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A photoreversible conformational change in 124 kDa *Avena* phytochrome

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Tryptophan (Trp) fluorescence quenching of phytochrome has been studied using anionic, cationic and neutral quenchers, I^-, Cs^+ and acrylamide, respectively, in an effort to understand the molecular differences between the Pr and Pfr forms. The data have been analyzed using both Stern-Volmer and modified Stern-Volmer kinetic treatments. The anionic quencher, I^-, was proven to be an ineffective quencher with Stern-Volmer constants, K_{sv}, of 0.60 and 0.63 M^{-1}, respectively, for the Pr and Pfr forms of phytochrome. The cationic quencher, Cs^+, showed about a 2-fold difference in the K_{sv} of Pr and Pfr, indicating a significant change in the fluorescent Trp environments during the Pr to Pfr phototransformation. However, only 25–37% of the fluorescent Trp residues were accessible to the cationic quencher. Most of the fluorescent Trp residues were accessible to acrylamide, but the quenching by acrylamide was indistinguishable for the Pr and Pfr forms. An additional quenching by acrylamide after a saturated quenching with Cs^+ showed more than 40% increase in the K_{sv} of Pfr over Pr. These observations, along with the finding of two distinct components in the Trp fluorescence lifetime, indicate the existence of Trp residues in at least two different sets of environments in the phytochrome protein. The two components of the fluorescence had lifetimes of 1.1 ns (major) and 4.7 ns (minor) for Pr and 0.9 ns (major) and 4.6 ns (minor) for Pfr. Fluorescence quenching was found to be both static and dynamic as the Stern-Volmer constants for the steady-state fluorescence quenching were higher than for the dynamic fluorescence quenching. Based on the quenching results, in combination with the location of Trp residues in the primary structure, we conclude that the Pr to Pfr phototransformation involves a significant conformation change in the phytochrome molecule, preferentially in the 74 kDa chromophore-bearing domain.

Introduction

Phytochrome, a blue-green protein, mediates several photomorphogenic and developmental responses in plants as per the scheme shown below:

$$Pr \underset{730\ nm}{\overset{666\ nm}{\rightleftharpoons}} Pfr \rightarrow \text{biological response}$$

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The quaternary structure of phytochrome determined by low-speed sedimentation, chemical cross-linking and size-exclusion high-performance liquid chromatography [1,2] has revealed that the phytochrome exists as a dimer in solution. The shape of the phytochrome molecule seems to change slightly upon phototransformation of the Pr to Pfr form [1,2]. The Stokes radius of phytochrome does not change drastically during the phototransformation [1–3]. Sedimentation velocities of the Pr and Pfr forms of large – 114/118 kDa; these species with truncated amino-terminal sequences arise from proteolysis during the isolation and purification; the full-length native phytochrome is 124 kDa [1,2] – oat phytochrome were found to be the same [4] and no differences were detected in the surface charges of the Pr and Pfr species [5]. Similar studies have also been reported for the 124 kDa native oat phytochrome [2]. Recently, secondary structural differences have been determined between the Pr and Pfr forms of phytochrome [6,7], but it has not been possible to specify topographical changes on the protein. For example, it is not known whether the changes in the peptide folding occur in the flexible polar region or in the relatively rigid hydrophobic core of the protein, or both. However, the changes can be suppressed upon binding of a monoclonal antibody specific to the N-terminal region on the chromophore domain [6].

Since the Pfr form of phytochrome is believed to be the physiologically active form, several studies have focused upon the molecular differences between the Pr and Pfr forms at the chromophore as well as at the protein topography level (for reviews, see Refs. 8, 9). Fluorescence quenching of Trp by I^- in the Pr and Pfr forms of degraded (118/114 kDa) oat phytochrome was carried out earlier [10], showing about 50% more fluorescence quenching in the Pfr form. This was explained in terms of an increase in the Trp exposure in the Pfr form. The native phytochrome (124 kDa) isolated thereafter has been shown to differ in spectroscopic [11–14], structural [6,7] and functional properties [15]. Therefore, it is important to re-evaluate the parameters such as Trp topography in the Pr and Pfr forms to reveal any change that occurs in the polypeptide folding during Pr → Pfr phototransformation. In this study, we have used a non-invasive technique of intrinsic fluorescence quenching to investigate the molecular differences between the Pr and Pfr forms of the native oat phytochrome protein.

