitation was initiated in microcrystals of PYP by fs pump laser pulses ($\lambda=450$ nm). Permanent bleaching of the chromophore was avoided by limiting the laser pulse energy to 0.8 mJ/mm² (5.7 GW/mm²). Laser pulse duration, spectral distribution and phase were characterized by ‘Second Harmonic Generation Frequency Resolved Optical Gating’ (SHG-FROG) (27). [We could add a reference here to the Hutchinson et al. ms. that is about to be submitted; it may well be accepted before the Science paper appears] The pulse duration was 140±5 fs and had both positive group delay dispersion and third order dispersion to maximize the conversion to the excited state (28). Offline spectroscopic experiments on thin crushed crystals of PYP had established that photoexcitation with fs laser pulses under comparable conditions could be as high as 10% without inducing damage (SM). The structural changes induced by the laser pulse were probed with 40 fs X-ray FEL pulses at 9 keV (1.36 Å). Both the pump-probe and the reference X-ray diffraction data (no pump laser pulse) were collected at the full 120 Hz pulse repetition rate of the LCLS to a resolution of 1.6 Å and 1.5 Å, respectively. To address concerns that the detector response might be influenced by the stray light of the intense fs laser pulse, the reference data

were collected as a negative time delay, where the fs laser pulse arrived 1 ps after the X-ray pulse. [Add a sentence here to describe exactly how/when the dark data were collected, to address Referee 2's new concern]

To assess whether fs laser pulses excited a sufficiently large number of molecules under these experimental conditions, we first performed a positive control experiment with a 200 ns pump-probe time delay, where large structural differences between the light and dark states have been well characterized (25, 29). A reference structure PYP_{ref} was determined from SFX data collected without optical excitation. From the pump-probe TR-SFX data and the reference data, DED maps were calculated (SM). Fig. 1C shows that the 140 fs laser pulses are able to initiate sufficient entry into the photocycle to produce strong, chemically meaningful features. The 200 ns DED map is essentially identical to maps determined earlier at both the LCLS (25) and at BioCARS (29) at a time delay of 1 μs , and can be explained with the same mixture of intermediates, pR_1 and pR_2 . The extent of reaction initiation is 12.6 % as determined by fitting a calculated ‘ $\text{pR}_1 \& \text{pR}_2$ minus pG ’ difference map to the 200 ns DED map, a value which agrees with the maximum extent of excitation determined spectroscopically (7 – 10%).

The fs time scale was explored by using nominal settings for the time delay of 300 fs and 600 fs. The timing jitter between the 140 fs laser pump and 40 fs X-ray probe pulses is \sim 280 fs (8). The jitter was measured for every X-ray pulse by a timing tool (30, 31), which was combined with adjustments that take longer-term experimental drift into account (see SM). Thus, each individual diffraction pattern was associated with a definite “time stamp”. However, the nature of the drift was such that the time stamps were non-uniformly distributed in time (Fig. S1). Since the quality of structure amplitudes and of the DED maps derived from them depends on the number of diffraction patterns, indexed, time-stamped diffraction patterns were binned into 8 different pump-probe delays with about 40,000 patterns in each bin, spanning the time range from 100 to 1000 fs (Tab. S1b). A set of diffraction patterns at a nominal time delay of 3 ps was also collected. Since the jitter and drift are much smaller than the delay, time stamping was not necessary for the 3 ps or the 200 ns time delays. The values of R-split for all datasets is 7.5 – 9.9% which indicates the high quality of the diffraction data, and results in DED maps of comparable, good quality for all time delays. Six [why don't we present all 8?] of the resulting eight maps are shown in Fig. S9. Visual inspection of these maps reveals an important qualitative result. The features in all maps at delays less than 455 fs are similar (compare Figs. S9, A-C);

and features in all maps at delays greater than 799 fs are also similar (compare Figs. S9, D-F), but differ from those in the first set. Consequently, there must be a structural transition between the 455 fs and 799 fs time delays that gives rise to the two distinct sets of features.

To identify with more precision the time delay at which this transition occurs, the time-stamped diffraction patterns were re-binned into 16 narrower time bins with about 20,000 patterns in each bin (Tab. S1A). The resultant time series of 16 DED maps in the fs time range (together with the map for the 3 ps time delay) were subjected to singular value decomposition (SVD; Fig. S2B) (32). The volume covered by the pCA chromophore, by the Cys69 sulfur and the Glu46 carboxyl was included in the analysis. When a time series exhibits a change, a corresponding change should be even more readily recognizable in the right singular vectors. This change is evident in the magnitude of both the first and second right singular vectors around 550 fs (red arrow in Fig. S2).

