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Femtosecond Studies of the Initial Events in the Photocycle of Photoactive Yellow Protein (PYP)

Matthew A. Horn, Julie A. Gruetzmacher, Jeongho Kim, Seung-Eng Choi, Spencer M. Anderson, Keith Moffat, and Norbert F. Scherer

Abstract

Primary photoinduced chemical events in the photocycle of photoactive yellow protein (PYP), a photoreceptor from the eubacterium *Ectothiorhodospira halophila*, have been probed by femtosecond pump-probe and anisotropy measurements. A transient absorption signal that relaxes with a bi-exponential decay (τ_1 = 760 fs and τ_2 = 2.8 ps) has been observed for the first time (400 nm pump and probe), the anisotropy of which remains constant over more than 10 ps. Kinetic modeling of the pump-probe data suggests an inhomogeneous population in the ground and initial photoexcited states. Complementary molecular dynamics simulations have highlighted the importance of hydrogene bond dynamics in the primary isomerization event of the chromophore.

23.1
Introduction

Photoreceptors are a class of molecules that have attracted great interest owing to their ability to transform the small structural changes of a photoisomerization event into large structural changes in the entire protein backbone, thereby facilitating cellular processes such as signal transduction.[1–8] The challenge is to understand the interactions relevant to function based on a detailed knowledge of the structure, dynamics, and photocycle kinetics. The recent development of a time-resolved X-ray diffraction/structure determination method has enabled a uniquely informative understanding of protein function through the direct connection of structural changes to kinetics.[9] At the same time, ultrafast optical spectroscopies provide an approach for elucidating the rate(s) of reactions even on the timescale of vibrational motions. Key issues that can profitably be addressed by these methods are how the dynamics of the chromophore influence the protein structure and motions, and the reciprocal influence of the protein.

One system well-suited for detailed study is photoactive yellow protein (PYP), a small (14 kDa), water-soluble, cytoplasmic photoreceptor found in halophilic phototrophic bacteria.[10,11] Action spectra suggest that PYP mediates a negative phototac-

tic response in *Ectothiorhodospira halophila.* [12] PYP, which contains a *p*-coumaric acid[13–15] chromophore, has a ground-state structure that is known to within an accuracy of 1.4 Å.[16] The chromophore is isolated from the surrounding solvent (in the ground state) and is connected to the protein through a thiol ester linkage to cysteine 69. Part of the photo-driven action is a *cis* → *trans* isomerization of the chromophore about its only carbon–carbon double bond.[14]

Several factors contribute to making PYP an excellent test case for the study of photoactive proteins.[17] The photocycle of PYP involves several kinetically and spectroscopically well-defined intermediates.[10,18,19] The ground state S_0(*trans*) exhibits a broad absorption (~60 nm) centered at 446 nm ($\varepsilon = 45\ mM^{-1}cm^{-1}$).[10] The first well-characterized intermediate S_0(*cis*) appears within 10 ns of photoexcitation from S_0(*trans*) to S_1(*trans*) and has an absorption centered at 464 nm, red-shifted relative to the *cis-trans* isomerized ground state.[20] The second intermediate, [pB], in which the chromophore is exposed to solvent and is protonated, gives rise to a blue-shifted absorption maximum (355 nm); it appears within 1 ms[21] and is very long-lived (τ = 150 ms). Furthermore, the [pB] intermediate has been observed by means of time-resolved X-ray crystallography, making PYP unique among the photo-sensory proteins.[22] Finally, since only one bond is involved in the isomerization, in contrast to the situation in rhodopsins, the isomerization step of the photocycle is less complicated.[23]

23.2 Methods

23.2.1 Molecular Dynamics Simulations

The molecular dynamics simulations were performed with a commercial simulation and visualization package (IMPACT, MSI) using the AMBER potential function with chromophore partial charges, angles, and bond lengths parameterized from He et al.[24] A complete description of the details of the simulation and the results are given elsewhere.[25]

23.2.2 Femtosecond Spectroscopy

Femtosecond spectroscopic measurements were made using a home-built regeneratively amplified gain-switched all-acousto-optic Ti:sapphire laser system. The design and performance specifications will be described elsewhere.[26] The system produces pulses of energy >100 μJ at a repetition rate of 4 kHz and with duration ca. 50 fs. The experimental set-up for pump-probe spectroscopy is described elsewhere.[25]

23.2.3 Sample

PYP apoprotein was cloned from the SL-1 strain of *Ectothiorhodospira halophila*. Although in an earlier publication[27] it was reported that the amino acid sequences from the SL-1 and BN9626 strains of PYP were identical, more recent results (S. M. Anderson, K. Moffat, unpublished results) have suggested that this is not the case; the sequences are very similar but not identical. The apoprotein was expressed in *Escherica coli* and then reconstituted with the chromophore *p*-coumaric acid to create the PYP holoprotein.[28] An *N*-terminal histidine tag was removed by cleavage with TEV protease to leave a residual glycine. The protein was purified by means of ion-exchange and size-exclusion chromatography until the optical purity (absorbance ratio 279 nm/446 nm) was 0.42. The purity was further assessed by mass spectrometry and isoelectric focusing.