Materials and Methods

Oat (*Avena sativa* L. cv. Garry oat) seeds were purchased from Stanford Seeds Co. (Buffalo, NY) and phytochrome was isolated as described earlier [13]. The chemicals used for phytochrome isolation were of the same grade as described in Chai et al. [13]. Acrylamide, potassium iodide and sodium thiosulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Cesium chloride was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Fluorescence lifetime measurements

Fluorescence lifetimes were measured using a single photon counting technique. A Coherent Radiation argon-ion laser (INNOVA 18) was mode-locked at 38.4 MHz, a 514.5 nm plasma line being used to pump a Coherent Dye Laser (CR-599) containing rhodamine-590. The dye laser was tuned to 584 nm and then the frequency was doubled by a temperature-tuned ADA crystal. The doubled 292 nm pulse having a 15 ps pulse width was used to excite preferentially the Trp residues in the sample. The emission at 360 nm and the scattered exciting light were discriminated by an ISA double monochromator (DH-10) at 90 °C to the exciting beam. Photons of selected frequencies were detected by a fast response photomultiplier (Amperex 2254B). The output of the photomultiplier was used as start signal and the sync output of the mode locker driver was used as the stop signal for the time-to-amplitude converter (TAC, ORTEC 457). The output pulses of different amplitudes from the TAC were then scaled and accumulated by a multichannel analyzer (MCA, ORTEC 7010). The final spectral data were then stored in a VAX 11/730 computer for analysis. The data were analysed by fitting the decay curves using a reconvolution technique [16]. The instrumental profile was obtained by light scattering from a dilute milky (Coffee-mate) solution. The stability of the system performance was checked occasionally and a full-width half-maximum

(fwhm) around 800 ps for the instrumental response was maintained. The temperature of the phytochrome solution was maintained at 273 K by a Berg-Warner LHP-150 heat pump, which was monitored by a TC-108 controller through an IC temperature sensor AD 590. For fluorescence measurements on the Pr form of phytochrome, the sample was irradiated simultaneously with continuous far-red light using an infrared cutoff filter (Ealing 26-4457) and for the Pfr measurements (12% Pr present as a photostationary mixture [13]), the sample was irradiated with continuous red light using a 666 nm interference filter (Oriel C572-6602), both from the direction opposite to the exciting beam. Spectral monitoring of the solution showed that the above arrangements had kept the phytochrome molecules in their respective spectral forms by minimizing the phototransformation of phytochrome affected by the fluorescence excitation beam at 292 nm.

Fluorescence intensity measurements were made on a Perkin-Elmer MPF-3 spectrofluorometer equipped with a 150 W xenon lamp at ice-bath temperature.

Fluorescence quenching studies

Steady-state and dynamic fluorescence quenching of Trp residues in phytochrome were carried out by measuring the fluorescence intensity and lifetime, respectively. The fluorescence lifetime or the fluorescence intensity was measured as a function of increasing concentrations of different quenchers (I^-, Cs^+ or acrylamide) according to the method of Eftink and Ghiron [17,18] at 273 K. The results obtained were plotted according to the Stern-Volmer equation [19].

$$\tau_0/\tau \quad \text{or} \quad F_0/F = 1 + K_{sv}[Q] \tag{1}$$

where, τ_0 is the Trp fluorescence lifetime in the absence of any quencher; τ is Trp fluorescence lifetime in the presence of a given concentration of quencher; F_0 is the steady-state fluorescence intensity in the absence of any quencher; F is the Trp fluorescence intensity in the presence of a given concentration of quencher, Q; K_{sv} is the Stern-Volmer constant; [Q] is the quencher concentration.

The data were also plotted according to the modified Stern-Volmer equation [20].

$$\tau/\Delta\tau \quad \text{or} \quad F/\Delta F = 1/f_{acc}K_Q[Q] + 1/f_{acc} \tag{2}$$

where, $\Delta\tau$ or ΔF is the difference in the fluorescence lifetime or in fluorescence intensity in the presence and absence of the quencher at a given quencher concentration; f_{acc} is the fraction of the maximum accessible Trp fluorescence; K_Q is the effective quenching constant. Other symbols have the same meaning as in Eqn. 1.

For quenching experiments, small aliquots of stock solutions of different quenchers (5 M KI, 5 M CsCl and 8 M acrylamide) were added separately to a 0.9 ml solution of phytochrome (Pr or Pfr) having an absorbance of less than 0.1 at the exciting wavelength, 292 nm.

Results

Intrinsic fluorescent probes such as aromatic amino acids can be used to probe alterations in the protein structure [8,21]. Quenching of Trp fluorescence by added solutes (acrylamide, cesium chloride and potassium iodide) has been used here to probe the conformational differences between the Pr and Pfr forms of phytochrome.

Oat phytochrome is shown to have 10 Trp residues in the native protein [11,22]. The emission maximum of Trp fluorescence in phytochrome

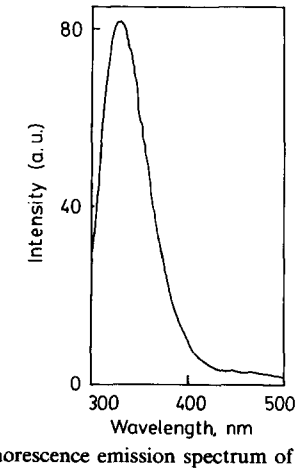

Fig. 1. Trp fluorescence emission spectrum of 124 kDa phytochrome (Pr) in 20 mM potassium phosphate buffer (pH 7.8)/1 mM EDTA at 276 K. The excitation wavelength and bandpass were 295 nm and 6 nm, respectively.

was observed at 330 nm for both Pr (Fig. 1) and Pfr forms (spectrum not shown), suggesting that the Trp residues are located in a relatively hydrophobic core [18,20].

