Tyrosine 263 in cyanobacterial phytochrome Cph1 optimizes photochemistry at the prelumi-R→lumi-R step

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ABSTRACT

We report a low-temperature fluorescence spectroscopy study of the PAS-GAF-PHY sensory module of Cph1 phytochrome, its Y263F mutant (both with known 3D structures) as well as Y263H and Y263S to connect their photochemical parameters with intramolecular interactions. None of the holoproteins showed photochemical activity at low temperature and the activation barriers for the Pr→lumi-R photoreaction (2.5-3.1 kJ mol^{-1}) and fluorescence quantum yields (0.29-0.42) were similar. The effect of the mutations on Pr→Pfr photoconversion efficiency ($\Phi_{Pr\rightarrow Pfr}$) was observed primarily at the prelumi-R S_0 bifurcation point corresponding to the conical intersection of the energy surfaces at which the molecule relaxes to form lumi-R or Pr, lowering $\Phi_{Pr\rightarrow Pfr}$ from 0.13 in wild type to 0.05 - 0.07 in the mutants. We suggest that the E_a activation barrier in the Pr* S_1 excited state might correspond to the D-ring (C19) carbonyl – H290 hydrogen bond or possibly to the hindrance caused by the $C13^1/C17^1$ methyl groups of the C- and D-rings. The critical role of the tyrosine hydroxyl group can be at the prelumi-R bifurcation point to optimize the yield of the photoprocess and energy storage in the form of lumi-R for subsequent rearrangement processes culminating in Pfr formation.

INTRODUCTION

Phytochromes are a family of photoreceptors ubiquitous in plants and widespread in microorganisms. They consist of a biliprotein dimer in which an open-chain tetrapyrrole is thioether-linked to the protein at a conserved cysteine residue. Light regulation of phytochrome action is based on a complex photocycle in which light-driven reactions in the chromophore initiate dark thermal relaxations of the protein culminating in one of two thermodynamically-stable products, namely the far-red-light-absorbing Pfr state in the forward route and the red-light-absorbing ground state Pr in the reverse route of the cycle (see reviews (1-4)). Whereas in Pr the chromophore adopts a *ZZZssa* conformation, Pfr is *ZZEssa* due to photoisomerization at the C15=C16 double bond although recent structural studies have shown that the A and particularly the D rings are twisted relative to the B-C ring plain (5-10). Interestingly however, Pr in various phytochromes is heterogeneous, the different substates being associated with spectroscopic, photochemical and/or structural differences (10-18). No such heterogeneity has been reported for Pfr, however.

The first intermediate following light activation of Pr is lumi-R. A hypothetical energy level scheme of the initial photoreaction in plant phyA (2, 12) postulated that initial structural changes of Pr already take place in the S_1 excited state Pr*, that there exists an S_1 activation energy barrier (E_a) for the $Z{\rightarrow}E$ isomerisation, and that the photoreaction (completed or uncompleted) is the only temperature-dependent route for deactivation of the excited state (Fig. 1). This scheme contradicts models in which an equilibrium between Pr* and lumi-R* on the S_1 surface is assumed (see (19-22) and discussion in Ref. 2). The former considers that at ambient temperature (T_a=293 K) the activation barrier is easily overcome and the quantum yield of the initial photoreaction is determined by the partitioning coefficients between the initial state Pr and photoproduct lumi-R at the "prelumi-R" S_0 transition point at the conical intersection of the S_1 and S_0 surfaces. Extensive work on plant phytochromes implies that as much as 150 kJ mol^{-1} is stored in lumi-R relative to Pr (ΔE; (23)), energy that is available to drive processes in the protein framework subsequently leading to Pfr formation. These have yet to be associated with specific structural changes, however.

<Figure 1>

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Recent progress in physicochemical and structural studies of phytochrome is connected largely with work on prokaryotic phytochromes (24-26). Cph1 phytochrome from the cyanobacterium *Synechocystis* 6803 is particularly valuable as it represents an excellent model for the plant phytochrome family. Cph1 attaches its phycocyanobilin (PCB) cofactor via a thioether bond to C259 (27, 28), closely reflecting the situation in plant phytochromes. The Pr extinction coefficient of 85 mM^{-1} cm^{-1} at λ_{max} is similar to that of plant phytochromes as are the photoconversion efficiencies of ~0.15 in both directions (29, 30). Furthermore, photoconversion of Cph1, whose chromophore is protonated both as Pr and Pfr, is associated with transient proton release (3, 30, 31), an effect seen in plant phytochromes after proteolytic attack (32, 33). Time-resolved UV-Vis absorbance analyses as well as vibrational spectroscopic (FTIR and FTRR) studies at cryogenic temperatures (T_c) (30, 34-36) revealed many additional features of Cph1 photoconversion in common with plant phytochromes as did T_c fluorescence and photochemical investigations (13, 37). In particular, time-resolved studies of the initial photoisomerization (Pr→lumi-R) of the cyanobacterial photoreceptor Cph1 (36, 38) performed at T_a supported the initial Pr photoreaction scheme proposed on the basis of low-temperature steady-state investigations of plant phyA (2, 12). This scheme also considers the Pr→lumi-R photoprocess as the mixed excited/ground state mechanism with a potential barrier for isomerization in the excited state, in contrast to the idea of isomerization exclusively on the S$_1$ surface requiring an equilibration of Pr* and lumi-R* (39). On the other hand, studies of amino acid substitution mutants (28, 40, 41) and finally crystal structures (7, 40) of the Cph1 sensory module (Cph1Δ2, comprising the PAS-GAF-PHY domains) provided insights into phytochrome molecular function, identifying roles for C259 (chromophore attachment), H260 (chromophore protonation), D207 (Pfr protonation), R254 (intramolecular signal transduction) and Y176 (photochemistry). The role of Y263 - which directly interacts with the D ring of the chromophore - was investigated in structural, spectroscopic and photochemical studies of the wild type, Y263F, Y263H and Y263S mutants (40). In that initial analysis, we considered that the aromatic character but not the hydroxyl group of Y263 was central to Pfr formation, although the reduced volume of the non-aromatic side chains represents an alternative explanation. The high resolution Y263F crystal structure (3ZQ5) presented in that paper revealed multiple side chain conformations for several residues near the chromophore, supporting the notion of Pr structural heterogeneity. MAS NMR at T_c revealed that the Pr ground state of Cph1

