VISCOSITY DEPENDENCE OF PRIMARY PHOTOPROCESSES OF 124 kDALTON PHYTOCHROME

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ABSTRACT To characterize the nature of primary photoprocesses of phytochrome which serves as the red-far red reversible photoreceptor for photomorphogenesis in plants, viscosity dependence of the fluorescence lifetimes of phytochrome isolated from etiolated oat seedling (*Avena sativa* L.) has been investigated. The fluorescence decay of phytochrome exhibited approximately two components, one with lifetime in the range of 50–70 ps and another with 1.1–1.2 ns in phosphate buffer with or without 40–67% glycerol. However, relative amplitudes of these decay components were found to be strongly viscosity dependent. Thus, the longer decay component increased from 2–5% in phosphate buffer to ~20% in 67% glycerol-phosphate buffer. These results have been interpreted in terms of primary reaction from the excited singlet state of phytochrome, yielding a photoreversible intermediate whose rate of formation and decay were apparently viscosity-dependent. Further, the viscosity dependence is consistent with the primary reaction involving conformational changes of the chromophore/its apoprotein environment.

INTRODUCTION

Phytochrome serves as an efficient photoreceptor for the red light-mediated morphogenic and developmental responses in higher plants (for recent reviews see reference 1–5). The red light-absorbing photoreceptor (Pr) functions to elicit such responses in the cell according to the following photoreversible scheme:

$$\mathrm{Pr} \underset{730\ \mathrm{nm}}{\overset{660\ \mathrm{nm}}{\rightleftharpoons}} \mathrm{Pfr} \rightarrow \rightarrow \rightarrow \text{Biological action}\ .$$

Scheme I

Like other primary photoreceptors in photobiological processes (e.g., rhodopsin, bacteriorhodopsin and photosynthetic reaction center chlorophylls), high efficiency of photoreception entails an ultrafast reaction from the excited singlet state of the photoreceptor in competition with other energy-wasting relaxation processes such as fluorescence and internal/intersystem conversions.

To probe the nature of the primary photoprocess of phytochrome, fluorescence properties of phytochrome have been examined (6–9). It has been suggested that either proton transfer (7, 8, 10, 11) or isomerization of the chromophore is involved in the primary photoprocess of phytochrome (Pr form). To ascertain the extent of movement of the chromophore and/or its proximal peptide chains in the primary photoreaction of phytochrome, we have examined the viscosity dependence of fluorescence lifetime in glycerol-buffer mixture, on the premise that any significant movement of the chromophore and peptide chains is subject to diffusion-control. For example, it has been observed that photoisomerization of bilirubin strongly depends upon microviscosity (protein) around the chromophore (12).

MATERIALS AND METHODS

Chemicals used for buffer preparation and phytochrome isolation and purification were of highest grade available commercially. Poly(ethyleneimine) and ammonium sulfate (ultra pure, special enzyme grade) were obtained from Eastman Kodak (Rochester, NY) and Schwarz/Mann (Orangeburg, NY), respectively. Glycerol (reagent grade) was purchased from Wako Chemical Co. Affi-Gel Blue gel (100–200 mesh wet, 75, 150 μ), Bio-Gel 0.5 m (200–400 mesh) and BioBeads (SM-2, 20–50 mesh) were purchased from Bio-Rad Laboratories (Richmond, CA). $CaCl_2 \cdot 2H_2O$ and K_2HPO_4 for hydroxylapatite preparation were obtained from MCB Manufacturing Chemists (Cincinnati, OH), and ethylene glycol from Fisher Scientific Co. (Fair Lawn, NJ). All other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO). Deionized, redistilled water was used for all buffer preparations and sample solutions.

The undegraded, 124 kD phytochrome was isolated from the etiolated oat seedling (*Avena sativa* L. cv Garry, supplied by Bahr's Hybrid, Rio, WI) grown on Vermiculite in a dark room at 298 K for 4 d. Phytochrome was isolated and purified according to the procedures essentially identical to the method of Vierstra and Quail (13). In brief, the refrigerated oat tissue with phytochrome as Pfr form was homogenized in a Tris buffer containing 50% (vol/vol) ethylene glycol and 4 mM phenylmethylsulfonyl fluoride (PMSF) for isolation work-up. After precipitation of the extract with poly(ethyleneimine) and then with ammonium sulfate, the crude phytochrome solution obtained by dissolving the pellets was chromatographed through a hydroxylapatite column, Affi-Gel Blue gel affinity column, and finally, Bio-Gel A 0.5 m molecular sieve column. For other details, see previous report (14).