Also, there is a substantial increase in the magnitude of the first right singular vector after 155 fs (Fig. S2B) which shows the earliest (fastest) evolution of the structure after excitation. We tentatively associate the structural transition at around 550 fs, qualitatively evident by inspection of the DED maps and more quantitatively in their SVD analysis, with the *trans* to *cis* isomerization of the pCA chromophore. The transition occurs within ~180 fs (Fig. S2B), but its exact duration needs to be further established. Nevertheless, as the time delay of ~550 fs is within the vibrational dephasing time (3, 33) this observation suggests that the structural changes at times shorter than ~550 fs arise from coherent excited state motion. We further propose that at ~550 fs the system lies at or very close to a conical intersection (20) (Fig. S8), a branch point from which molecules either continue towards the *cis* configuration and enter the photocycle, or revert to the *trans* configuration and return to the resting (dark) state.

To further identify the isomerization, refined structures before and after the transition are required. This in turn requires the best structure amplitudes, based on the largest number of diffraction patterns. Initially data in the 8 bins with 40,000 indexed diffraction patterns each were used. Preliminary PYP structures were refined against these data. Refinement details are in the SM. The 3 bins with the fastest delays can be interpreted with chromophores in a twisted *trans* configuration. After 700 fs the configuration after is near *cis*. The time-course of the refined ϕ_{tail} torsional angles can be fit with the same transition time as observed in the rSV2 (Fig. S2A). We further took advantage of the similarity of the DED maps for extended time ranges before and

after the transition, and of the similarity of the refined underlying structures to increase the accuracy of the data. We combined the diffraction patterns into two bins: the fast time scale (100-400 fs with 81,237 patterns) and a slower time scale (800-1200 fs with 157082 patterns) (Tab. S1B). The structure denoted PYP_{fast} was refined against the 100-400 fs data; that denoted PYP_{slow} was refined against the 800-1200 fs data. The refinement statistics are presented in Tab. S2. The DED maps are shown in Fig. 2B (100-400 fs bin) and Fig. 2D (800-1200 fs bin), with the corresponding, refined structures of PYP_{fast} and PYP_{slow} in pink and light green, respectively. The 3ps DED map and the refined PYP_{3ps} structure are also shown (Fig. 2E). Note that the PYP_{fast} (100-400 fs) and PYP_{3ps} (3ps) structures are refined against roughly the same number of diffraction patterns while that for PYP_{slow} is refined against roughly twice that number (Tab. S1b). Consequently the structures differ in refinement quality (Tab. S2).

We emphasize that refinement of transient structures populated on an ultrafast timescale is challenging, since these structures are very far from equilibrium and likely to be highly strained. Restraints in standard libraries are derived from structures at equilibrium and are therefore not applicable. In order to provide restraints more appropriate for this refinement, we employed excited state quantum mechanics/molecular mechanics (QM/MM) calculations on PYP (20, 34) (SM). In addition, we employed an iterative procedure, in which improved difference phases $\phi^{F,\text{calc}}$ were obtained and used with observed difference structure factor amplitudes during refinement (SM). The structural results of the refinement are summarized in Tab. 1.

For the shortest time delays (up to about 450 fs), the PYP chromophore tail adopts a highly strained, twisted *trans* configuration, in which the C₁-C₂=C₃-C_{1'} torsional angle ϕ_{tail} (shown by the red line spanning these four atoms in Fig. 1B) is $\sim 140^\circ$ (Fig. 2A-B, PYP_{fast}). The position of the C₂=C₃ double bond in PYP_{fast} is displaced by $\sim 1\text{\AA}$ behind the chromophore plane (loosely defined by the Cys69 sulfur, the tail carbonyl oxygen and the atoms of the phenyl ring, Fig. 2A-B and Fig. S9A-B). Hydrogen bonds to Glu46 and Tyr42, which are unusually short in the reference (dark) structure (24), are substantially elongated from 2.5 \AA to 3.4 \AA (Tab. 1). This structure is primed for the transition to *cis*. During the structural transition, substantial rotation about the double bond takes place. The head of the chromophore pivots about tail atom C₂ thereby aligning the C₂=C₃ bond along the tail axis. Simultaneously, the head rotates about the C₃-C_{1'} single bond (the complex motions can be effectively illustrated by using an educator's stick model set, see Fig. S3). Its hydroxyl oxygen (Fig. 1B, O_{4'}) moves even further away (3.6 \AA ,