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comprises two isoforms, Pr-I and Pr-II, the latter being equivalent to the 2VEA crystal structure (10). A similar dimorphism is seen for oat phyA3 in the Pr state (14). It should be noted that a potential effect of the transmitter domain is negligible: the C-terminus has no effect on spectroscopic and photochemical properties of the pigment as compared with Cph1Δ2; full-length Cph1 was also shown to be heterogeneous (13).

In this work, we further investigated the Cph1Δ2 sensor module and its Y263F, Y263H and Y263S substitution mutants using the T_c fluorescence / photochemical approach employed earlier (12) but now exploiting knowledge of the 3D structures of Cph1Δ2 wild type and Y263F (7, 40). The two isotypes of wild-type Cph1Δ2 showed much smaller differences at T_c than at T_a. This was also true for Y263 mutants, which in any case showed much smaller inhomogeneity. The substitutions brought about changes both in the Pr fluorescence and photochemical properties. We show that the critical parameter affected by the mutations in lowering the overall quantum yield of the Pr→Pfr photoconversion is at the bifurcation point of the routes from prelumi-R to lumi-R or back to Pr. The conserved Y263 residue was thus shown to play an important role in optimizing the photochemical efficiency of the photoreceptor.

MATERIALS AND METHODS

Protein expression of the histidine-tagged Cph1 photosensory module (Cph1Δ2; comprising the first 514 amino acids), production of its site-directed mutants Y263F, Y263H and Y263S,sample preparation, manipulations and absorption and fluorescence spectroscopy were carried out as described (13, 37, 40, 42). Briefly, absorbance spectra of phytochrome in solution were recorded in a quartz cuvette on an Agilent 8453 UV–Vis diode detector-array spectrophotometer suitably modified to allow the sample to be irradiated laterally by either FR or R light. Absorbance spectra of phytochrome were taken after saturating FR and R irradiation. Kinetics of phytochrome absorption changes upon red illumination for determination of the quantum yield of the Pr→Pfr phototransformation ($\Phi_{Pr \to Pfr}$) were measured with an UVmini-1240 spectrophotometer (Shimadzu). The photoconversion was monitored at λ_{max} of the Pfr state to measure the increase of the Pfr fraction by irradiating the sample at its respective Pr λ_{max} (photon fluence rates 6.7–7.9 µmol · m^{-2}· s^{-1}) using appropriate interference filters (wild-type: λ = 660 nm, T = 37 %, FWHM = 16 nm; Y263F: λ = 651 nm, T= 45 %, FWHM = 13 nm; Y263H / Y263S: λ = 643 nm, T=

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41 % FWHM = 10 nm; Schott). The photoconversion rate constant k and the quantum efficiency of photoconversion $\Phi_{Pr \to Pfr}$ were calculated as described (43, 44), integrating with respect to wavelength from 620 nm to 680 nm to account for the spectral distributions of the light sources (measured with an Ocean Optics spectroradiometer (30)). The photoconversion rate constant k was determined by extrapolating the initial slope of the resulting curves of phototransformation (44). The measuring light did not induce significant photoconversion relative to the actinic source.