The molecular weight of phytochrome isolated was determined to be 124,000 by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). No contaminant protein bands were seen when ~5 μg of phytochrome preparations with specific absorbance ratios (A_{660}/A_{280}) > 0.84 was

applied on the electrophoresis gel (We thank Dr. I. -S. Kim and Mr. Y. G. Chai for running the electrophoresis). Fluorescence lifetime data were obtained with phytochrome preparations ranging from absorbance ratio 0.84 to 0.93, but results were not noticeably dependent upon the purity of phytochrome preparations. Nonetheless, data presented in this paper were obtained with those preparations with absorbance ratios greater than 0.87.

Absorption spectra were recorded on a Lambda-3 (Perkin-Elmer Corp., Instrument Div., Norwalk, CT) or Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, MD) spectrophotometer, whereas steady state fluorescence emission and excitation spectra were taken on a Spex Fluorog-2 spectrofluorometer (Spex Industries, Inc., Edison, NJ) equipped with a cooled red-sensitive photomultiplier tube (31034; RCA, Lancaster, PA). Fluorescence decay measurements were performed using a synchronously pumped, cavity-dumped dye laser and a time-correlated, single photon counting system. Details of the experimental apparatus have been described elsewhere (15). Excitation laser power was 6.8×10^{-4} W/cm^2 at 800 kHz, corresponding to 10^8 photons/cm^2/pulse. Excitation pulse width set at 637 nm was shorter than 10 ps; $6 \approx 7$ ps for most runs. Emission was detected at 680 nm, using a microchannel-plate photomultiplier (R1564U; Hamamatsu Corp., Middlesex, NJ), which allows us to obtain an instrument response function with 50-ps pulse width for the scattered laser light.

Fig. 1 shows the sketch of optical arrangement for excitation, detection and phytochrome regeneration. For the phytochrome regeneration during pulse excitation, a 500 W xenon source with one 730 nm interference filter was used. In addition, phytochrome solution was constantly stirred with a Tri-R micro-submersible magnetic stirrer (model MS-7, Tri-R Instruments, Rockville Centre, NY) with the stirring bar placed inside the cuvette. Under these conditions, negligible amounts of Pfr were produced during each data acquisition lasting 30–60 s for 2,000 counts. Only after an extended period of measurement for more than 10 min, excitation pulses (beam diameter $\approx$ 1.5 mm) produced appreciable amounts of Pfr in the cuvette (~10% conversion from Pr). For steady state fluorescence measurements, phytochrome solution was stirred with a multistirrer (model M-3, Iuchi Co., Tokyo, Japan).

To prepare solutions with varying viscosity, phytochrome in phosphate buffer, pH 7.8, was mixed with glycerol (40 or 67%, vol/vol) and the ambient temperature of the solution with or without glycerol was read (±1°C). Relative viscosities of water and water-glycerol mixtures were taken from Handbook of Chemistry and Physics (16), and temperature dependence of the viscosity of glycerol-water mixture was graphically estimated by assuming that the temperature variation of the mixture is predominantly determined by glycerol, as the viscosity of glycerol varies from 12,110 at 0°C to 1,490 centipoise at 20°C, whereas the water viscosity decreases from 1.787 to 1.002 over the same temperature interval. The relative viscosities of 40 and 67% glycerol (vol/vol) are 3.1 and 15 at 20°C, respectively (16).