Tab. 1) from Glu46 (Fig. 2C-D and Fig. S9D), thereby breaking the hydrogen bond. [Tab. 1 does not offer this info. Further, we've just said that the DED maps and structures are pretty similar throughout the 100–400 fs time range. If so, we can't identify any differences in structure among time points such as 142, 269 or 455 fs (Fig. S9)]. At time delays longer than about 700 fs, ϕ_{tail} has decreased to $\sim 50^\circ$ (structure PYP_{slow}), which is characteristic of a *cis* configuration. PYP_{slow} relaxes further towards the 3 ps structure (PYP_{3ps}), in which the hydroxyl oxygen of the head re-establishes its hydrogen bond with Glu46 (Fig. 2e). ϕ_{tail} changes slightly to $\sim 35^\circ$. The PYP_{3ps} structure is already very similar to the early structures derived with 100 ps time resolution by independent, synchrotron-based approaches (Tab. 1; PDB entries 4I38 and 4B90) (22, 23), and evolves only slightly by establishing shorter hydrogen bonds to Tyr42 and Glu46.

The refinements confirm that the structural transition at around 550 fs is indeed associated with a *trans* to *cis* isomerization. Theoretical considerations (20) (Fig. S8) suggest that during isomerization the PYP chromophore relaxes through a conical intersection between the electronically excited state PES and the ground state PES. Accordingly, structures between 100 and 400 fs can be identified as electronically excited, whereas the structures at time delays > 700 fs can be identified with the electronic ground state. In both the excited and ground states, structural changes i.e. translation of atoms may also have occurred. Our experiments identify the ultrafast dynamics of both the excited state structures and the ground state structures (Fig. 2 and Figs. S7-S9). Since we restricted our pump laser pulses to moderate power we avoid damaging non-linear effects (e.g. two photon absorption) and most excited molecules populate the excited state surface S1 (5). Part of the stored energy is used to rapidly displace the chromophore by about 0.7 Å within the crowded molecular environment in the interior of PYP (Fig. 2a, Tab. 1). If this initial displacement is complete after 250fs [Why is this time given so precisely? Safer not to tie it to a particular time delay], the chromophore must have experienced an acceleration of $\sim 2 \times 10^{15}$ m/s² and attains a final velocity of 500 m/s (SM). Fig. 1b shows that 9 carbon atoms, two oxygens and 7 hydrogen atoms (molecular mass = 147 g/mol) are displaced. During the first few hundred fs the force on the chromophore is ~ 500 pN, which is enormous compared to forces in single molecules at thermal equilibrium which are usually only a few pN (35). The energy required to displace the chromophore is ~ 0.2 eV which is $\sim 10\%$ of the blue photon energy (2.76 eV) that starts the reaction. It appears that by rapidly evolving down the excited state PES, part of the photon energy is initially converted into kinetic energy that is then released by collision of

the chromophore atoms with the surrounding protein atoms comprising the chromophore pocket. The excited chromophore loses 0.12 eV energy by intramolecular vibrational energy redistribution on sub-100 fs time scale (39) which can be roughly estimated from the Stokes' shift by comparing absorption and fluorescence spectra(3). Accordingly,~85% of the photon energy remains stored as strain and electronic excitation in the chromophore before isomerization occurs.

On passing through the conical intersection (20), the molecules either revert towards the initial dark state (30% of the excited molecules, Tab. 1, see also Tab. S3) or continue relaxing towards the *cis* isomer (70%), gradually releasing the excess energy as heat. Because the chromophore pocket tightly restricts the chromophore head displacements, further relaxation must be volume-conserving i.e. must minimize the volume swept out by the atoms as they move. Accordingly, the chromophore performs the complex motions described above (Fig. S3). Although the energy stored in the chromophore is sufficient to break the hydrogen bonds (~0.1 eV), the spatial constraints imposed by the chromophore pocket direct the reformation of the hydrogen bonding network at longer time delays (Tab. 1). This is a ‘macromolecular cage effect’ reminiscent of the ‘solvent cage effect’ in liquid chemical dynamics (36). The ‘macromolecular cage’ in PYP, however, is soft enough to allow certain specific, relatively large (up to 1.3 Å, Tab. 1) structural changes that we observe. This contrasts with crystals of small molecules, where the stronger crystal lattice constraints usually do not support such large structural displacements. As a result, biological macromolecular crystallography aimed at elucidating biological function may also provide insight into the reaction mechanisms of small molecules.