Fluorescence lifetimes of phytochrome along with component analyses are given in Table I. For lifetime measurements, emission was monitored at 360 nm (excitation at 292 nm) to avoid any inter-

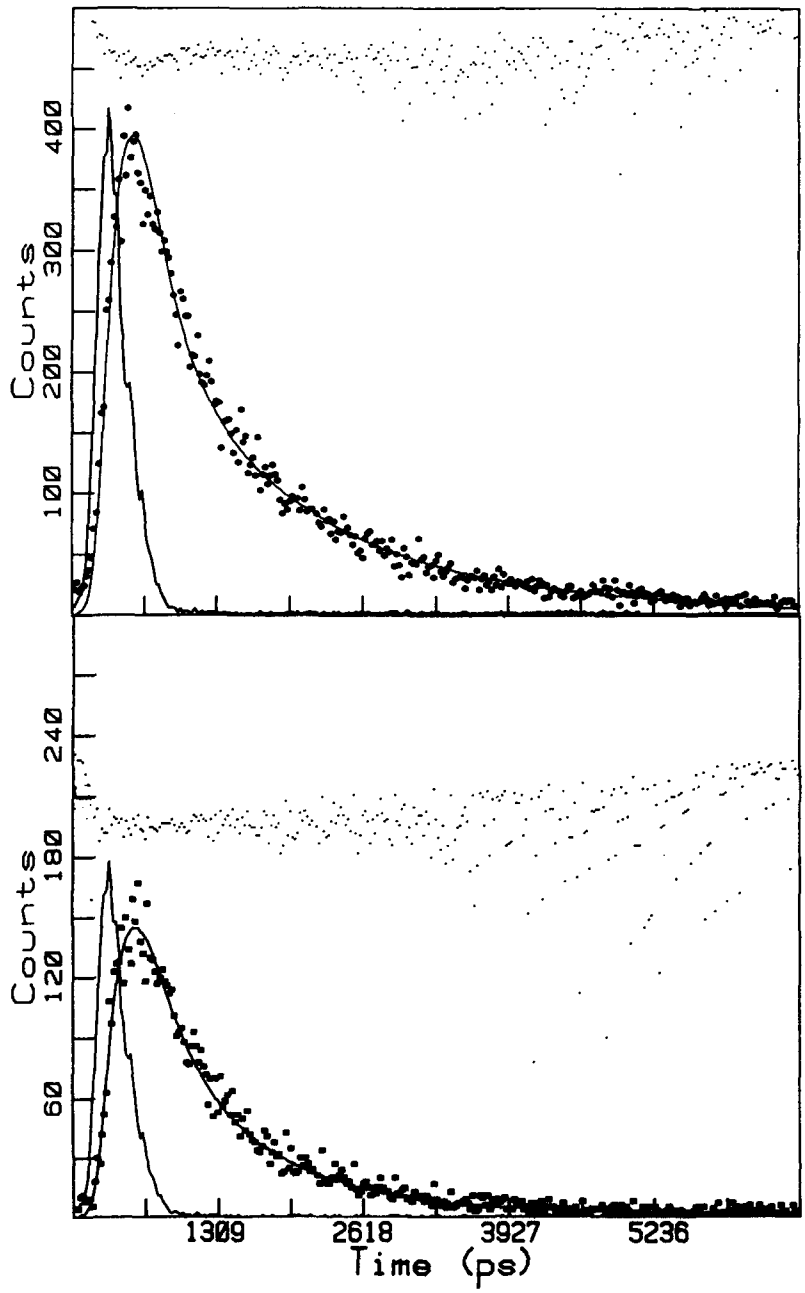

Fig. 2. Trp fluorescence decay curve in Pr (upper panel) and Pr+0.25 M Cs^+ (lower panel) in 20 mM potassium phosphate buffer (pH 7.8)/1 mM EDTA. The excitation wavelength was 292 nm and the emission was monitored at 360 nm. All the measurements were carried out at 273 K. Solid dots are the experimental counts and the two component reconvolution fit is shown by the solid line. The residuals are shown on the upper part (small dots). The laser pulse profile is shown preceding the fluorescence decay curve.

TABLE I

Trp FLUORESCENCE LIFETIMES OF FREE Trp AND THE Trp RESIDUES IN THE Pr AND Pfr FORMS OF PHYTOCHROME.

Phytochrome solutions were made in 20 mM potassium phosphate buffer (pH 7.8)/1 mM EDTA. Fluorescence lifetime measurements were carried out at 273 K as described in Materials and Methods. Excitation wavelength was 292 nm and the emission wavelength was 360 nm.