Determination of the fluorescence parameters and kinetics and energetics characteristics of the initial processes in the pigments were done as described in detail elsewhere (2, 12, 13, 37). Briefly, fluorescence spectra and its kinetics associated with the Pr→Pfr photoconversion of the pigments were recorded using a Fluoromax4 spectrofluorometer (HORIBA Jobin Yvon). To minimize spectral artifacts deriving from self-absorption and scattering a protein concentration of approximately 0.1 mg / ml (absorbance at $\lambda_{max} <$ 0.1) was used. Emission spectra were measured using an excitation wavelength of 610 nm, a slit bandwidth of 0.3 nm and a red interference filter ($\lambda_{max} = 610$ nm, T = 40 %) to minimize phytochrome photoconversion during the scan. To exclude possible interference of scattered exciting light with the fluorescence spectra measurements, a 600 nm cut-off filter was used and the spectra were recorded from 630 to 750 nm with a slit bandwidth of 10 nm and an integration time of 0.1 s. The low protein concentration and the reduced integration time necessitated three separate scans, which were then averaged and smoothed (by adjacent averaging method) for the final emission spectrum. To determine the fluorescence quantum yield (Φ_f), chlorophyll <u>a</u> (Chl<u>a</u>) solution with a defined $\Phi_f = 0.3$ was used as a reference (45). The fluorescence intensity was determined by integrating the emission spectrum from 630 to 750 nm. Excitation spectra were recorded in the region 500–700 nm and 350–700 nm with a slit bandwidth of 1 nm and using 1 % grey transmission filter to minimize photoconversion. The emission was measured at 720 nm with a slit bandwidth of 10 nm and using a 680 nm cut-off filter to avoid interference from scattered light. The integration time was 0.1 s and excitation spectra were averaged and smoothed from 3 measurements. To determine the yield of the Pr→Pfr photoconversion $\Phi_{Pr \to Pfr}$ by fluorescence spectroscopy, a kinetic measurement was done with excitation light at the respective λ_{max} (I_0 ~ 5 µmol m^{-2} s^{-1}), with a slit bandwidth of 5 or 10 nm, a 0.2 % transmission grey filter and an integration time of 0.1 s. Emission was recorded for 600 s at 720 nm, slit bandwidth of 10 nm and a cut-off filter of

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680 nm. The resulting initial slope of the curve was used to determine the rate constant of photoconversion (k_p) and $\Phi_{Pr \to Pfr}$ of the samples as described in detail in (40): $\Phi_{Pr \to Pfr}$ of the mutants was obtained by comparison with the wild type. Action spectra of the Pr→Pfr photoconversion were determined for the fluorescent species of Cph1Δ2 based on similar kinetic measurements of the Pr fluorescence decay during Pr→Pfr photoconversion under exciting / actinic light of different wavelengths. Three kinetic curves were recorded for each exciting wavelength with a slit width of 5 nm at 3–10 µmol m^{-2} s^{-1}(I_0 below). A diffuser was inserted in the light path to reduce the fluence rate and ensure that the entire sample was irradiated uniformly. The initial slope (during the first 2-10 s of registration) of averaged kinetic curves of the Pr fluorescence decay resulting from Pr→Pfr photoconversion was linearly approximated to obtain the tangent of the angle (α) of the decay as a measure of its rate for different wavelengths of the exciting / actinic light. ($\sqrt{\tan\alpha}$)/I_0 is proportional to the absorption coefficient of the investigated fluorescing and photoconverting Cph1Δ2 species and was plotted as the action spectrum of photoconversion according to the procedure suggested in Ref. (46). Fluorescence excitation spectra of the samples used to obtain the Pr→Pfr action spectra were constructed by plotting the photon fluence rate of the fluorescence emission recorded at 720 nm taken at time t=0 on the same fluorescence decay kinetics and divided by the photon fluence rate of the exciting light measured using a spectroradiometer (Ocean Optics).

Low-temperature fluorescence measurements were performed in a quartz tube placed in a Dewar flask coated with a mixture of 50% glycerol and 50% ethanol to avoid condensation on the outer surface. The samples were irradiated with saturating FR before being placed in the Dewar. Upon thawing, either fluorescence spectra or single wavelength measurements at λ_{max} of the emission at 85 K were taken in 10 or 20 K steps, respectively. Emission spectra were essentially recorded as mentioned above using excitation slit bandwidth of 1 nm and emission slit bandwidth of 5 nm. Temperature was monitored using a thermocouple. The Φ_f values in the temperature interval from 85 K to T_a were determined based on the Φ_f of the respective protein at T_a and the coefficient of its temperature dependence. The later was derived by integration of the fluorescence emission spectrum or simply from the fluorescence intensity maximum - each yielding essentially similar results. The determination of the fluorescence decay activation energies (E_a) and the temperature-independent excitation energy dissipation, k_d, were determined as described (12)

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by linearization of the temperature-dependent Φ_f in the Arrhenius coordinates. The fluorescence constant, k_f, was determined from the oscillator strength f obtained from the absorption spectra of the samples according to established principles (47). The temperature-dependent constant of the excitation energy deactivation along the photochemical route (see the scheme in Fig. 1), k_p, as well as the quantum yield of this process, $\Phi_{Pr \rightarrow prelumi-R}$, was calculated based on the determined values of the temperature-independent constants k_d, k_f and of the Φ_f values at T_a and T_c. Finally, from the quantum yield of the primary photoreaction, $\Phi_{Pr \rightarrow lumi-R}$, which is assumed to be the same as $\Phi_{Pr \rightarrow Pfr}$ (48, 49) and from $\Phi_{Pr \rightarrow prelumi-R}$ we obtained the coefficients (probabilities) of partitioning of the routes from the prelumi-R (virtual or real) state either to the ground state of the photoproduct lumi-R or back to the ground Pr state: $\Phi_{Pr \rightarrow lumi-R} = \Phi_{Pr \rightarrow prelumi-R} \cdot [k_{ab}/(k_{ab}+k_{ba})]$ as described (12). We discuss the photophysical and photochemical processes in Pr and its initial photoproducts in the framework of the energy level scheme suggested earlier (2, 12) for plant phytochromes and related photochromic isomerizing pigments, (bacterio)rhodopsins and biliproteins (see Fig. 1). This approach is justified, in particular, by the appearance of a similar scheme of the initial Pr→lumi-R photoreaction proposed by Kim et al. (38) based on multi-pulse pump-dump-probe transient spectroscopy of Cph1. Technical details of the spectral measurements and derived parameters are also presented in the text and in the figure legends.

