Out of the blue: the photocycle of the photoactive yellow protein

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A first real glance at the structural, spectral and temporal interplay that constitutes the photocycle of the photoactive yellow protein (PYP) has been obtained from a combination of time-resolved crystallography with mutational analysis and spectroscopic studies.

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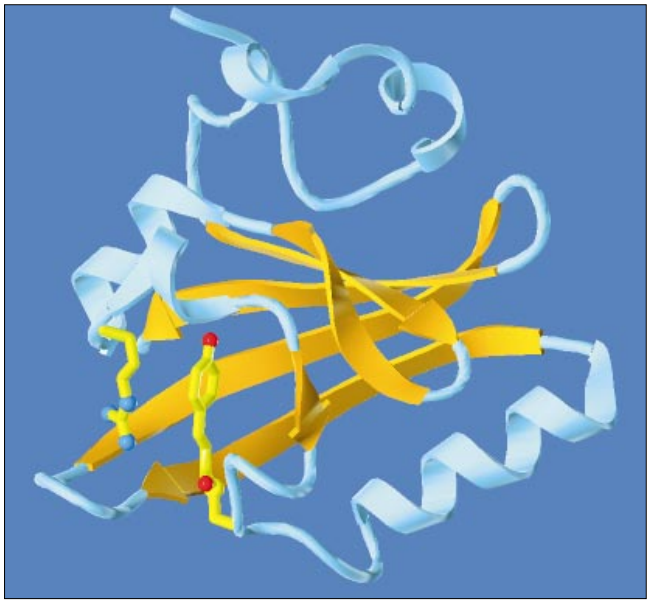
As one's plane slips down into San Francisco airport, an alert traveler might notice a multicolored patchwork of drying basins at the margins of the bay below. These ponds, which are used to dry seawater for salt production, range in hue from muddy brown to brilliant red and even light purple, depending on the microorganisms living there. Among these are *Ectothiorhodospira halophila*—purple bacteria that are not only attracted to light to do an honest day's photosynthesis, but also swim away from the damaging effects of blue light. The photosensors that initiate this negative phototactic response are the photoactive yellow proteins (PYPs) [1] or Xanthopsins [2,3], so called for their brilliant yellow color. (PYPs should not be confused with the old yellow enzyme, a flavoprotein for which the structure was also solved recently [4].)

Photoactive proteins are excellent systems in which to investigate the functional consequences of protein dynamics, because the chromophore (light-absorbing group) acts as an inherent 'trigger' that permits synchronization of events on timescales as short as tens of femtoseconds. Also, their remarkable optical properties—their absorption spectra are broad, intense and commonly shift by about 50 nm upon absorption of a single photon—offer potential technological applications to nonlinear optics and optical computing. At present, only a handful of photoactive proteins have been characterized, and far fewer have been crystallized. PYP from *E. halophila*, despite its relatively recent discovery [5], is in many ways the most attractive model system because it is small (14 kDa) and soluble, and its high-resolution (1.4 Å) structure has been determined.

PYP has an α/β fold (Fig. 1) with strong similarity to parts the actin-binding regulatory protein profilin and the Src homology domain 2 (SH2 domain) that is common in proteins involved in signal transduction, inviting one to speculate on the possibility of a 'signal-transduction fold' [6]. PYP contains a 4-hydroxycinnamate chromophore [7,8] that is covalently attached to the thiol group of Cys69, located in the hydrophobic core of the protein. The phenolic oxygen of the cinnamate hydrogen bonds with the hydroxyl group of Tyr42 and the carboxylate oxygen of Glu46, which is most probably protonated [9]. These two groups are indirectly linked by the hyroxyl group of Thr50, which hydrogen bonds to the hydroxyl of Tyr42 and the carbonyl of Glu46. In addition, the interaction of the carbonyl oxygen of Thr50 with the guanidinium group of Arg52 positions the Arg52 sidechain such that it shields the chromophore from solvent (see Fig. 2). The stabilization of the chromophore's negative charge in the hydrophobic binding pocket red-shifts the absorption of the PYP-bound chromophore relative to the non-protonated form, rendering PYP bright yellow.