To assess global conformational changes of PYP on the fs time scale, we calculated the radius of gyration R_g from each refined structure (SM). R_g fluctuates by only 0.2% in all structures from 200 fs to 200 ns (Tab. 1). An increase of R_g by up to 1 Å determined by others using X-ray scattering in solution upon photo-dissociation of CO from CO-myoglobin (Mb-CO) in solution (9) is not observed in our PYP crystals. Concomitant, systematic large volume changes are also not apparent in PYP crystals over the first 3 ps that our data span. A protein quake (9, 10, 37) characterized by an ultrafast and large change in R_g that occurs significantly before a large volume change is not evident in our data. The reason for this is unclear and will require further experiments.

Ultrafast fluorescence and transient absorption spectroscopy of PYP has shown that excited state decay is multi-phasic (3, 5, 38). It is well established that the fast time constants are significantly more productive to create the *cis*-like photoproduct whereas the long-lived excited state population is unproductive (5, 39). With 450 nm femtosecond excitation at least 50% of the total isomerization yield is generated with a dominant ~600 fs time constant (5), which agrees with the present observation of a transition at ~550 fs. It should be noted that a ‘ground state intermediate’ with 3-6 ps life time has been proposed by ultrafast spectroscopy (39). However, under the conditions employed here the peak concentration of this intermediate is expected to be small (5) and the ground state intermediate is not further considered here. In contrast to selective spectroscopy techniques that have reported vibrational coherence with 50 cm⁻¹ and 150 cm⁻¹ frequency (3, 40), no oscillations could be unambiguously identified in our structural data. Vibrational amplitudes may be too small for our methods to detect, and the periodicities of vibration may be masked by the non-uniform sampling of time delays. Intense femtosecond optical pumping of PYP crystals necessarily generates both excited state and ground state vibrational coherences within the 3.15 THz experimental bandwidth(41). As a result, the time dependent molecular dynamics before de-phasing involve complex coherent ground and excited state motions that evolve under different initial phases and frequencies. It will be an important goal of future experiments to structurally characterize these coherences using fs TR-SFX. Nevertheless, our data show that before 400 fs there are large distortions corresponding to a Franck-Condon (FC) intermediate (42). [Referee 1 asks for an explanation of what is meant by this FC intermediate] The existence of such an intermediate agrees with the conclusions from ultra-fast spectroscopy (3, 42-45)-that also suggest a distortion of the C₂=C₃ double bond on similar timescales, which we observe in the PYP_{fast} structure. The isomerization at 550 fs through the conical intersection between the excited state and ground state PES is in reasonable agreement with the timescales for isomerization reported by others (3, 5, 42, 46). After passing through the conical intersection, the chromophore is *cis*-like and still highly strained. The transiently broken hydrogen bond is re-established quickly as the structure relaxes, exemplified by the PYP_{3ps} structure. Further relaxation on the ground state PES completes the initial phase of the isomerization.

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Author Contributions.

M.S. prepared the proposal with input from J.J.vT., K.M., V.S., J.C.H.S., H.N.C., A.O. and P.F.; A.A., S.B., M.L., J.S.R. and J.E.K. operated the CXI instrument including the time-tool and the fs-laser. K.P, A.B., J.T., S.B., T.A.W., N.Z., O.Y. and T.D.G. analyzed the SFX data. C.D.M.H and J.J.vT. set up the FROG at the CXI instrument; G.G. and D.M. performed QM/MM calculations; J.T., J.B., D.O., P.L.X. , C.G., C.K. and M.S. prepared protein and grew nano- and microcrystals; D.DeP., C.K., C.C., S.R-C., J.D.C., M.M., G. K., and U.W. provided and operated the injector system; M.F., R.F., M.S., J.T., P.F., D.O. and C.G. wrote the electronic log; M.F., M.S, J.T., J.S.R., J.J.vT. and K.M. discussed fs laser excitation; J.T., M.S, V.S, R.H, C.D.M.H. and J.J.vT. performed preliminary ultra-fast experiments on crystals; M.S. calculated and analyzed the difference maps; M.S., K.P., K.M., G.G., P.F. and J.J.vT. wrote the manuscript with improvements from all authors.