RESULTS AND DISCUSSION

Spectroscopic studies at ambient temperature

Optical studies of the wild-type Cph1Δ2 sensory module and the Y263 mutants at T_a reported in our previous paper (40) revealed the following λ_{max} values for emission / excitation (absorption) spectra: wild-type Cph1Δ2^{676}/$_{648(660)}$ Y263F^{678}/$_{654(651)}$, Y263H^{669}/$_{644(644)}$ and Y263S^{663}/$_{640(643)}$, giving λ_{max} (em) - λ_{max} (ex) spectral differences ($\Delta\lambda_{em-ex}$; We use this in place of "Stokes shift" whose formal definition as the emission - absorption spectral difference ($\Delta\lambda_{em-abs}$) *for the fluorescing species* is ambiguous in the present context) of 28, 24, 25 and 23 nm, respectively. Thus Y263 specifically provides for long-wavelength absorption as the mutations induce considerable (9-17 nm) blue shifts of Pr and Pfr as well as changes in the extent of the Pr→Pfr conversion (data summarized in Table 1). The emission spectra too are blue-shifted in Y263H and Y263S but not Y263F. Interestingly, the Pr absorption and excitation spectra do not

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always coincide. The trivial explanation that the differences result from a wavelength calibration error in the instrumentation was ruled out by measurements with pure Chl$\underline{a}$ solutions in which we observed close coincidence of the absorption and fluorescence excitation spectra (+/- 1 nm). The discrepancy is particularly marked in the wild type, which shows a 12 nm discrepancy. This emission corresponds to a minor, strongly fluorescent, blue-shifted species (Cph1$\Delta 2_{648}$) whose existence is not apparent from the UV-Vis absorbance spectrum. The major species (Cph1$\Delta 2_{660}$), on the other hand, does not efficiently emit at T_a. In the case of the mutants, these discrepancies are smaller and there is no systematic relationship between the relative positions of the excitation and absorption maxima and the amino acid substitutions. Nevertheless inhomogeneity is still apparent.

<Table 1>

Spectroscopic studies at cryogenic temperatures

At 85 K we observed the following fluorescence emission/excitation λ_{max} values: wild type Cph1$\Delta 2^{679}/_{659}$, Y263F$^{677}/_{659}$, Y263H$^{674}/_{658}$, and Y263S$^{672}/_{654}$, giving $\Delta\lambda_{em\text{-}ex}$ of 20, 18, 16 and 18 nm, respectively (see Fig. 2 and Table 1). The calculated Φ_f values are much higher and more similar than at T_a. Thus the spectra are generally displaced to longer wavelengths as expected and the $\Delta\lambda_{em\text{-}ex}$ values significantly smaller than those at T_a, whereas the high Φ_f reflects the absence of photochemistry at 85 K. Interestingly, with the exception of the Y263S mutant, the absorption and fluorescence λ_{max} values are closely similar at 85 K. Thus the spectra of the different samples at 85 K are much more similar than those observed at T_a. We propose that these effects result from different temperature dependences of the different species involved in regard to positions of their spectra and fluorescence yields. At 85 K we expect, firstly, Φ_f for different species to reach its maximum because the excitation energy cannot be funneled to photochemistry and temperature-dependent thermal relaxation associated with it and thus appears as fluorescence of the bulk absorbing species poorly fluorescent at T_a (see Fig. 1). Secondly, the variety of emitting/absorbing species decreases because the thermoequilibrium between different species will be shifted towards those with the lowest energy level. This would result in a more homogeneous emitting species at T_c. Conversely, the fluorescence and absorption spectroscopic diversity seen at T_a arises from (i) considerable differences in the fluorescence yields of the individual emitting species and (ii)

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differences in the proportions of various ground state populations due to different barriers separating them (see (2)).

<Figure 2>

Wild-type Cph1 comprises two species

Considering further the properties of the wild-type sensory module at T_a (Fig. 3), the absorption spectrum reflects the dominant species corresponding to $\lambda_{max} = 659$ nm whereas the corresponding fluorescence excitation measurements correspond to a shorter-wavelength species (λ_{max} for excitation and emission at 648 nm and 670 nm, respectively). Action spectra for photoconversion corresponded to the shorter-wavelength species. For Cph1Δ2 the $\Delta\lambda_{em-ex}$ value of 22 nm is much greater than $\Delta\lambda_{em-abs}$ of 10 nm and also of the 14 - 16 nm derived for plant phytochromes (2) and close to the 19 nm for *in vitro*-assembled full-length Cph1 reported earlier (37). At the same time, for a number of mutants including Y263F and Y263H, $\Delta\lambda_{em-ex}$ is again higher (24 and 25 nm, respectively) than in the aforementioned phytochromes, but close to their $\Delta\lambda_{em-abs}$ values (27 and 25 nm). This indicates that the $\Delta\lambda_{em-ex}$ value of 22 nm obtained for the wild type at T_a may be actually close to the $\Delta\lambda_{em-abs}$ value of its predominantly fluorescently active species, implying that the emission band measured here might belong primarily to the species absorbing maximally at 648 nm. This is consistent with the emission peak being of approximately of the same width as that of the mutants (FWHH 50, 44 and 60 nm for wt, Y263F and Y263H, respectively). Since the emission and excitation spectra were found to be independent of the wavelengths of fluorescence excitation and monitoring, respectively, the longer-wavelength species absorbing at 660 nm is likely to be only very weakly fluorescent and is not apparent in fluorescence measurements at T_a.