The photocycle of PYP includes a transient proton uptake [10], and a conformational change that presumably communicates 'news' of the blue light to the next element in the signal transduction pathway. Absorption of a visible

Figure 1



Overall fold of PYP showing in yellow stick representation the location of the 4-hydroxycinnamyl chromophore and the active-site arginine, Arg52, relative to the six-stranded β sheet; β sheets are shown in gold and α helices in blue.The upper side of the β sheet is shielded by the N-terminal α-helical lariat, and the bottom side is shielded by a loop containing Cys69, to which the chromophore is covalently attached through a thioester linkage.

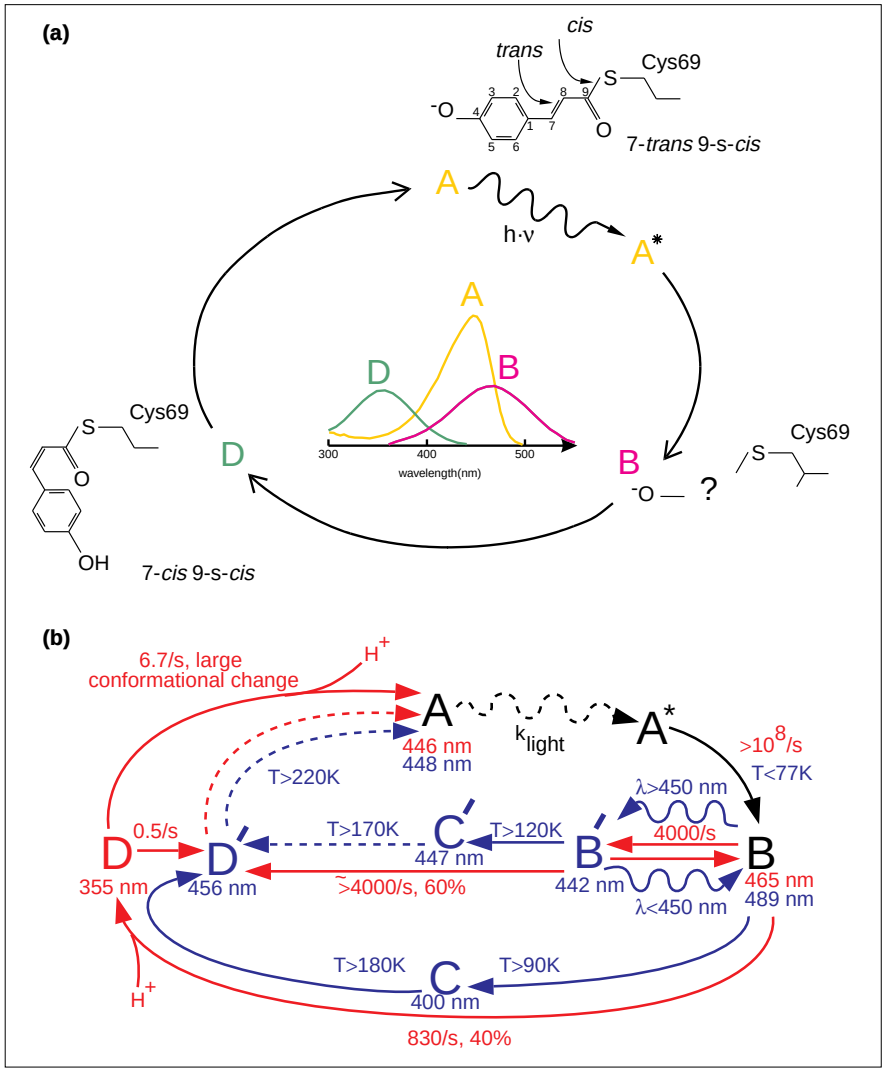
Figure 2

PYP in the *A* and *D* states. (a) Schematic of the yellow ground state *A*, in which the *trans*-chromophore is shielded from solvent by Arg52, and a hydrogen-bonding network (red dotted line) stabilizes its deprotonated phenolic oxygen atom. (b) Schematic of the bleached state *D*, in which the chromophore has isomerized to the *cis* form. The protonated phenolic oxygen of the chromophore has displaced the guanidinium group of Arg52, and forms a hydrogen bond with it. In its new position, the chromophore becomes solvent exposed. (c,d) Stereoviews of the active sites of *A* and *D*, respectively. The stereoviews are rotated approximately 45° about the vertical axis from Figure 1. (Figures 2c and d were reproduced from [22] with permission.)