Supplementary Materials.

Materials and Methods

Supplementary Text

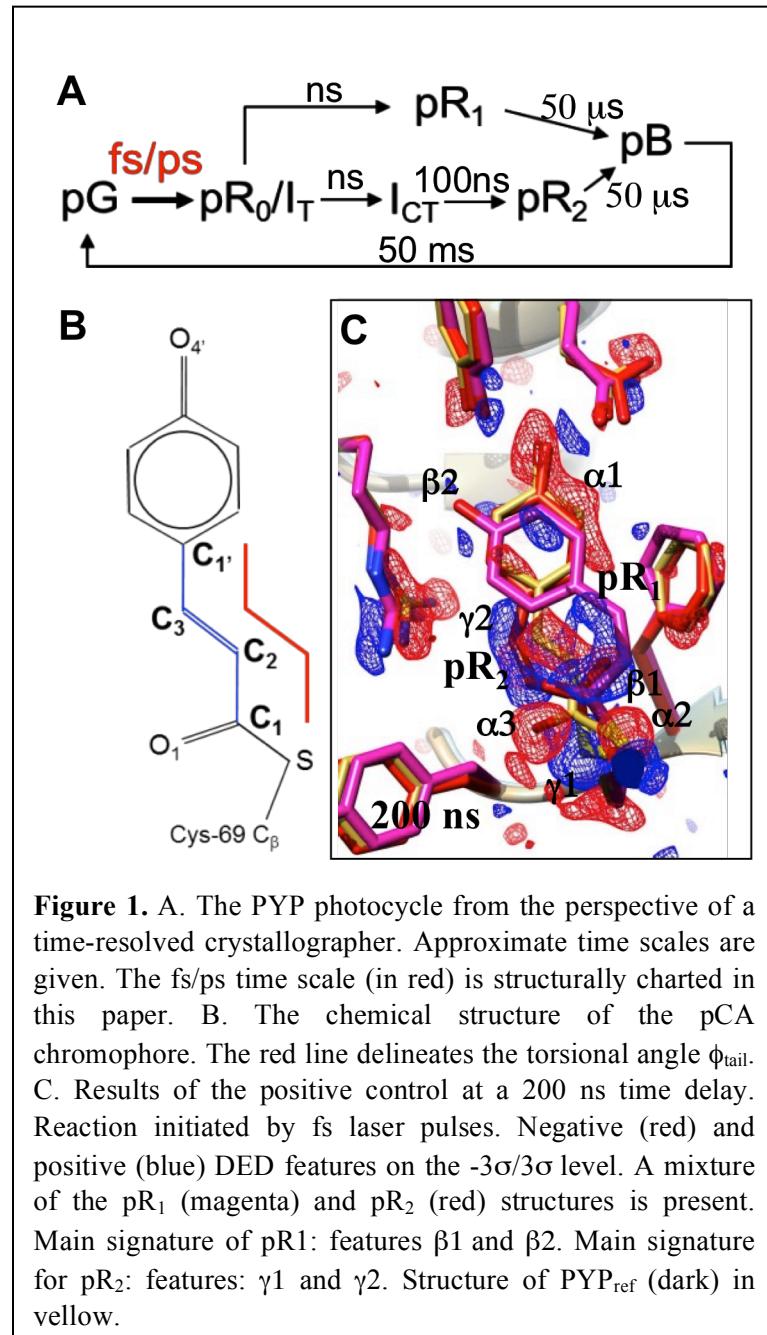