Fluorophore	Major component lifetime (ns) (%)	Minor component lifetime (ns) (%)
Pr	1.1 ± 0.1 (71)	4.7 ± 0.2 (29)
Pfr	0.9 ± 0.2 (72)	4.6 ± 0.4 (28)
Trp	3.7 (85)	9.6 (15)

ference from the anionic forms of tyrosine residues. The anionic forms of tyrosine residues are known to fluorescence at 340–350 nm [23]. The fluorescence decay curve for the Pr form of phytochrome is shown in Fig. 2. The Trp fluorescence lifetimes, monitored at 360 nm, were 1.1 ± 0.1 ns (major component, 71%) and 4.7 ± 0.2 ns (minor component, 29%) for Pr and 0.9 ± 0.2 ns (major component, 72%) and 4.6 ± 0.4 ns (minor component, 28%) for Pfr (Table I). Fig. 2 also shows the fluorescence decay curve of the Pr form of phytochrome after addition of Cs^+. Free LTrp in 20 mM potassium phosphate buffer (pH 7.8)/1 mM EDTA had fluorescence lifetimes (excitation 292 nm and emission monitored at 360 nm) of 3.7 ns (major component, 85%) and 9.6 ns (minor component, 15%). For comparison, the fluorescence decay curve for LTrp in aqueous solution is shown in Fig. 3.

For the Trp fluorescence quenching experiments three different quenchers (KI, CsCl and acrylamide) were used either separately or in combination. I^- is an anionic quencher, whereas Cs^+ is a cationic quencher. Acrylamide is a neutral molecule which has been shown to be a quencher for Trp fluorescence in hydrophobic domains of proteins [17,24]. The results were analyzed using Stern-Volmer [19] and modified Stern-Volmer kinetics [20]. Fluorescence quenching of Trp residues by I^- was ineffective up to about 0.2 M

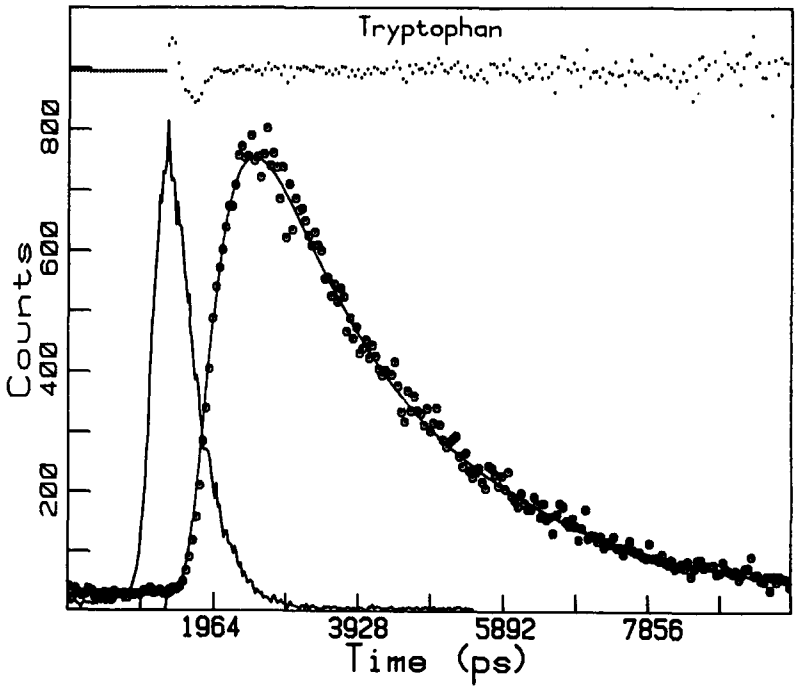

Fig. 3. Fluorescence decay curve for Trp in 20 mM potassium phosphate buffer (pH 7.8)/1 mM EDTA under the same conditions as in Fig. 2. Excitation wavelength was 292 nm and emission was monitored at 360 nm. The explanation of experimental points and curves are same as in Fig. 2 except for the experimental counts that are represented by open instead of solid circles.

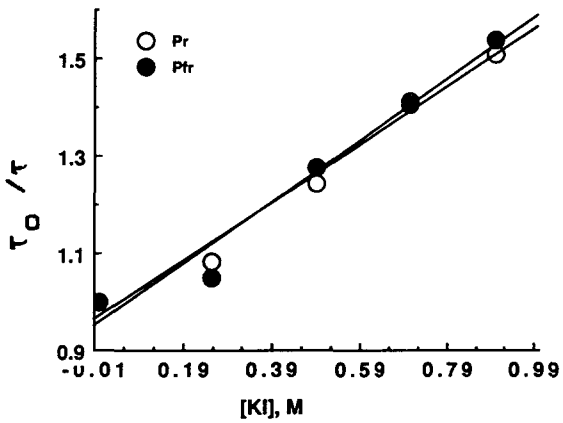

Fig. 4. Stern-Volmer quenching plots of Trp fluorescence lifetime in the Pr and Pfr forms of phytochrome for I^- quenching. Phytochrome samples and KI were dissolved in 20 mM potassium phosphate buffer (pH 7.8)/1 mM EDTA. Excitation wavelength for the fluorescence was 292 nm and the emission was monitored at 360 nm. τ_0 and τ are the fluorescence lifetimes in the absence and presence, respectively, of the added quencher, I^-.