The PYP sample was prepared by diluting a concentrated stock solution with a tris-HCl buffer of pH ~ 8.0 to obtain an optical density of 0.3 at 400 nm. The sample was spun (~15 Hz) in a cell to mitigate photobleaching effects by providing a new sample for each laser shot. The sample path length in the cell was 0.5 mm.

23.3 Results

23.3.1 Pump-Probe Measurements

Pump-probe measurements were performed for pump-probe delays from –5 ps to 125 ps with a 400 nm pump and probe pulses between 400 nm and 490 nm. These measurements allow the determination of kinetic processes such as excited-state absorption, ground-state bleaching, and photoproduct formation. Short-time data with parallel pump and probe polarizations (400 nm pump and probe) are given in Figure 23.1a.

An excited-state transient absorption (negative signal) is seen at early delay times and rapidly converts to a bleach/stimulated emission signal. The rate of this conversion is wavelength-dependent. To the best of our knowledge, this is the first time that an excited-state absorption has been observed from the S_1(*trans*) state of the PYP chromophore. A least-squares fit of the data, as shown in Figure 23.1a, yields a bi-exponential decay of the transient absorption (time constants: τ_1 = 760 fs and τ_2 = 2.8 ps) and a bleach component that does not decay within the 125 ps measurement window, in agreement with previous measurements.[29,30]

A pump-probe measurement taken from –1 ps to 19 ps following excitation with a 400 nm pump and probing at 490 nm is shown in Figure 23.2. This measurement, in agreement with that at 487 nm in ref. [29], offers a spectral window on the bleach recovery kinetics of the S_0 states.

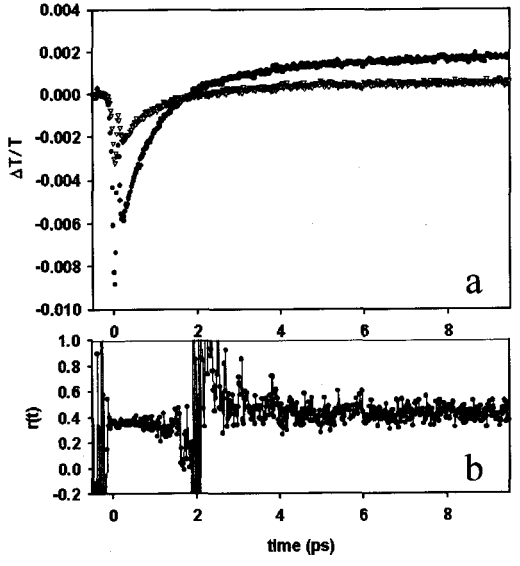

Fig. 23.1 Pump-probe measurements (a) and the calculated transient anisotropy (b) of PYP for 400 nm pump and probe pulses; parallel (•) and perpendicular (∇) polarizations.

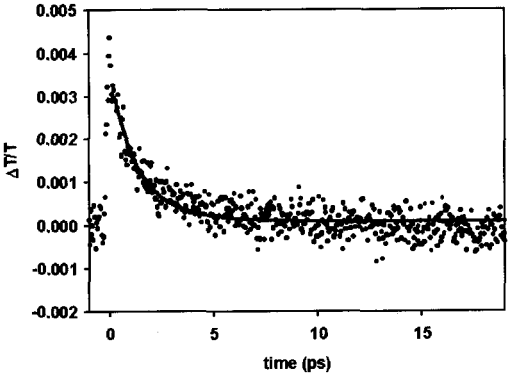

Fig. 23.2 Pump-probe signal of PYP for pump = 400 nm, probe = 490 nm, and the kinetic model fit.

23.3.2 Anisotropy

The anisotropy, $r(t)$, as indicated in Figure 23.1b, was constructed from the results of pump-probe experiments performed with parallel and perpendicular polarizations.[31] The derivative-like shape is due to a singularity at a delay of ~1.8 ps. Rotational motion makes a negligible contribution to the anisotropy as the protein is too large to rotate on these timescales and the chromophore is locked in its interior. The lack of a decay of the anisotropy indicates that the transition dipole direction of the probed transition does not change with time as detected in the 400 nm window.