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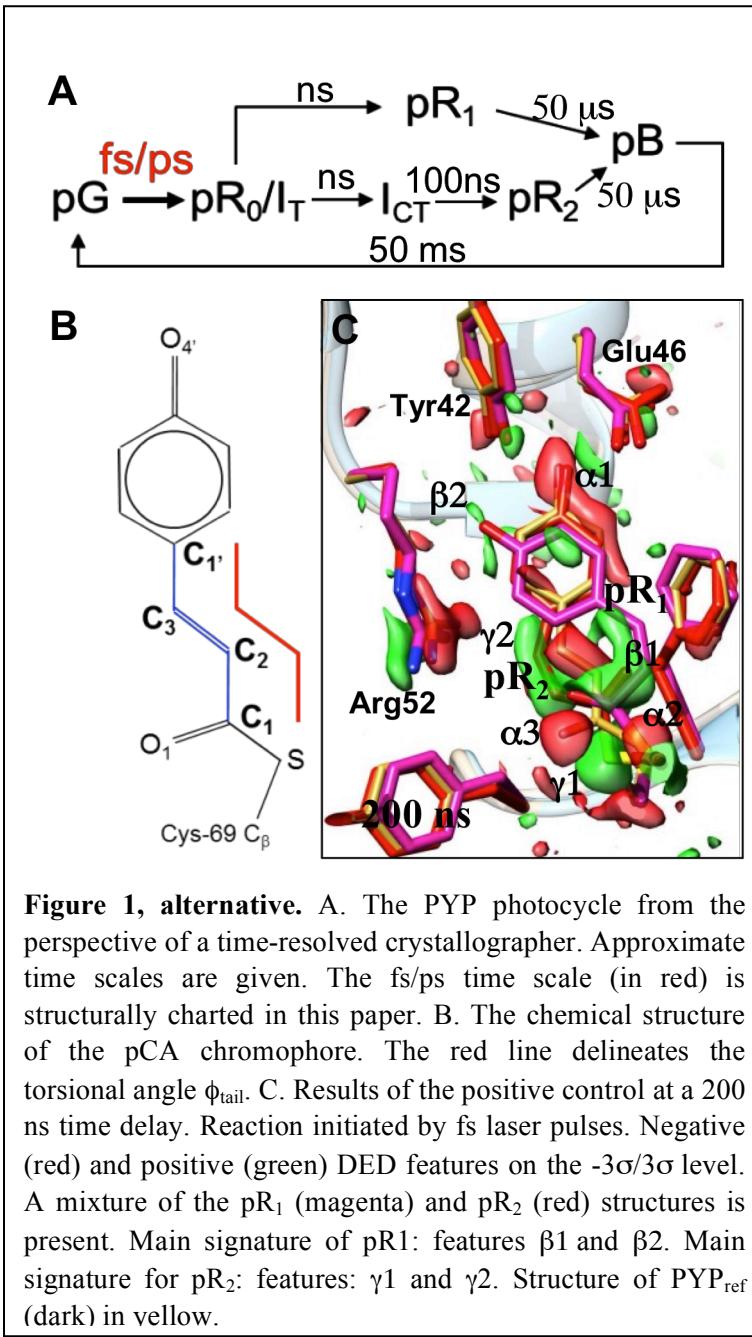
Figs. S1 to S11