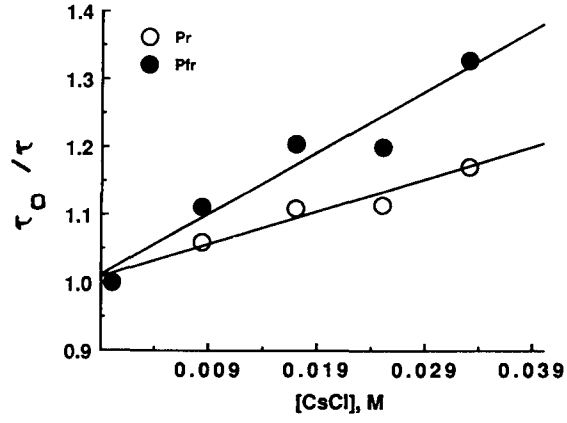

Fig. 5. Stern-Volmer quenching plots of the Trp fluorescence lifetime in the Pr and Pfr forms of phytochrome for Cs^+ quenching. Conditions same as in Fig. 4.

(data not shown, see Ref. 25), indicating that fluorescence Trp residues are not in a positively charged environment. However, a slight quenching was observed at/above 0.2 M KI. Stern-Volmer plots for the I^- quenching are shown in Fig. 4 and Stern-Volmer constants (K_{sv}; 0.60 M^{-1} for Pr and 0.63 M^{-1} for Pfr) are listed in Table II.

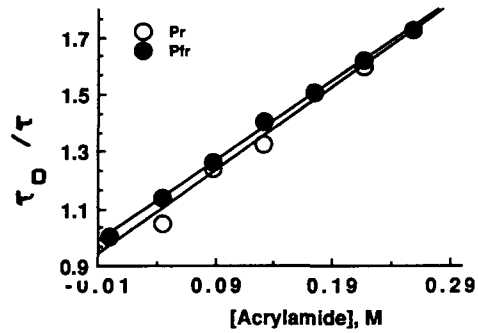

Fig. 6. Stern-Volmer quenching plots of the Trp fluorescence lifetime in the Pr and Pfr forms of phytochrome for acrylamide quenching. Conditions same as in Fig. 4.

TABLE II

THE K_{sv}, K_Q, k_q AND f_{acc} FOR THE Trp FLUORESCENCE QUENCHING BY I^-, Cs^+ AND ACRYLAMIDE IN THE Pr AND Pfr FORMS OF PHYTOCHROME

Units used in expressing the quenching parameters are as follows: K_{sv}, M^{-1}; K_Q, M^{-1}; f_{acc}, fraction; k_q, 10^9 $M^{-1} \cdot s^{-1}$.

Quencher	Pr				Pfr			
	K_{sv}	K_Q	f_{acc}	k_q [e]	K_{sv}	K_Q	f_{acc}	k_q
KI	0.60	n.d. [a]	n.d.	0.19	0.63	n.d.	n.d.	0.19
CsCl	4.80	33.33	0.24	1.48	9.04	50.00	0.37	2.77
Acrylamide	2.94	2.38	1.10 [b]	0.91	2.81	3.33	0.92	0.86
Acrylamide [c]	4.19	4.08	0.87	1.29	4.54	5.16	0.92	1.39
Acrylamide [d]	1.02	0.51	0.60	0.42	1.46	1.82	0.92	0.61

[a] Not determined, as the intercepts in the modified Stern-Volmer plots gave negative values.

[b] Fraction of the maximum accessible fluorescence should not exceed 1.0. f_{acc} of 1.10 is arising due to the least-squares fit of the data while taking into account of the lowest concentration of the quencher added.

[c] Quenching parameters in terms of fluorescence intensity measurements.

[d] Acrylamide quenching after saturated quenching by Cs^+. For k_q calculations, Trp fluorescence lifetimes used were 2.43 ns for Pr and 2.39 ns for Pfr. These lifetimes refer to the respective lifetimes after the saturated quenching by Cs^+.

[e] k_q was calculated using the relationship $K_{sv} = k_q \cdot \tau_0$, where τ_0 is the Trp fluorescence lifetime. The single component fluorescence lifetime was 3.24 ns for Pr and 3.26 ns for Pfr.

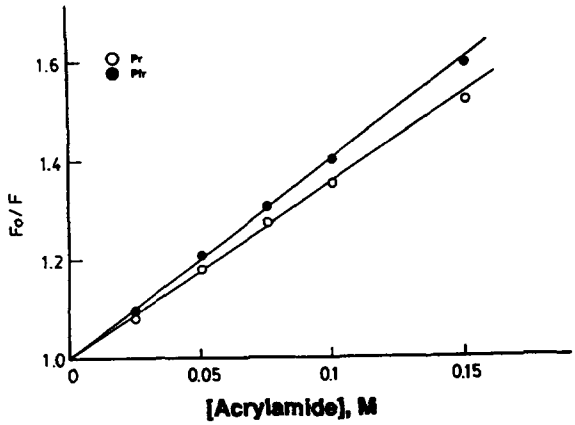

Fig. 7. Stern-Volmer quenching plot of Trp fluorescence intensity in the Pr and Pfr forms of phytochrome for acrylamide quenching. Fluorescence recordings were carried out at 276 K. Excitation wavelength, 295 nm; emission wavelength, 330 nm. F_0 and F are the fluorescence intensities in the absence and presence, respectively, of the added quencher.