23.3.3
Molecular Dynamics Simulations

Analysis of a series of trajectories lasting ~200 ps revealed unusual H-bond dynamics between the carbonyl oxygen on the *p*-coumaric acid "tail" and a backbone amine, specifically that on tyrosine 92. This bond must be broken for isomerization to occur. An autocorrelation function analysis of the trajectories indicated significant partitioning between the two H-bond conformations (that is, formed *versus* broken), which would be expected to lead to different barriers for isomerization. The time required for H-bond breaking is ~30 ps. By contrast, the autocorrelation function of virtually all other H-bonds lost correlation in less than 1 ps.

23.4
Discussion

23.4.1
Energetics

The excited-state transient absorption presented here has not been seen in previous femtosecond experiments on PYP.[29,30] In these previous studies, only probe wavelengths between 420 nm and 570 nm were used. The lack of absorption at 420 nm, in contrast to that seen at 400 nm and 410 nm in 400/410 nm pump-probe data (not shown), suggests that the Franck–Condon region for the excited-state absorption is narrow. The excited-state absorption seen here cannot be accounted for in terms of a two-electronic state model; a hitherto unobserved higher-lying state must also be invoked. Transient absorptions have been noted previously in photoisomerization reactions and have been used to probe reaction dynamics.[32,33]

The relatively slow dynamics of the transient absorption decay suggests that the S_1(*trans*) energy surface in the Franck–Condon region is not repulsive but is instead flat or a shallow well. A model developed by Schulten[34] to explain similar observations in rhodopsin was invoked by Anfinrud et al.[35] to explain their experimental observation that, in bacteriorhodopsin, the energy surface of the photoexcited state cannot be repulsive. Glasbeek and coworkers[36] also made reference to this model in explaining their time-resolved fluorescence results for PYP.

23.4.2
Isomerization

The rapid rate out of the S_1(*trans*) Franck–Condon region (that is, the initial phase of the isomerization) is in accordance with photoisomerization measurements made on stilbene in solution. The *cis* → *trans* photoisomerization of stilbene has been interpreted as occurring on a potential surface with a very small energy barrier (<1.5 kcal/mol) and to take place within 2 ps.[37,38] This rapid photoisomerization of *cis*-stilbene (nearly 100 times faster than the photoisomerization of *trans*-stilbene)

suggests that the *cis* → *trans* stilbene photoisomerization is analogous to *trans* → *cis* photoisomerization of PYP; only a small energy barrier needs to be surmounted in S_1. Rhodopsins are believed to undergo a rapid photoisomerization very similar to that of PYP,[4] hence comparisons can be expected to be quite informative. The rhodopsin *cis* → *trans* photoisomerization occurs on a barrierless energy surface and is complete within 200 fs.[39,40] An extremely rapidly decaying excited-state absorption is also observed.[33,41]

Although the bi-exponential decay observed here is detected by probing to a higher-lying excited state, bi-exponential kinetics for electronically-excited PYP have been observed previously in time-resolved fluorescence measurements.[36] It was concluded that this was due to an inhomogeneous distribution of the protein environments that affect the dynamics of the chromophore in the S_1 state. The excited-state absorption presented in this work is likewise sensitive to an inhomogeneous distribution of chromophore environments in the S_1 state. Furthermore, the bi-stable hydrogen-bond dynamics of the hydrogen on tyrosine 92 with the carbonyl oxygen on the chromophore observed in our molecular dynamics simulations is indicative of the existence of two distinct ground-state populations.[25] This is strongly supportive of an inhomogeneous distribution in the S_1 state upon photoexcitation.

The kinetic scheme shown in Figure 23.3 has been used to simulate our data. An initial photoexcited population exists in a pair of states ($S_{1a/b}$(*trans*)) that differ in the hydrogen-bonding behavior revealed by the molecular dynamics studies. They can only be distinguished spectroscopically from the different absorption decay time constants; their spectra overlap in the probed region. From the $S_{1a/b}$(*trans*) states, PYP proceeds to a species "I", a twisted conformation in S_1 that lies intermediate between *cis* and *trans*. From this well, PYP can proceed to either the *cis* or *trans* S_0 configuration.