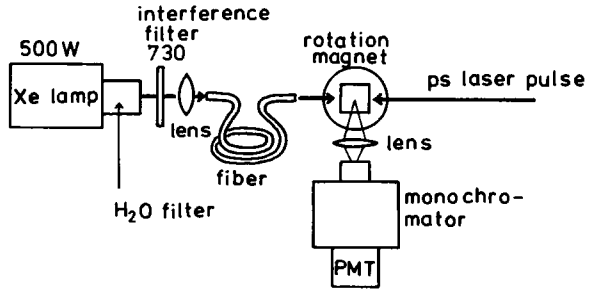

FIGURE 1 Optial layout for fluorescence excitation (ps pulses) and actinic irradiation (730 nm) of phytochrome solutions for lifetime measurements. The sample solution was stirred with a rotating magnet during excitation. Intensity of the excitation laser light is decreased (3.0 W/m^2) by using a neutral density filter so as to minimize the degradation of Pr.

Phytochrome solutions with and without added glycerol were first degassed by vacuum and then flushed with water-saturated nitrogen (99.998%). This procedure eliminated air bubbles in the solutions introduced during solution preparation and minimized any irreversible photobleaching of phytochrome during pulse excitation.

RESULTS

Fig. 2 shows the steady state fluorescence spectra of phytochrome at two different viscosities. The corrected fluorescence maxima are at 689 and 688 nm in buffer and buffer-40% glycerol, respectively. Thus, within noise levels seen in Fig. 2, the two fluorescence spectra are virtually identical. Fig. 3 shows examples of fluorescence decays of phytochrome in phosphate buffer and buffer-glycerol mixtures. It is clear that the decays are nonexponential, with the short component being the major contributor. A satisfactory fit can be obtained in terms of two exponential decay components, according to the least squares fit procedure used. Decay curves were reproducible well within 10% variations. In fact, several runs at two slightly different temperatures yielded nearly perfect superimposable decay curves, suggesting that the decay is not strongly temperature-dependent.

Table I summarizes the fluorescence decay data obtained in three different media containing 0, 40, and 67% glycerol, respectively. It can be seen that the short lifetime (τ_1) of the fluorescence decay of phytochrome are slightly viscosity-dependent, and the longer component (τ_2) is definitely viscosity-independent. However, the amplitudes of both components are strongly viscosity-dependent.

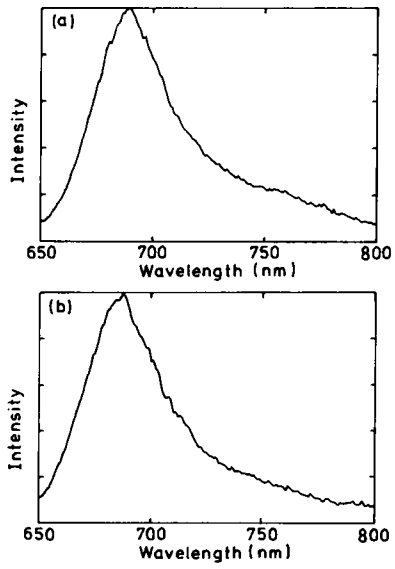

FIGURE 2 Fluorescence emission spectra (corrected) of phytochrome (Pr) in potassium phosphate buffer pH 7.8 (*a*) and in 40% glycerol-buffer mixture at ~13 and 15°C, respectively. Concentrations, 1.7 μM (*a*) and 1.0 μM (*b*). Excitation wavelength, 640 nm. Excitation bandwidth, 5.4 nm. Emission bandwidth, 5.4 nm.

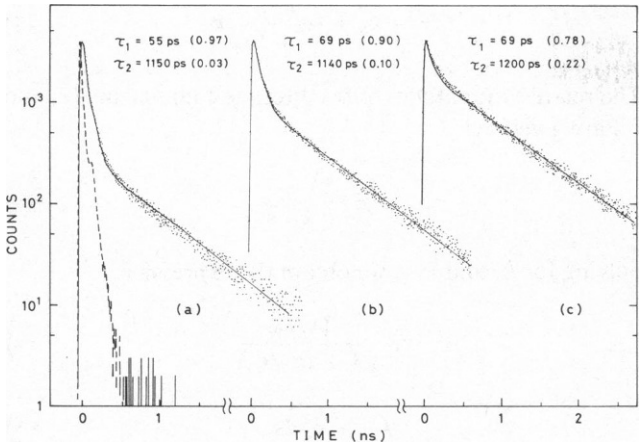

FIGURE 3 Fluorescence decay curves (. . .) and best-fit curves (—) calculated by convolution with the instrument response function (---) for phytochrome (Pr) in glycerol-buffer mixture at ~288 K. Concentrations: (*a*) 1.8 μM; (*b*) 1.2 μM; (*c*) 0.7 μM. Excitation pulse wavelength, 637 nm, and emission wavelength monitored, 680 nm (Slit width ~3–6 nm).