Trp fluorescence quenching by Cs^+ was very strong. The Stern-Volmer plots for the Pr and Pfr fluorescence quenchings are given in Fig. 5. The Stern-Volmer constants for the Cs^+ quenching were 4.80 M^{-1} and 9.04 M^{-1}, respectively, for the Pr and Pfr forms of phytochrome.

Acrylamide quenched Trp fluorescence effectively but without showing any significant difference between the Pr and Pfr forms of phytochrome. The Stern-Volmer plots for the acrylamide quenching are shown in Fig. 6. A similar observation was made when the fluorescence quenching was monitored in terms of fluorescence intensity rather than fluorescence lifetime (Fig. 7; see Table II for Stern-Volmer constants).

In one set of experiments, the fluorescence was first quenched with a saturable concentration of Cs^+ (see Fig. 8 for saturation curve) and acrylamide was added subsequently to demonstrate that the acrylamide additionally quenches Trp fluorescence not accessible to Cs^+ ions in the protein. It was found that the acrylamide could further quench the Trp fluorescence as shown in Fig. 9. The effective quenching constants, K_Q, and the fractional maximum accessibility, f_{acc}, derived from the modified Stern-Volmer plots (a representative plot shown in Fig. 10) are listed in Table II. The value of f_{acc} for the fluorescence quenching by acrylamide in combination with Cs^+ ions

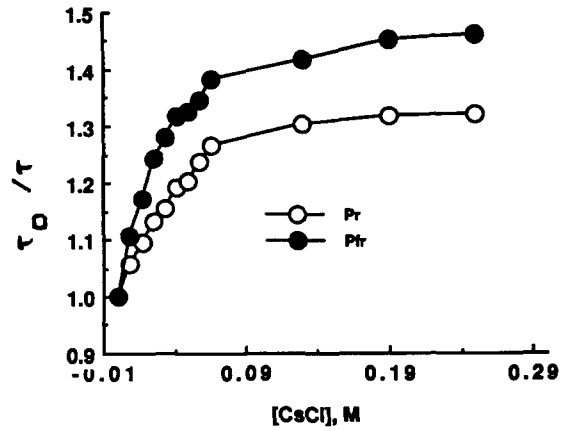

Fig. 8. Quenching of the Trp fluorescence lifetime in the Pr and Pfr forms by Cs^+. Conditions same as in Fig. 4. Note the saturation of fluorescence quenching beyond 0.05 M Cs^+.

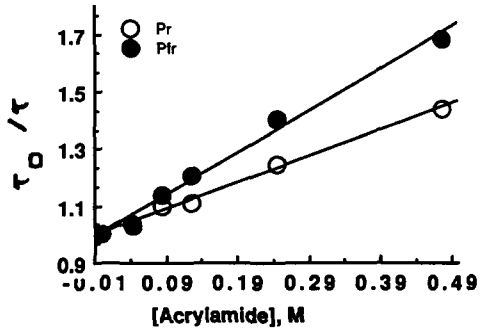

Fig. 9. Stern-Volmer quenching plots of the Trp fluorescence lifetime in the Pr and Pfr forms of phytochrome for acrylamide quenching after a saturating quenching by Cs^+. Conditions same as in Fig. 4.

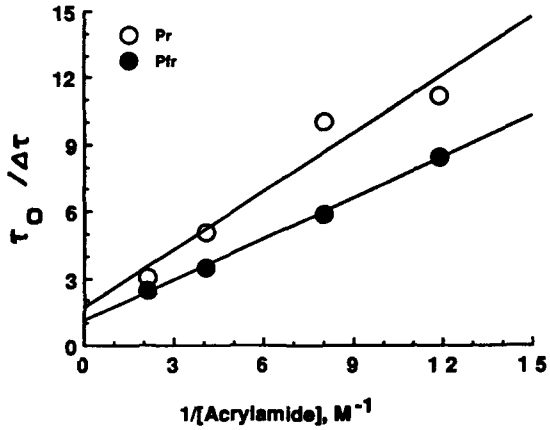

Fig. 10. Modified Stern-Volmer quenching plots of the Trp fluorescence lifetimes in the Pr and Pfr forms of phytochrome for acrylamide quenching after a saturating quenching by Cs^+ ions. Conditions same as in Fig. 4.

remained high, as was the case for acrylamide alone. The K_{sv} for fluorescence quenching by acrylamide after a saturating Cs^+ quenching was smaller than the K_{sv} for quenching by Cs^+ and acrylamide, separately (Table II). This indicates that, in the absence of Cs^+, acrylamide quenches the fluorescence of Trp residues both at the surface and deep inside the protein matrix. However, in the presence of saturating quenching with Cs^+ ions, the fluorescence of the surface trp residue(s) is already quenched by Cs^+ and acrylamide quenches only the remaining fluorescence of the Trp residues buried inside the protein matrix.