<Figure 3>

At T_c, however, fluorescence from the longer-wavelength species is obvious. Figure 4 describes the wavelength-dependencies of excitation and emission at T_c. Pronounced variations in the position of the excitation spectra at different emission monitoring wavelengths was found: λ_{max} was at 652 nm (satellite at 594 nm) and 664 nm (satellite at 606 nm) when monitored at 660 nm and 676 nm, respectively. Small variations in the position of the emission spectra were also observed: 674 nm and 676 nm when the excitation wavelengths were at 652 nm and 594 nm, respectively. These dependencies imply that at T_c there are at least two fluorescing species, one of which can be characterized as $\text{Cph1}\Delta2^{676}/_{664}$.

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Determination of the position of the emission spectrum of the species with excitation (absorption) λ_{max} at 652 nm is more difficult because of the overlap of the absorption spectra. However, taking into consideration (a) the large $\Delta\lambda_{em-abs}$ value of 22 nm for this species at T_a and the fact that it may decline at lower temperatures, and (b) the $\Delta\lambda_{em-abs}$ value of around 12-15 nm at T_c of Cph1Δ2^{676}/$_{664}$ in this and earlier papers (13, 37) and plant phyA (2), we postulate that the shorter-wavelength species with $\Delta\lambda_{em-abs}$ = 15 - 20 nm is Cph1Δ2$^{670-674}$/$_{652}$.

<Figure 4>

Temperature-dependency of fluorescence

Emission spectra were recorded sequentially upon warming the samples with 20 K temperature steps (Fig. 5a). The emission λ_{max} was constant below 220-240 K but steeply blue-shifted at higher temperatures for all the samples (Fig. 5b). The fluorescence yield (Fig. 6, left panel) showed a monotonous decline up to 220-240 K and a steep drop above this was seen in all cases except Y263F. These dependences are linearized in the Arrhenius coordinates (Fig. 6, right panel) giving two parts – 80-220 K and 220-273 K – corresponding to different activation energies for the photoreaction in the Pr excited state. This was also seen in earlier work (13). The 80-220 K interval primarily reflects changes in Φ_f of the weakly fluorescent bulk species, as in the case of Cph1Δ2^{676}/$_{664}$ wild-type species because the yield of the minor fluorescent Cph1Δ2$^{670-674}$/$_{652}$ is high already at T_a and is likely to saturate in the interval 220-273 K (as demonstrated by the deconvolution of the experimental curves of the Pr fluorescence temperature dependence into the sum of individual curves of different species of plant phyA (12)). For Cph1Δ2 in the 80-220 K interval, E_a was derived as ~3.0 kJ mol^{-1} for the wild type, Y263H and Y263S and only slightly lower (2.5 kJ mol^{-1}) for Y263F. This coincidence provides further support for the notion that we are dealing primarily if not exclusively with changes of the fluorescence of the bulk relatively weakly fluorescent species of Cph1Δ2, although the input of the highly fluorescent species in the fluorescence of the wild type and the mutants is different. This is also implied by the good linearity of the Arrhenius plot in this temperature interval. The higher T interval (220-273 K) reveals, however, complex changes, probably due to at least three factors: firstly, formation of highly fluorescent species even under relatively weak monochromatic exciting light (13, 37); secondly, operation of the Pr→Pfr photocycle and formation of intermediates stable near T_a; and thirdly, possible redistribution of the conformers of Cph1Δ2 in thermoequilibrium with each other. The

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latter is supported by the fact that redistribution of the conformers with temperature takes place in the case of phyA (15, 23). This complexity may explain the scatter of the data in the Arrhenius plot, which gives more variable and higher values of 6.5-17.6 kJ mol^{-1} in the interval 220-273 K (Table 1). Thus, we may conclude that below 220 K the Y263 substitutions do not dramatically change the excited state activation barrier.

<Figure 5>

<Figure 6>

Photochemical parameters

Evaluations of Φ_f at T_c from that at T_a gave *ca.* 0.30 for the wild type and Y263H and somewhat higher values of 0.35 and 0.41 for Y263F and Y263S, respectively (Table 1). The heterogeneity of the emitting species whose proportion could change with temperature is not taken into consideration in these initial approximations. The experimentally determined fluorescence constants, k_f, at T_a derived from the integral under the absorption curve correlate with Φ_f at T_c (Table 1). The derived values for k_d (the temperature-independent emission-less excitation energy dissipation constant obtained according to (12, 50) were higher than those for k_f. The two temperature-independent constants (k_f and k_d) and the Φ_f values at T_a and T_c allowed evaluations of k_p for the transition from Pr* via E_a to prelumi-R and its yield ($\Phi_{Pr \to prelumi-R}$ or Φ_p) as well as the yield of the prelumi-R→lumi-R transition ($\Phi_{prelumi-R \to lumi-R}$ or Φ_{ab}) at the two temperatures (Table 1). The efficiency of the photochemical route from Pr* to prelumi-R at T_c is surprisingly low in the wild type and is further lowered sequentially in the H, F and S mutants. Assuming in the first approximation the partitioning coefficients for prelumi-R→lumi-R at T_c to be the same as those at T_a (see Table 1 and below), the yields of formation of lumi-R from Pr at T_c were derived. The values obtained were very low, especially in the mutants. Were the lumi-R→Pr reverse photoreaction to be significant, lumi-R yield would be even lower under steady-state conditions, explaining the lack of formation of lumi-R at T_c. Due to the activation character of the Pr→lumi-R photoreaction via Pr* and prelumi-R at T_c, the rate and the yield of the photoreaction increase more than 10-fold upon warming to T_a while Φ_f for Pr falls (Table 1): $\Phi_{Pr \to prelumi-R \text{ or }} \Phi_p$, in particular, approaches unity (0.93) in the wild type and 0.76-0.79 in the mutants. Since $\Phi_{Pr \to Pfr}$, the quantum yield of the overall Pr→Pfr photoconversion, at T_a is 0.13 (40) and as there is probably no lumi-R→Pr dark reversion (51) (this has been shown for plant

