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Photophysics and photochemistry of phytochrome, a chromoprotein in plants

Kurt Schaffner, Silvia E. Braslavsky and Alfred Holzwarth

Max–Planck–Institut für Strahlenchemie, D–4330 Mülheim a. d. Ruhr, Fed. Rep. Germany

Abstract – The photophysical properties of 124–kDa phytochrome from etiolated oat shoots and some aspects of its photochemical reactivity are reviewed in terms of the working scheme depicted in Fig. 3. A review discussing these results in greater detail – with the exception of the interaction of the P_r form of phytochrome with ubiquitin – has been recently published in ref. 1e.

INTRODUCTION

Phytochrome is a light receptor in green plants, which converts absorbed light energy into physiological signals (ref. 1). It thereby exerts photomorphogenic control functions in response to the spectral composition of the light by way of a photochromic $P_r \leftrightarrows P_{fr}$ equilibrium between its physiologically inactive red absorbing (P_r; λ_{max} 665 nm) and active far–red absorbing (P_{fr}; λ_{max} 730 nm) forms.

During the first years of phytochrome research, facile endogenous proteolysis had precluded the isolation of the native–size protein. Rather, partially degraded preparations of ca. 60 kDa and 114/118 kDa (the so–called "small" and "large" phytochromes) were obtained which, however, still exhibited the proper absorption and photochromic ("photoreversible") properties. When isolated from etiolated oat shoots, undegraded native phytochrome has a molecular weight of 124 kDa (the size varies only slightly with the plant source), with a polypeptide chain of 1128 amino acid residues. The protein dissolves as a dimer in aqueous buffers, and electron microscopy has shown the dimer to be Y shaped, with the amino terminal domains occupying one branch each and the carboxyl domains combining to form the third (ref. 4).

Phytochrome possesses a single bilatriene chromophore which is bound covalently to cystein–321 (Fig. 1), and which is responsible for the absorption in the red visible region. The bilatriene chromophores of P_r and P_{fr} are constitutionally identical, but the C(15) double bond configuration has been established to be different: P_r possesses the 15*Z* and P_{fr} the 15*E* configuration. Configuration and conformation around the other double bonds and around the single bonds of the methine bridges are still unknown.

Our results obtained in studies at physiological temperatures (≥ 275 K) shall be discussed in terms of the working scheme shown in Fig. 2 for the phototransformation of P_r into P_{fr}.

PHOTOPHYSICAL PROPERTIES OF P_r

The stationary fluorescence of the P_r bilatriene chromophore is compatible with the stretched alignment shown in Fig. 1 (ref. 5). Single–photon–timing (SPT) measurements with global data analysis

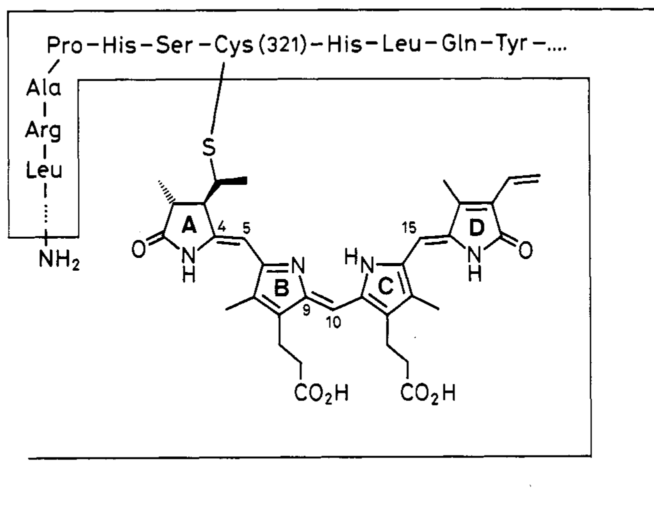

Fig. 1. Bilatriene chromophore of P_r phytochrome. A "stretched" conformation with *Z,Z,Z* configuration of the double bonds was chosen in analogy to that of the phycocyanobilin chromophores α–84 and β–84 in C–phycocyanin of cyanobacteria (ref. 2). Furthermore, absorption (ref. 1e) and resonance Raman evidence (ref. 3) is in favour of N–protonation.