Discussion

Several studies have shown distinct conformational changes in the phototransformation of 124 kDa oat phytochrome [1,6,8,11,26–28]. The present study further probed the conformational aspect of phytochrome in terms of the disposition of Trp residues in the protein molecule.

The Trp fluorescence lifetime with two distinct components may represent two groups of Trp residues in phytochrome. The major lifetime component (1.1 ns) is drastically different from any of the components of free LTrp. The longer component group may be in an environment similar to the free Trp, i.e., fully exposed Trp residue(s) (see Table I). The fluorescence lifetimes of Trp in various proteins have been reported to range from 1.9 to 6.0 ns [24,29]. The group of Trp residues in phytochrome with a short fluorescence lifetime (1.1 ns) appears to be located near the tetrapyrrole chromophore. This conclusion is derived from the fact that the sensitized fluorescence of the chromophore observed at 680 nm upon excitation at 292 nm showed a lifetime of 0.7 ns [13,25]. Differential energy transfer, in the Pr and Pfr forms, between Trp residue(s) and the chromophore also occurs (Ref. 30; Moon and Song, unpublished data), indicating a conformational change during the Pr to Pfr phototransformation.

A significant fraction of the fluorescence intensity or the fluorescent Trp residues is located inside the protein matrix, as they are accesible only to acrylamide, which is capable of penetrating the protein matrix [17]. However, it is the Trp residues accessible at/near the protein surface that are reflected by high K_{sv} for Cs^+ quenching. A 2-fold difference between the K_{sv} of the Pr and Pfr fluorescence quenchings by Cs^+ (Table II) indicates a significant change in the folding of the protein during its phototransformation. The Stern-Volmer constants obtained indicate that the fluorescent Trp residue(s) in the Pfr form are more accessible to the cationic quencher than those in the Pr form. This is an indication for a photoreversible alteration in the folding of the protein in such a way that either the fluorescent trp residues become more exposed to the surface or the environment around the Trp residues become more negatively charged, or both. Thus, the Cs^+ quenching of the Trp fluorescence in phytochrome contrasts with the acrylamide and I^- quenchings (Table II), but is similar to another cationic quencher, thallium acetate [25].

Acrylamide quenched Trp fluorescence beyond the quenching by Cs^+ (vide infra). However, acrylamide showed lower K_{sv} values than Cs^+ ions. This suggests that there is a significant fraction/number of Trp fluorescence intensity/Trp residue(s) located near the negatively charged group(s). The quenching efficiency of Cs^+ ions is usually low in other proteins [18], but this ion is very effective in quenching the Trp fluorescence in both the Pr and Pfr forms of oat phytochrome.

The fraction of fluorescent Trp residues accessible to the cationic quenchers is 24–37% based on f_{acc} values (Table II). This is further supported by a maximum of 25–30% quenching of the Trp fluorescence by the saturation quenching by Cs^+ ions (Fig. 8). Results shown in Table II also suggest that the Trp residues not quenched by Cs^+ ions are accessible to acrylamide. While the fluorescence quenching by acrylamide alone does not distinguish between the Pr and Pfr forms of phytochrome, the fluorescence quenching by acrylamide in the Pr and Pfr after a saturating fluorescence quenching with CS^+, shows significantly different K_{sv} values for the Pr and Pfr forms (Table II), indicating a conformational alteration in the hydrophobic domain(s) of the phytochrome protein. An examination of the hydropathy profile of the amino-acid sequence of the phytochrome shows that the Trp residues at positions 475 and 522 have hydrophobic stretches in their closest vicinity. Trp-475 is in the middle of a