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phytochrome A although not for Cph1), we can evaluate the coefficient of partitioning of the routes Pr←prelumi-R→lumi-R (Φ_{ba} and Φ_{ab}) as 0.86/0.14 for the wild type and 0.92/0.08; 0.90/0.10 and 0.89/0.11 for Y263H, Y263F and Y263S, respectively at T_a (Table 1). On the other hand, the fact that $\Phi_{Pr\rightarrow Pfr}$ obtained by absorbance (i.e. for the longer-wavelength species with low fluorescence) and fluorescence (i.e. for the shorter-wavelength fluorescent species) measurements were almost identical ((40), see Table 1) suggests that Φ_p and Φ_{ab} are also similar for the two species. This and the fact that the extent of Pr→Pfr transformation in red light is quite high (*ca.* 0.70-0.75 in the wild type) supports the notion that all molecules (i.e. both the predominant low fluorescence and the minor high fluorescence species) have either similar $\Phi_{Pr\rightarrow Pfr}$ values or are in rapid, dynamic equilibrium. The latter assumption is, however, unlikely as the action spectrum of Pr→Pfr photoconversion obtained from the kinetics of Pr fluorescence decay coincides with the fluorescence excitation spectrum of the shorter-wavelength Cph1Δ2 species (λ_{max} = 648 nm; Fig. 3). The nature of the minor form is unclear. It is not associated with the Pr' / Pr'' distinction: although in plant phyA a large fraction of the Pr population – denoted Pr' – can photoconvert to lumi-R even at 85 K, this is not the case for Cph1, whose dark state comprises exclusively Pr'' in which photochemistry is minimal below 85 K because of the relatively high E_a barrier (2, 37, 52). MAS NMR has indicated, however, that two isoforms of Cph1Δ2 Pr (I and II) exist, Pr-II representing the crystal structure and Pr-I showing a different position and charge of the H260 side chain and the hydrogen bonding of chromophore ring D (10). These differences might be associated with different fluorescence properties deriving from different E_a values and Pr S_1 energy levels. Given that the yield of the Pr de-activation along the photochemical route (that is, via the E_a barrier towards prelumi-R) almost reaches unity at T_a in all the species under investigation, the decisive functional characteristics are the partitioning coefficients at the orthogonal prelumi-R state.

$\Phi_{Pr\rightarrow Pfr}$ is lower in the mutants than in the wild type. However this is not directly reflected by an increase in Φ_f because the decline of $\Phi_{Pr\rightarrow prelumi-R}$ is only *ca.* 20 %, a difference that cannot account for the much more pronounced drop in $\Phi_{Pr\rightarrow Pfr}$. The changes associated with the mutations are thus likely to affect the system primarily at the point of the prelumi-R relaxation either into lumi-R (and further into Pfr) or reverse into Pr (in contrast to the differences between the fluorescing and non-fluorescing wild-type species). Mutations might also change the distribution of the pigment molecules between different

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conformers, an effect that manifests itself particularly at T_a. At 85 K the molecule tends to "freeze" in one dominating state, which is not so prominently modified by the mutations. Under these conditions, the initial stages of the photochemical process prior to the activation barrier are likely to be similar in the wild type Cph1$\Delta2^{675}/_{662}$ and the mutants judging by the similarity of their absorption and emission spectra at 85 K and their E_a values. All the differences between them at T_a are thus related primarily to the states and transitions after crossing the E_a barrier and at the prelumi-R partitioning point.

Possible structural interpretations

Explanation of phytochrome function relies upon structural, physical, biochemical and cell biological approaches including experiments in which the system is experimentally perturbed by mutations or chemical modifications of the chromophore. In the three mutants described here and in (40), the Φ_p value for the Pr to Pfr conversion is less than half that of the wild type, despite the fact that E_a ~3 kJ mol^{-1} from the Pr* excited state remains the same.. The key point in Pr→lumi-R→Pfr conversion is the bifurcation point of the routes from the orthogonal prelumi-R to the lumi-R ground state or back to that of Pr: we observe pronounced differences in the Φ_{ab} yields between the wild type and the mutants in harmony with the changes in $\Phi_{Pr\rightarrow Pfr}$. This means that in terms of the chromophore-binding pocket around the D ring, Y263 promotes forward relaxation of prelumi-R to lumi-R. The principle thermal relaxation route of the excited state is via the E_a barrier. We hypothesize that rings A, B and C are held in such a way as to channel the energy into this essential photochemical process. Since there is no appreciable change in E_a in our mutants, Y263 does not directly affect this funneling process. Thus, it likely plays its role at the latter stages of the photoprocesses, i.e. at the prelumi-R partitioning.