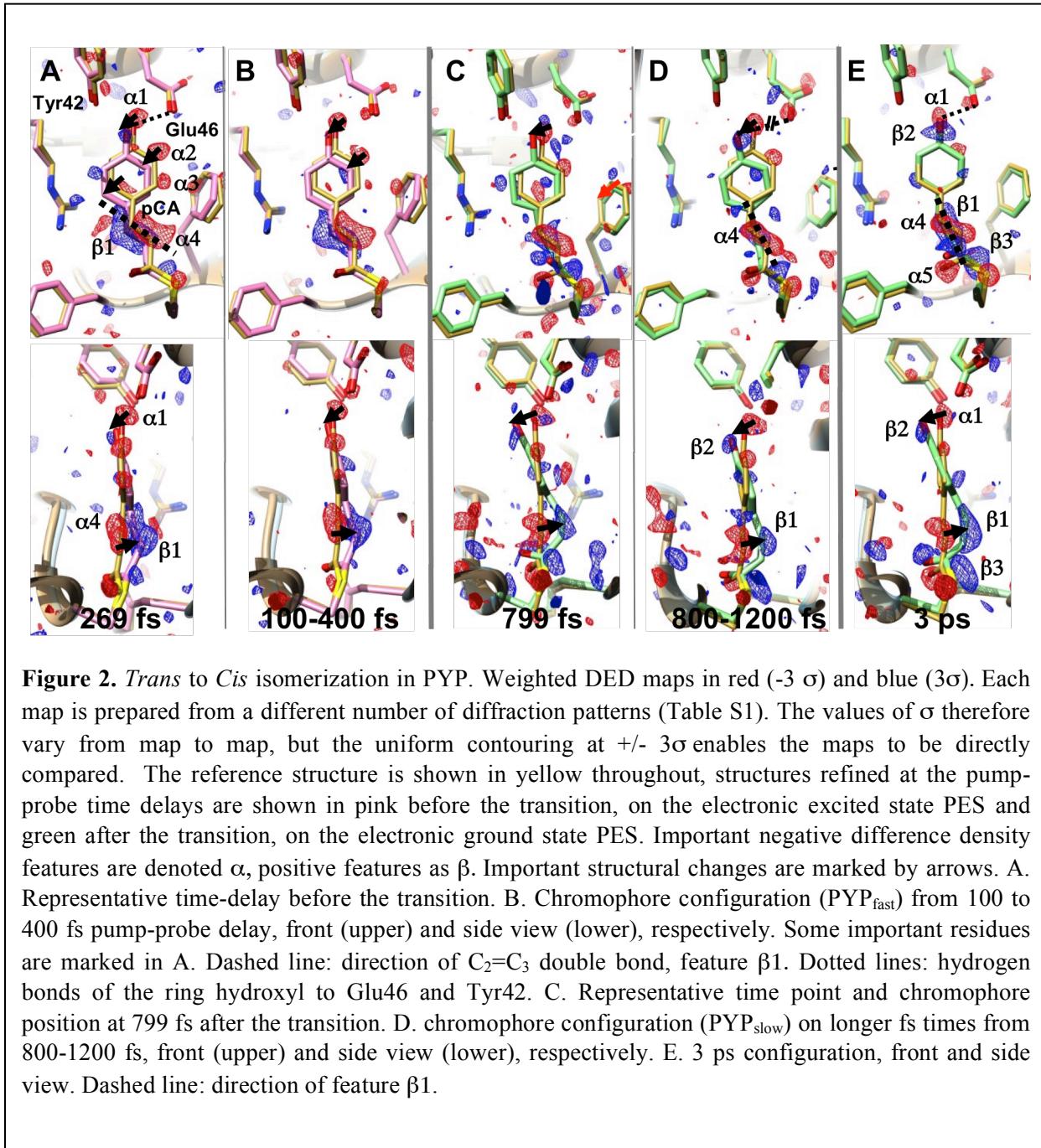
Tables S1 to S5

Figures

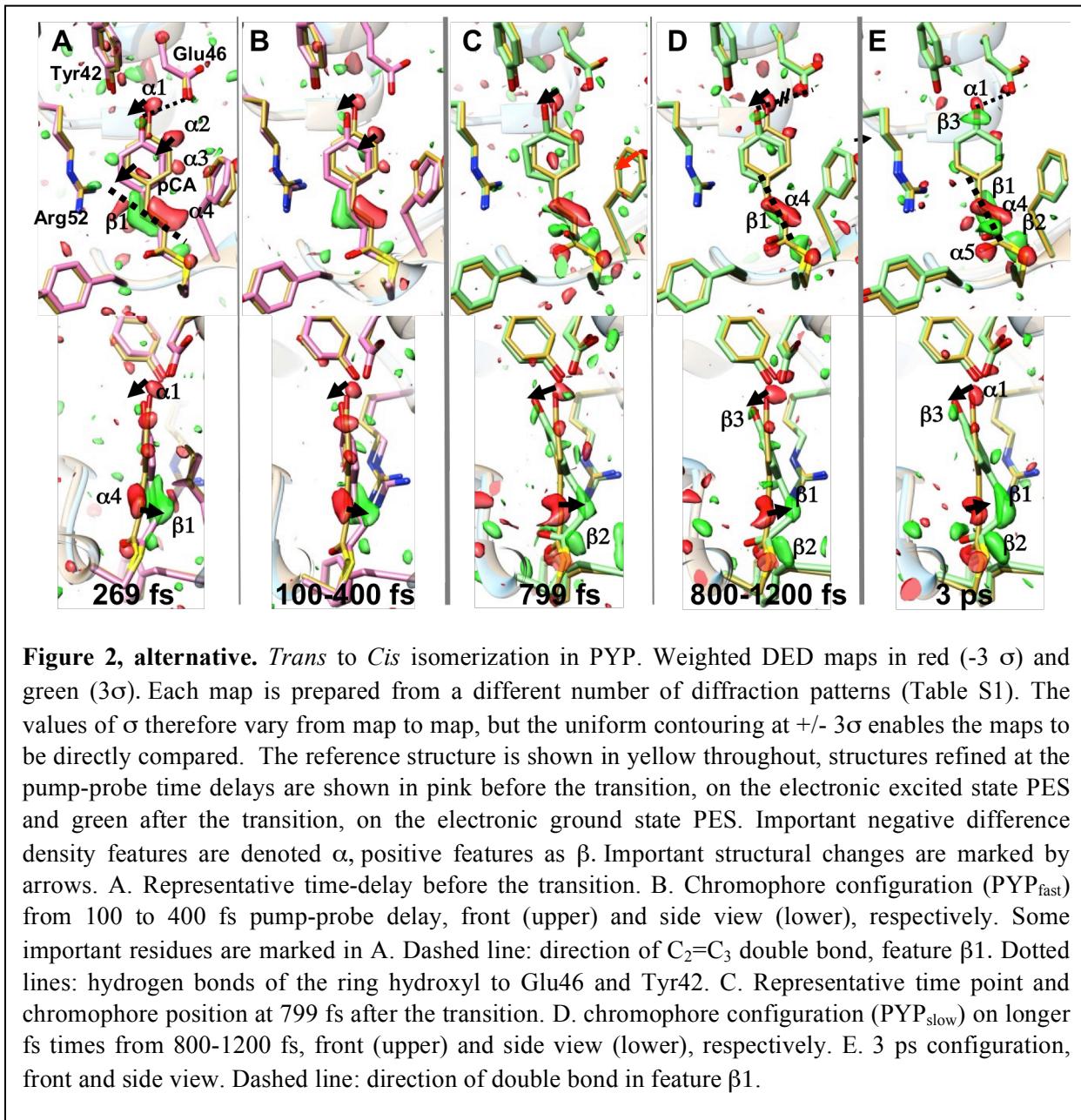
- Figure 1 -







- Figure 2 -



Tables

Table 1. Geometry of PYP structures. The PYP_{fast} structure was refined using a data bin spanning 100–400 fs with 81327 snapshots, and the PYP_{slow} structure from a bin spanning 800 – 1200 fs with 157082 snapshots (Tab. S1b). Structures of I_T, pR₀ and pB₁ from Protein Data Bank, code listed in brackets (22, 23, 47). Uncertainties of the torsional angles can be estimated to be +/-20° by displacing the 4 atoms that define the angle with the coordinate error (0.2 Å). [Need to add estimate of errors in these entries]

	PYP _{ref} (dark)	PYP _{fast}	PYP _{slow}	PYP _{3ps}	PYP _{200ns} (fs-laser) pR1/pR2	I _T (4I38)	pR ₀ (4B90)	pB ₁ (1TS0)
Time Delay	0	100- 400 fs	800 - 1200 fs	3 ps	200 ns	100 ps	100 ps	ms
Torsional Angles [°]								
C1-C2=C3-C1' (ϕ_{tail})	172	136	53	35	3/-8	90	33	-27
O1-C1-C2=C3	-15	-21	28	30	12/-6	11	29	-10