photon causes PYPs to undergo *trans–cis* isomerization [11], in which the phenylate anion on the chromophore [12] picks up a proton from its protein environment [9]. This isomerization is accompanied by large shifts in PYP's absorption spectrum — a process reminiscent of the famous *trans–cis* isomerization of retinal in bacteriorhodopsin with its concomitant deprotonation of the Schiff base [13].

The photocycle proceeds at room temperature via two intermediates, *B* and *D*, with distinct spectral and kinetic properties (see Fig. 3); *B* has been denoted in the literature as I_1, *pR* and PYP_B, whereas *D* has been known variously as I_2, *pB*, and PYP_M. It turns out, however, that the photocycle is not as simple as initially believed (Fig. 3a), but it is in fact branched and light-dependent (Fig. 3b) [14]. The findings from kinetics measured on fast timescales at room temperature and slow timescales at cryogenic temperatures can be summarized as follows: *B*, which is characterized by a red-shift in its absorption spectrum of approximately 40 nm from the ground state,

Figure 3



The PYP photocycle. **(a)** Simplified version of the PYP photocycle. The *A*, *B*, and *D* states are shown along with their visible absorption spectra and the structures of the 4-hydroxycinnamate chromophore. Single photons of visible light raise the ground state of the protein, *A*, to the excited state, *A**. In the *B* state, the chromophore is believed to have photo-isomerized into the 7-*cis* 9-s-*trans* isomer [9] or into the deprotonated 7-*cis* 9-s-*cis* form. **(b)** Detailed kinetic and spectroscopic features of the PYP photocycle. Features found only in low-temperature studies are shown in blue, those found in studies near room temperature are shown in red and features seen in both are shown in black. Absorption maxima, rates and temperatures are shown where known. This scheme is a synthesis of results from a room temperature study over the time range of 10 nanoseconds to a few seconds [7], a slow spectroscopic study over the temperature range of 77K to room temperature [17] and a photoacoustic study [15]. There is evidence for a parallel pathway, shown here with primes, which may differ from the unprimed pathway in protonation of the chromophore as discussed in the text. The transition between *B* and *B′* may be driven reversibly at 77 K by application of light of different wavelengths. *C* and *C′* are not seen in room temperature studies; presumably low temperatures prevent the structural relaxations that would otherwise lead to formation of *D* and *D′*. None of the printed intermediates have been observed in room temperature studies for kinetic reasons.

is formed within 10 ns and decays bi-exponentially on a millisecond time scale. A fraction of *B* decays by a slower route that corresponds to the quantum efficiency of conformational change, which has been measured by photoacoustic spectroscopy to be near 40% [15]. Photolysis of PYP at liquid nitrogen temperature produces a mixture of the red-shifted intermediate observed at room temperature, *B*, and another species that is very slightly blue-shifted [16,17], which we denote in Figure 3b as *B′*. The ratio of *B* and *B′* obtained upon photolysis was found to be wavelength-dependent, with wavelengths lower than 450 nm favoring the *B′* state [17]. Perhaps most striking is that *B* and *B′* can be interconverted by illumination with light of wavelengths on alternate sides of 450 nm. Such effects are known in physical chemistry as 'nonphotochemical hole burning', and in organic glasses they are commonly associated with photon-induced tunneling of a proton between two alternate positions in a hydrogen bond [18–20]. It seems likely that such a mechanism could be operating in PYP between Glu46 and the phenylate ion of the chromophore.

At room temperature, the more slowly decaying component of *B* is transformed into a strongly blue-shifted intermediate, *D*. The decay of *D* in solutions near room temperature is also bi-exponential, with rate coefficient and fractions that were compatible with a simple branching scheme without back-reaction. Decay of *D* in the crystal is noticeably different [21].