We have solved the crystal structures of both the wild type ((7); 2VEA) and Y263F ((40); 3ZQ5) in the Pr state and obtained corroborative evidence for the wild-type structures both as Pr and Pfr from solid-state NMR (10) using the crystal structure of *Pa*BphP, an unusual bacteriophytochrome, as a template for the Pfr state ((8); 3C2W). The 3ZQ5 high resolution structure of the Y263F mutant shows rather modest changes around the D ring, providing a good basis for understanding several physical aspects of phytochrome photoreaction.

Probably in all phytochromes in the initial Pr state the chromophore D ring lies above (α-facial) the plane of the B-C rings, such that its C19 carbonyl oxygen forms a ~2.8 Å hydrogen bond with Nε of the nearby

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histidine (H290 in Cph1) as well as a 3.2 Å hydrogen bond between its pyrrole nitrogen N24 and a water molecule within the chromophore binding pocket (supplementary Fig. S1a). The α-facial disposition arises from these interactions and a rotation about the C14-C15 single bond of the methine bridge. The structural changes following photon absorption are still unclear. Interestingly, CD spectroscopy shows opposite changes upon Pfr formation in bacteriophytochromes and plant/Cph1 type phytochromes. As the D ring in *Pa*BphP Pfr is α-facial, it is likely that the D ring in plant phytochromes and Cph1 Pfr is below (β-facial) the B-C plane ((53), see supplementary Fig. S1c). In view of the C13[1] and C17[1] hindrances and the angular distances involved, this in turn was interpreted as the D ring rotating clockwise in bacteriophytochromes and anti-clockwise in plant/Cph1-type phytochromes during Pr→Pfr photoisomerization (53). There is, however, no obvious explanation for such a fundamental difference despite various characteristic structural features of the two families. Even if 3C2W represents a general model for bacteriophytochrome Pfr, it certainly would not be applicable to Cph1/plant-type Pfr if the D-ring adopts the opposite disposition (54).

Whichever direction the D-ring rotates, both H-bonds associated with the pyrrole nitrogen and carbonyl have to be broken, a process which might explain the E_a barrier. The measured E_a values for all plant/Cph1 type phytochromes, including the mutants described here (Table 1), are close to 3 kJ mol^{-1}, about a quarter that of a typical H-bond. Interestingly, the D-ring flip following photon absorption requires 30 ps in Cph1 and plant phytochromes, remarkably slow when compared to the <1 ps isomerization seen in photoreceptors such as bacteriorhodopsin (BR) or rhodopsin and, indeed, Pfr (21, 55, 56). We note, however, that the initial photoreactions in these photoreceptors were also shown to be activation processes with E_a barriers for isomerization in the excited state similar to that in Pr (see comparative analysis of phytochromes and (bacterio)rhodopsin in (2)). In the case of bacteriorhodopsin, E_a is comparable with that in Pr of Cph1Δ2 for the fluorescent BR species, iso-BR, for instance, with E_a in the region of 1.2-4.2 kJ mol^{-1}, and much lower for nonfluorescent photoactive BR species. There is also an inverse correlation between the fluorescence yield and photoactivity of (bacterio)rhodopsins. Pfr, on the other hand, which does not fluoresce even at liquid helium temperature (Sineshchekov, Deeg & Rüdiger, unpublished data), also has an E_a value close to that of Pr (57). The existence of the barrier in Pfr for isomerization in the excited state seems to be in contradiction with the lack of Pfr fluorescence and

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also with the notion that the initial isomerization of the chromophore in Pfr proceeds without steric hindrance by the protein moiety. In the case of Pr*, however, it is much more clear that the D-ring might experience changing interactions during its photoflip. The potential energy surface of the Pr* state would thus be fairly flat but also rugged due to dynamic changes in the protein framework, which slow down chromophore isomerization and might explain the observed E_a values as well. Additionally, the S_1 potential surface might be affected by interactions between the $C13^1$ and $C17^1$ methyl groups of rings C and D.

The role of Y263 during photoisomerization contrasts to that of another tyrosine in the D-ring pocket, Y176. Y176H is highly fluorescent but photochemically inactive in Cph1/plant-type phytochromes, but not the bacteriophytochrome family (41, 58). This implies that although Y176 itself is conserved in different phytochrome clades, its function is not. This difference was associated with the clockwise *vs.* anticlockwise photoflip of the D-ring. In terms of prelumi-R partitioning, an anticlockwise D-ring rotation predicts that a transient hydrogen bond might be formed between N24 of the D-ring and the Y263 hydroxyl (see Fig. S1b) in the orthogonal excited state. Given that all chromophore nitrogens are protonated in both Pr and Pfr, such a hydrogen bond formation might be associated with proton transfer, for example to D207 via Y263 (see Fig. S3c). Our findings for Y263F imply that despite the 2- to 3-fold reduced photoconversion efficiency, Pfr formation does not depend on this interaction. Why then is Y263 perfectly conserved within the phytochrome superfamily?