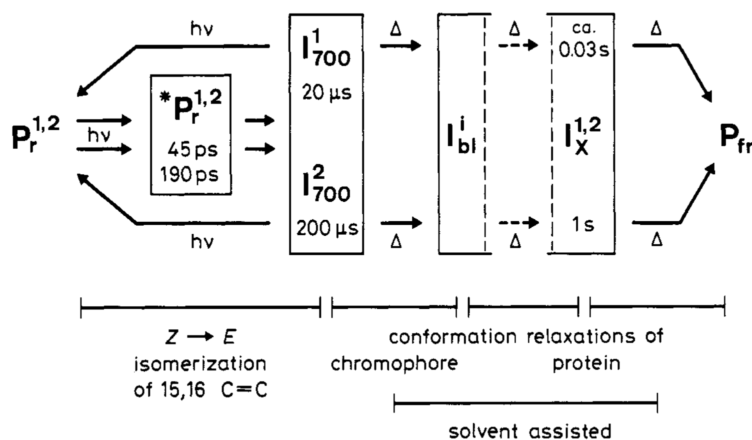

Fig. 2. Tentative working hypothesis of the mechanism of the $P_r \rightarrow P_{fr}$ transformation.

exhibit three spectrally overlapping decay components (Fig. 3). The two predominant shorter–lived of these components [P_r^{*1}: $\tau^1 \approx 44$ ps, ca. 91%; P_r^{*2}: $\tau^2 \approx 163$ ps, ca. 8%; at 275 K] possess the $P_r \leftrightarrows P_{fr}$–photochromic properties characteristic of phytochrome. The long–lived minor component P_r^{*3} [$\tau^3 \approx 900$ ps, ca. 1%] is not fully "photoreversible". The criterium of photochromicity therefore qualifies only P_r^{*1} and P_r^{*2} as functional phytochrome species, whereas no biological function can be attributed as yet to the P_r^{*3} component (ref. 6).

The fluorescence quantum yield of the P_r^{*1} and P_r^{*2} components at 275–293 K is only $\Phi_f \approx 3{\cdot}10^{-3}$ (refs. 6,7), and deactivation proceeds predominantly through nonradiative channels, such as internal conversion back to ground–state P_r and primary photoreaction(s) such as the $P_r^{1,2*} \rightarrow P_{fr}$ transformation.

An SPT study of the 10 tryptophan residues, which are situated within the central third of the polypeptide chain of 1128 amino acids (residues 366 – 790), showed that the fluorescence falls into four lifetime classes with distributions ranging from ca. 30 ps to 5 ns at 277 K. The changes in

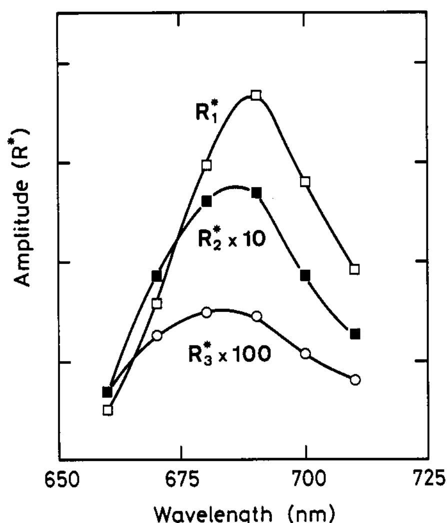

Fig. 3. Time–resolved decay–associated emission spectra of the fluorescence components P_r^{1-3*} with the relative amplitudes R^*_{1-3} (124–kDa phytochrome) at 275 K. The decay amplitudes and lifetimes were obtained by global analysis, i.e., they were simultaneously calculated at variable excitation wavelengths and constant emission wavelength, assuming that the lifetimes are independent of the former. (Taken from ref. 1e.)

the decay pattern occurring in the $P_r \rightarrow P_{fr}$ transformation are insignificantly small (Fig. 4). Evidently the central protein domain does not undergo any gross overall conformational change in the reaction (ref. 9).

THE $P_r \rightarrow P_{fr}$ PHOTOTRANSFORMATION

Primary photoreactions and the first set of parallel thermal secondary steps

Nanosecond flash photolysis, absorption (refs. 10–12) and time–resolved optoacoustic spectroscopy (refs. 13–15) reveal that in an apparent first step P_r simultaneously affords two products, I^1_{700} and