Expressions were derived for the time-dependent populations of each state shown in the scheme.[25] With this kinetic scheme, the measured time constants, and the constraint that the *cis*/*trans* quantum yield must be in agreement with that reported in ref. [29], the 400 nm probe data could be fitted by adjusting the inhomogeneous distribution in the S_1(*trans*) states, the kinetic time constants, and the absorption coefficients of the states involved. The best fit was achieved with the inhomogeneous distribution of the S_1(*trans*) (and hence also the S_0 (*trans*) ground state) populations of 0.5 each and a quantum yield of S_0(*cis*) of 0.6. The fit to the data in Figure 23.1a is excellent. The absorption coefficient of the S_1(*trans*)→S_n transition was

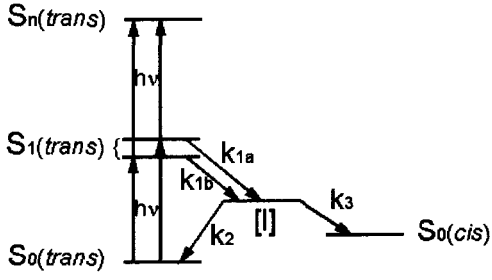

Fig. 23.3 Proposed mechanism accounting for inhomogeneous kinetics. Arrows indicate the optical transitions and the reaction "pathway".

found to be approximately five times greater than that for the $S_0(trans) \rightarrow S_1(trans)$ transition. The absorption coefficient for the $S_0(cis) \rightarrow S_1(cis)$ transition proved to be zero, which is consistent with previous work suggesting that the *cis* absorption does not extend to these wavelengths. This kinetic scheme differs from that of Tollin et al.[29] in that in their scheme the two $S_1(trans)$ states (Tollin's P^* and $P^{*\prime}$) decayed to two different intermediates.

The kinetic parameters from the 400 nm fit were used to fit the 490 nm data; only the absorption coefficients were allowed to change. The very good fit of the 490 nm data is shown in Figure 23.2. The time constants for the evolution of the two $S_1(trans)$ states to I are 1.8 ps and 0.6 ps; for the reversions from I to $S_0(trans)$ and $S_0(cis)$ the values are 1.4 ps and 0.9 ps, respectively. The absorption coefficients for the 490 nm data are approximately equal, both being around half of that for the $S_0(trans) \rightarrow S_1(trans)$ transition at 400 nm.

23.4.3 Anisotropy

The transition dipole direction has been measured in a single crystal of PYP.[43] The electronic structure of the PYP chromophore in an amino acid environment has been calculated.[24] Initial results suggest that the transition dipole of the PYP chromophore from the *trans* ground state to the lowest excited state, in an environment of seven amino acid residues represented by point charges and water molecules to allow hydrogen bonding, is largely contained within the plane of the chromophore. It is also roughly collinear with the axis of the carbon chain. It is assumed that the transition dipole of the *cis* form is rotated with respect to that of the *trans* form.

A transient anisotropy measurement determines the change in direction of the transition dipole vector between the initial *trans* ground to S_1 state absorption and any optical transitions from the various photointermediates. It gives a measure of the angle between the transition dipole of the $S_0(trans)$ to $S_1(trans)$ initial excitation at time zero and the same transition moment probed at time t and the transition moment of $S_1(trans) \rightarrow S_n$. The initial anisotropy is indicative of the excited-state absorption and stimulated emission of the *trans* conformation; the anisotropy of the long-time increased transmission is indicative of the remaining ground-state bleaching that persists due to photoreaction and the transient absorption from the intermediate and $S_0(cis)$ states that are formed. Thus, the timescale of the decay of the instantaneous anisotropy level to the long-time anisotropy level will be indicative of the formation of the intermediates (I and $S_0(cis)$). Hence, the lack of a change in the anisotropy, as shown in Figure 23.1b, suggests that these intermediates are not detected within this spectral window. Only bleaching and ground-state recovery are observed, which is at variance with other reports.[42] However, further anisotropy measurements over a broader range of probe wavelengths are necessary to confirm this tentative conclusion. Such efforts are in progress.

23.5 Conclusions

The results presented here suggest that the photoisomerization reaction of PYP shows a striking similarity to that of rhodopsin. The Franck–Condon region of the ground-state absorption is relatively flat and the chromophore proceeds rapidly from this region. An excited-state absorption is observed, which decays bi-exponentially. This is argued to be the result of an inhomogeneous distribution in the S_1(*trans*) photointermediate because of two populations in the ground state caused by hydrogen bonding of the chromophore, as observed in molecular dynamics simulations.

The energy level scheme used here is analogous to that proposed by Anfinrud for bacteriorhodopsin.[35] This model entails several predictions for spectroscopic measurements. First, no immediate stimulated emission spectral shift to lower energies should be seen (or seen as a Stokes shift in fluorescence up-conversion measurements), as the excited-state population does not immediately slide down an excited-state well to the product conformation. To date, no such shift has been observed.[29,30,36,44,45] Second, as the small excited-state energy barrier is surmounted, low-temperature (below the thermal activation barrier) measurements should indicate a population trapped in the excited state. Finally, the stimulated emission spectrum should not match the fluorescence spectrum as an excited-state absorption should cancel some of the emission.

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