CB-S-C1-C2	-185	-171	-164	-137	163/-165	-136	-123	180
Hydrogen bonds [Å]								
pCA-O _{4'} - Glu46-O _a	2.50	3.40	3.60	2.94	4.97/2.88	2.73	2.73	8.03
pCA-O _{4'} - Tyr42-O _a	2.54	2.92	2.63	2.88	2.97/2.66	2.57	2.59	5.19
pCA-O ₁ - Cys69-N	2.77	3.11	2.50	3.12	3.37/4.29	3.04	3.05	2.88
others								
<pCA> ^a [Å]	0	0.66	0.78	0.60	1.55/0.81	0.67	0.68	2.39
<global> ^b [Å]	0	0.20	0.19	0.24	0.13	0.13	0.19	0.17
Radius of gyration ^c [Å]	13.32	13.33	13.30	13.34	13.29	- nd -	- nd -	- nd-
Volume [Å ³]	17831	17856	17833	17838	17672	17830	17683	17807
ΔV to dark [Å ³]	0	25	2	7	-159	-1	-148	-24
Photoactivation Yield [%] ^d	- na -	15.2	9.6	10.1	12.5 ^e	(5%) ^e	(10%) ^e	(10%) ^e

^aMean displacement of equivalent chromophore atoms relative to dark (SM).

^bMean displacement of equivalent c_a atoms relative to dark (SM).

^cSee SM for the calculation.

^dDetermined by fitting calculated DED maps to the experimental DED maps in the chromophore region.

^eEstimate