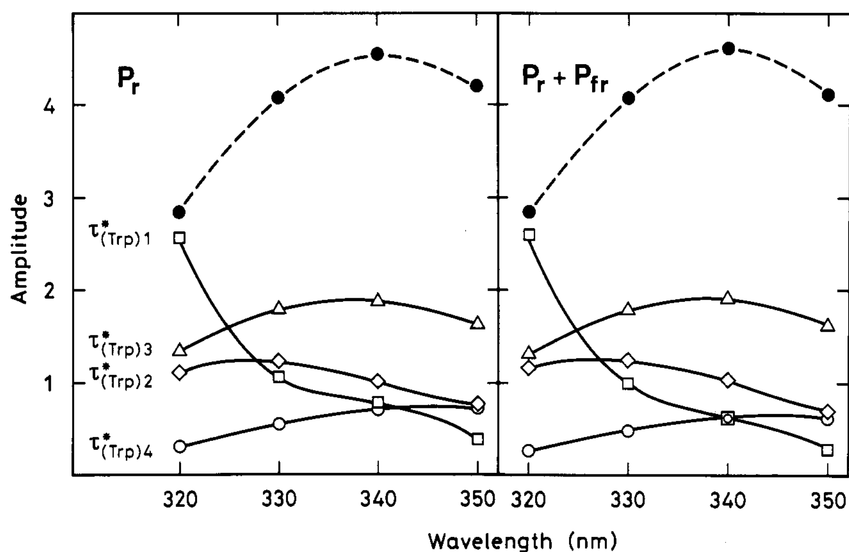

Fig. 4. Time–resolved decay–associated spectra of the UV (protein) fluorescence components $\tau^*_{(Trp)1-4}$ of the P_r and P_{fr+r} forms of 124–kDa phytochrome, obtained by global analysis; λ_{exc} = 297 nm, 277 K. The spectral amplitudes can be compared on an absolute basis.

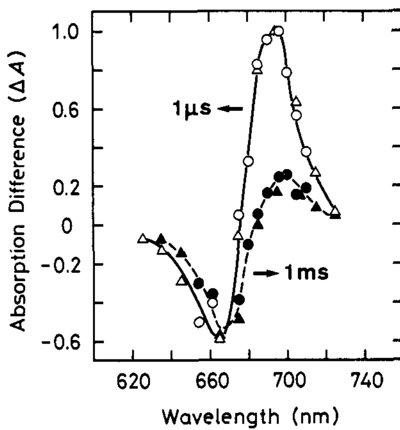

Fig. 5. Time-resolved relative difference spectra of the I^{i}_{700} absorption decay curves of P_r (124-kDa phytochrome) in phosphate buffer solution 1 μs (–Δ–Δ–) and 1 ms (–▲–▲–) after excitation with a 15 ns laser flash at 650 nm, and of 124-kDa P_r covalently bound to the surface of soybean lecithin liposomes after 1 μs (○ ○) and 1 ms (● ●); 275 K. The ΔA values are normalized to the 1-μs signal amplitude at 695 nm. (Taken from ref. 18.)

I^{2}_{700}, along parallel reaction pathways. Both exhibit a new absorption maximum close to 700 nm (λ_{max} 695 nm; Fig. 5). The proportion and lifetimes of the two transients are 38% and $\tau^1 \approx 21$ μs for I^{1}_{700}, and 62% and $\tau^2 \approx 200$ μs for I^{2}_{700}, at 275 K in ethylene glycol-free buffer solution. The dynamic photoequilibrium $P_r \leftrightarrows I^{1,2}_{700}$ (ref. 16) is established within the 15-ns period of the laser flash, in competition to the thermal forward reaction of I^{i}_{700} [$\rightarrow I^{i}_{bl}$, as witnessed by the bleaching of the I_{700} absorption around 700 nm without concurrent recovery of the P_r absorption at 665 nm; cf. Fig. 5]. The total quantum yield of this first reaction step is $\Phi_{r\rightarrow 700} \geq 0.5$, i.e., more than three times greater than the total yield of the P_{fr} formation, $\Phi_{r\rightarrow fr} = 0.15$ (ref. 17). In other words, P_r is partly recovered from intermediates of the $P_r \rightarrow P_{fr}$ transformation, such as I^{i}_{bl}, via still unknown routes.

It is still an open question whether $P_r \rightarrow I_{700}$ indeed represents the primary photoreaction of 124-kDa P_r. Pump-probe absorption measurements by Hermann *et al.* (ref. 19) of 120-kDa P_r from oat suggest that there is a pre-I_{700} formed with a lifetime of ≈ 10 ps, a result which has not yet been duplicated with the 124-kDa chromoprotein from the same plant material.

The endogenous proteolytic elimination of the 6-, 10- and 60-kDa fragments from the native protein affects neither photophysics nor photochemistry of P_r, nor the thermal reactivity of the I_{700} intermediates (ref. 7). This insensitivity of the sequence $P_r \rightarrow I^{1,2}_{700} \rightarrow I^{i}_{bl}$ suggests that the transformations involved are confined to the bilatriene chromophore and its close surroundings. This is further supported by the invariance of the properties of 124-kDa P_r when it is covalently bound to liposomes composed of various different lipids and investigated in different buffer solutions (refs. 20,21).