The crystal structure of the *D* intermediate of the PYP photocycle was recently solved by the groups of Elizabeth Getzoff and Keith Moffat [22], thus adding a fourth dimension — time — to the structural studies. This was made possible by using polychromatic X-rays provided by a synchrotron for data collection. This 'Laue method' allows one

to collect diffraction data with a time resolution that can be as fast as nanoseconds [23]. In order to seen an intermediate by any kinetic technique, it is necessary to use a timescale in line with the formation of that intermediate. At the working temperature of 261K in this experiment, the rapidity of the trigger (given by k_{light} in Fig. 3b) and the timescale of data collection of 10 ms was sufficient to populate and determine the structure of the slow-decaying *D* state, but not the first intermediate, *B*. A 1.9 Å electron-density map was calculated from the data set collected within 12 ms after photolysis and showed that only about 50% of the chromophores had changed conformation. The cause for this partial occupancy might be decay of the rest of the *B* population through the pathway $B \rightarrow C' \rightarrow D'$ of Figure 3b, in which the phenylate is unprotonated.

The structures of the intermediate *D* and the ground state *A* differ substantially in the region of the chromophore. The 4-hydroxycinnamate undergoes a *trans–cis* isomerization around the vinyl double bond that is conjugated with, and located between, the phenolic ring and the thioester bond to Cys69 (see Fig. 2 and Fig. 3a). The ensuing movement of the phenolic ring moves its oxygen atom into the ground state position of Arg52, which consequently has to swing outwards. The phenolic oxygen atom of the chromophore becomes protonated [9], solvent exposed and hydrogen bonds to Arg52 (Fig. 2b). This changes the molecular surface and electrostatic potential of PYP, and is presumably the mechanism by which the signal is transmitted to the next element in the transduction cascade.

These structural findings, in combination with spectroscopic data, led to a model of the photocycle in which the first very rapid transition to the intermediate *B*, following the photon absorption, occurs with the isomerization of the chromophore. The following slower transition to *D* was identified with the concomitant displacement of Arg52 (resulting in exposure of the chromophore to the solvent environment) and proton uptake by the chromophore from solvent [22] or from Glu46 [9] (thereby blue-shifting its absorption maximum). The bleached, blue-shifted, *D* state would then slowly return to the ground state by re-isomerization of the chromophore accompanied by the corresponding rearrangement of the protein.

A time-resolved spectroscopic study of mutants of the key residue Glu46 which interacts with the chromophore phenolate oxygen in the ground state *A* and Arg52, which interacts with the phenyolic oxygen in the bleached state D, was performed to identify their roles in the photocycle [24]. Arg52 was believed to stabilize the negative charge on the 4-hydroxycinnamate, which red-shifts its absorbance maximum relative to the non-protonated chromophore [12]. This seems not to be the case, however, as the removal of the positive guanidinium group in the Arg52→Ala mutant not only has little influence on the absorption maximum, but even slightly red-shifts it. The $B \rightarrow D$ transition is accelerated three to fourfold, whereas the $D \rightarrow A$ transition is slowed six to eightfold in this mutant.

The Glu46→Gln mutant is isosteric with the wild type, but it lacks the ability of the chromophore to take up a proton from this residue [9], because deprotonation of the Gln46 sidechain's nitrogen is unlikely. A slight red-shift, when compared with the wild type, in the absorption spectrum of the Glu46→Gln mutant indicates that some hydrogen-bonding interaction with the phenolate oxygen of the chromophore must have been retained. In this mutant at neutral pH, the $B \rightarrow D$ transition is accelerated five to sixfold, and the $D \rightarrow A$ transition is accelerated three to fourfold over the wild type. However, upon a pH change from 5 to 10, the rate of the $D \rightarrow A$ transition of the mutant increases by 700-fold compared with a 16-fold increase for wild-type PYP. It seems that an intricate interplay of ionizable groups modulates the photocycle of PYP.

PYP offers a unique combination of biochemical, spectroscopic and atomic-resolution crystallographic data that together can provide new insights into protein photocycles. Not only can PYP serve as a paradigm for more complex systems, such as bacteriorhodopsin and the rhodopsins, but stands in its own right as a biophysical laboratory for studying the interactions of light and live.

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