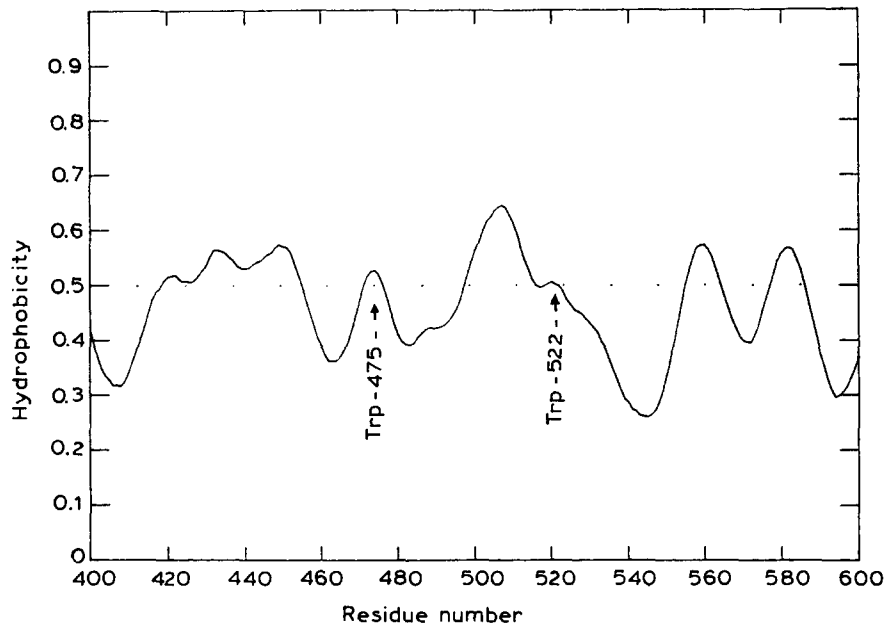

Fig. 11. Hydropathy profile of phytochrome between residues 400 and 600 according to the method of Kyte and Doolitte [36]. Hydrophobicity parameters are scaled to the range 0–1, where 0 is the most polar and 1 is the most hydrophobic. For the original hydropathy index values, the following equation can be used: $Y = (X - 0.5)/(0.111111)$, where Y represents original values and X represents the values plotted in this figure.

weak and short hydrophobic stretch, whereas Trp-522 is at the edge of about a 25-amino-acid-long stretch with significant hydrophobicity (Fig. 11). Furthermore, Trp-522 is surrounded by several non-polar amino acids [22]. Other Trp residues (Trp-366, Trp-458, Trp-533, Trp-569, Trp-645, Trp-774, Trp-778 and Trp-788) are located in the middle of charged amino-acid residues/hydrophyllic domains, and therefore, may not be the site of the alteration in the protein folding that is detected by the quenching with a combination of Cs^+ ions and acrylamide. Since the Trp-475 is in a short and low hydrophobic stretch, region 498–522 is at least one segment that is likely to undergo alteration in the folding during the Pr → Pfr phototransformation.

A comparison of k_q, the bimolecular quenching rate constant, of the Trp fluorescence quenching by I^- ions and acrylamide shows that the fluorescent Trp residues are significantly more accessible to acrylamide (k_q; $9.1 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ for Pr and $8.6 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ for Pfr) than to I^- (k_q; $1.9 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ for both Pr and Pfr).

Trp-366, which has four consecutive lysine residues in its vicinity [22], is likely to be quenched preferentially by an anionic quencher. Since I^- has low K_{sv} and no significant difference is found for I^- quenching in the Pr and Pfr forms, it is likely that the conformation of phytochrome in that region is not significantly affected by the phototransformation. It is likely that Trp-366 is non-fluorescent or very weakly fluorescent because it has an -SH group in its vicinity (Cys-371). In this case, the changes monitored by fluorescence quenching may involve residues 458–645 of the phytochrome sequence. Trp residues beyond residue 645 may not contribute to the observed fluorescence (vide infra) and therefore the changes detected by the fluorescence quenching do not refer to that segment of the phytochrome protein. This conclusion can be supported by the differential accessibility of peptide bonds for proteolysis in the 69–72 kDa region from the N-terminus [1].

The total fluorescence quantum yield of Trp residues in phytochrome is 0.06 [31]. Considering the fact that the phytochrome protein has 10 Trp residues [22], this is very low compared to free Trp [32]. The majority of the Trp residues are either non-fluorescent with only a small fraction of the Trp residues being moderately fluorescent, or very

weakly fluorescent. In the former case, both static and dynamic quenching (Figs. 4–7, 9 and 10) of Trp fluorescence reflect changes that occur in the vicinity of one or two fluorescent Trp residues. Seven of the ten Trp residues in the phytochrome are in the chromophore domain (74 kDa N-terminal domain [1,33,34]) and the other three in the 55 kDa C-terminal domain [22]. Trp-774 and Trp-778 have an -SH group (a strong quencher of Trp fluorescence) in their vicinity at Cys-776 position and thus are likely to be nonfluorescent. Trp-788 is in a polypeptide segment which seems to be an antigenic determinant according to Hopp and Woods prediction [35]. This segment is exposed to the solvent. Therefore, if Trp-788 were strongly fluorescent, its emission maximum would have shifted to 350 nm. Since this was not observed (emission maximum, 330 nm; Fig. 1), Trp-788 is either non-fluorescent or only very weakly fluorescent. It appears, therefore, that the fluorescent Trp residues are likely to be present in the chromophore domain of the phytochrome protein and thus the changes in fluorescence quenching observed reflect a conformational difference in this region between the Pr and Pfr forms of phytochrome.

In conclusion, the data presented above indicate that, in phytochrome, the following apply. (i) There are at least two different groups of Trp residues representing different sets of environments; one group is exposed to the surface of the protein and the other is buried inside the protein matrix. (ii) There are significant conformational changes at the surface as well as in the hydrophobic core segments of the protein molecule accompanying the Pr → Pfr phototransformation. (iii) Trp residues on the surface of the protein are in a negatively charged environment which changes significantly upon Pr → Pfr phototransformation. (iv) The environmental changes, detected by acrylamide quenching of the Trp fluorescence, appear to occur in the 74 kDa chromophore domain of the phytochrome.

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