The terminal steps of the P_{fr} formation

The increase of P_{fr} 20 ms after the excitation of P_r again proceeds in two phases, which suggests two immediate precursors of P_{fr} [viz., $I^{1,2}_{X}$] with lifetimes of $\tau^1 \approx 0.03$ s and $\tau^2 \approx 1$ s at 275 K (ref. 22). Changing from H_2O to D_2O buffer affects the P_{fr} formation only slightly, which excludes any kinetic H/D isotope effect.

The influence of ethylene glycol and ubiquitin

Addition of 20–25% ethylene glycol does not alter the $P_r \leftrightarrows P_{fr}$ photochromicity, and lifetimes and amplitudes of the $P^{1,2}_{r}$ fluorescence components (ref. 9) as well as the absorption decay of $I^{1,2}_{700}$ are hardly affected. This is in accord with a confinement of the sequence $P_r \rightarrow\rightarrow I_{bl}$ to the bilatriene

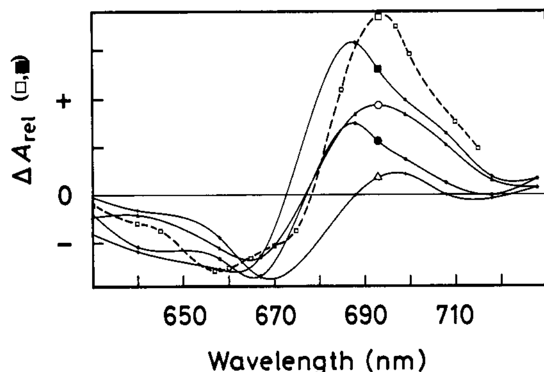

Fig. 6. Comparison of the difference spectra of I^i_{700} at zero time and 275 K for the ubiquitin-free P_r dimer (□; 124-kDa phytochrome) and for ubiquitin-P_r 5:1 (■), and component spectra for the ubiquitin-P_r 5:1 complex (●, I^1_{700}; ○, I^2_{700}; Δ is a constant function required in the equation

$$\Delta A(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + \text{constant}$$

which fits the decay of the I^i_{700} absorption. (Taken from ref. 22.)

chromophore-protein domain without any far-reaching assistance by other domains. Interference by ethylene glycol in this domain is reflected by the formation rates of I^1_{700} and I^2_{700}: while the ratio I^1_{700}/I^2_{700} is clearly dependent on temperature in the absence of ethylene glycol, it does not vary any more in its presence.

Results like this initiated a search for other agents to modify the dynamics of the complex $P_r \rightarrow P_{fr}$ transformation, and in particular to affect differentially the various intermediates. The search focused especially on cellular constituents which presumably interact *in vivo* with phytochrome. Ubiquitin, an 8.5-kDa protein claimed to undergo covalent binding *in vivo* to the physiologically active P_{fr} (ref. 23), has now also been found to interact *in vitro* with the dormant P_r form in the absence of any other cellular constituent (ref. 24). Association causes dissociation of the protein dimer and formation of ubiquitin-P_r complexes which contain one P_r monomer and in which the ubiquitin and P_r components are not covalently bound. These complexes are still fully photoreversible. In addition to the monomerization effect, the complexation shifts the 695-nm absorption maximum of I^i_{700} differentially to shorter wavelengths by ca. 10 nm, with the shorter-lived component I^1_{700} being affected at a lower ubiquitin-P_r ratio than I^2_{700} (Fig. 6). The results altogether point to a reversible interaction of ubiquitin with the protein pocket domain housing the bilatriene chromophore, which thus constitutes – in contrast to previous transformations with non-biological reagents – a tool to differentially monitor the complex reaction dynamics without a permanent chemical change in the domains involved directly.

The chemical nature of the individual reaction steps

Photochromicity as well as fluorescence lifetimes, relative amplitudes and quantum yields of P^1_r and P^2_r, which account for ≥ 99% of the total fluorescence decay amplitude, are the same in H_2O and D_2O at 275 and 293 K (ref. 6). The appearance of the I^i_{700} absorption does not reveal either a kinetic H/D effect on the efficiency of the primary photoreaction (ref. 12). A proton transfer is therefore unlikely in the photoreaction or any other deactivation of $P^{1,2}_r$. The most probable process for $P_r \rightarrow I_{700}$ is therefore a $Z \rightarrow E$ double bond isomerization.

The subsequent steps leading to P_{fr} exhibit no more than mere solvent-assisted H/D effects on the reaction rate constants, which excludes rate-determining proton transfer processes.

Step $I^{1,2}_{700} \rightarrow I^i_{bl}$ comprizes a conformational relaxation of the chromophore, which leaves room in the still insufficiently explored "grey" zone between I^i_{bl} and P_{fr} for relatively slow reorganizations of the protein structure.

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