(i)   Y263 might be important in intramolecular signal transduction, e.g. by disrupting the salt bridge between D207 and R472 from the tongue region (7). Recent data indeed imply that this might be true in plant phyB because, although photoconversion is inefficient as expected, its physiological responses are exaggerated (59).

(ii)   Y263 might represent a flip/flop gate either trapping the energy in lumi-R necessary for the rearrangements associated with Pfr or, more often, losing it as heat. In Y263F, however, lumi-R formation is less likely, possibly because of a loss of an N24 – hydroxyl H-bond.

(iii)   The hydroxyl group of Y263 might allow only anti-clockwise D-ring rotation by steric interference with the $C17^1$ methyl group (2VEA: 2.5 Å). In this scenario clockwise rotation would be non-productive for the formation of the lumi-R state.

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On the basis of the present work we propose that interactions between the D ring and Y263 optimize the probability of direct Pr*→lumi-R conversion. Photoswitching between Pr and the photoproduct lumi-R (and thence Pfr) depends on the bifurcation point – the point of intercrossing of the two ground state potential curves of Pr and lumi-R as a function of the C15=C16 torsional angle – and its interaction with the S_1 potential curve (see Fig. 7). In other words, if we assume that the relaxation of the pigment from S_1 to S_0 occurs via a conical intersection at the twisted (90°) state of the molecule, then we can speculate that the Y263 residue provides the most optimal shape to yield the highest efficiency of the direct route from this point to the lumi-R photoproduct. The Y263 mutants described here obviously lowered the probability of transitions along the forward branch of this crossing, revealing the importance of the wild type Y263 residue. Interestingly, Pfr (and lumi-R) formation has a similar low probability of ~0.15 in all known wild-type plant phytochromes and Cph1. In addition to the conformational heterogeneity of Cph1 and plant phytochromes in the Pr state, the low quantum yields for photoconversion in both directions is remarkable. Y263 is clearly a crucial factor in the optimization of this seemingly impaired photochemistry.

<Figure 7>

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FIGURE CAPTIONS

Figure 1. Energy level scheme of the photoreaction of Pr into the first photoproduct (lumi-R) stable at low temperatures via the "hot" S_0 crossover point prelumi-R. I_0, fluence rate; ε, molar extinction coefficient; k, rate constants (k_f, fluorescence; k_d temperature-independent degradation of excitation; k_p, initial photoreaction; k_{ab}, phototransformation to lumi-R photoproduct and k_{ba}, return to Pr); E_a, activation energy barrier to forward photoreactions; ΔE, lumi-R – Pr ground state energy difference. After (12).

Figure 2. Fluorescence emission (em) and excitation (ex) spectra of Cph1Δ2 and Y263 mutants at T_c (solid lines) and T_a (dotted lines, from (40) for comparison). The wild-type maxima at T_c are indicated.

Figure 3. Absorption (1), fluorescence emission (2) and excitation (3) and photoconversion action ($\circ$, $\square$) spectra of Cph1Δ2 in the Pr form at T_a. The action spectra were obtained from the kinetics of the Pr fluorescence decay during Pr→Pfr conversion in actinic light for two different samples as described in the Materials and Methods section. The maxima in the spectra are indicated. Wavelengths of fluorescence excitation and monitoring were 610 nm and 720 nm, respectively.

Figure 4. Fluorescence emission and excitation spectra of Cph1Δ2 at T_c. Excitation spectra were recorded at $\lambda_{em} = 660$ nm (1) and 676 nm (2), emission spectra, upon excitation at $\lambda_{ex} = 652$ nm (3) and 594 nm (4).

Figure 5. Temperature dependence of the fluorescence emission spectra and of their λ_{max} values of wild-type Cph1Δ2 (■), Y263F ($\circ$), Y263H (Δ) and Y263S (•). The spectra were taken in steps of 20 K from 80 K (1) to 300 K (12).

Figure 6. Temperature dependence of the fluorescence intensity of Cph1Δ2 wild type and Y263 mutants (left panel) and their linearization in Arrhenius coordinates (right panel). The breaking point (bp) of the linearized curve of Cph1Δ2 at 219 K is indicated.

Figure 7. Hypothetical potential energy curves and quantum yields of Pr photoactivation and associated processes following Figure 1 and (2, 12). Note that the reaction coordinate is the C-D ring angle rather

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than the C15=C16 dihedral. The ring angle reaction coordinate cannot approach 180° at C15=C16 isomerization because of the $C13^1$ - $C17^1$ clash (dotted line) but is accompanied by a rotation of the C14-C15 single bond.

Supplementary Figure S1. Stereo pairs of the chromophore D ring environment.

(a) 2VEA structure of Cph1 Pr (Essen *et al*. 2008) showing the interactions of the pyrrole N24 and C19 carboxyl oxygen; (b) hypothetical structure of prelumi-R at C15=C16 isomerization angle of 117°; (c) Hypothetical Pfr structures with β-facial PCB D-ring in Cph1 (cyan) and the α-facial biliverdin D-ring in PaBphP (green). Y263 and D203 in Cph1 Pr (2VEA; (7)) and their homologs *Y250* and *D194* in PaBphP Pfr (3C2W; (8)) are shown in gold and green, respectively. PyMol molecular images.