DISCUSSION

When phytochrome is irradiated in frozen matrix at liquid nitrogen temperature, a photoreversible intermediate is formed, suggesting that the primary process occurring from the excited singlet state (^1Q_y) of phytochrome is extremely fast (10). Both low temperature and ambient temperature studies of phytochrome phototransformation suggested the same conclusion, although these studies used proteolytically degraded oat or rye phytochrome preparations which qualitatively retained the spectroscopic and photochemical properties of undegraded 124 kD phytochrome (17–20). It is now established that the latter exhibits a higher photostationary concentration of the Pfr form when the Pr form of phytochrome was excited at 660 nm and the absorption maximum of the Pfr form is red shifted by 5–10 nm relative to that of degraded phytochromes (13, 21). However, the spectral maximum of the Pr form is not significantly different from that of degraded proteins. Thus, it is not surprising that the fluorescence decay characteristics of degraded and intact phytochrome preparations are essentially identical (9).

Although the fluorescence decay of phytochrome in phosphate buffer in the absence of glycerol may be better fit with three exponentials, the two component analysis appears to be satisfactory. This is also true for phytochrome solutions in buffer-glycerol mixtures as shown in Fig. 2. We have also performed three-component analysis, with the results tabulated in Table II. As can be seen from the values listed in Table II, the third component does not significantly contribute to the overall decay amplitudes. The fitting is better in the three component analysis (X_r^2 = 1.05–1.1) than in the two component analysis (X_r^2 = 1.5–2.0), as shown in Fig. 4. It is seen from values for the fastest and slowest components correspond to the results in the two component analysis (Table I), and that the lifetime and the contribution of the intermediate component is changed in parallel with those of the slowest component. The results of the three component analysis are qualitatively in agreement with the previous work (9), if the different phytochrome preparations and solution conditions employed are taken into account.

Although a complicated model can be proposed to account for the three component decays observed, a modeling based on the two component decays would be satisfactory and more useful for a qualitative understanding of the viscosity dependence investigated here. The latter can be justified on the ground that the relative contribution of the third component to the overall fluorescence intensity in Fig. 4 and Table II (τ_2) is negligible; i.e., τ_1(ns) × A_1 (% amplitude) = 4.47, $\tau_3 \times A_3$ = 2.43 and $\tau_2 \times A_2$ = 0.48 at viscosity η/η_o = 0.98. Even at the highest relative viscosity examined, this component is not a significant one, compared to the strongly viscosity-dependent component (τ_3).

TABLE I
FLUORESCENCE LIFETIMES OF PHYTOCHROME (Pr) AS A FUNCTION OF VISCOSITY

Medium* (Pr)	η/η_0	T°C	τ_1		τ_2		k_1, $s^{-1} \times 10^{-10}$	k_{-1}, $s^{-1} \times 10^{-8}$
			ps	%	*ns*	%		
Buffer	0.98	21	53	(98)	1.14	(2)	1.84	4.4
(0 % glycerol)	1.10	16.5	55	(97)	1.15	(3)	1.77	4.8
	1.20	13	55	(97)	1.15	(3)	1.77	4.8
	1.47	6	61	(96)	1.16	(4)	1.58	6.9
40 % glycerol	3.1	21	62	(93)	1.13	(7)	1.49	11.9
in buffer	4.7	15.5	58	(93)	1.14	(7)	1.61	11.3
	7.2	10.5	69	(90)	1.14	(10)	1.30	15.1
	16.1	2	73	(89)	1.14	(11)	1.22	15.2
67 % glycerol	19	19	64	(78)	1.17	(22)	1.29	26.1
in buffer	21	15.5	69	(78)	1.20	(22)	1.12	32.6
	21	15	69	(80)	1.20	(20)	1.16	29.0
	34	9	68	(81)	1.14	(19)	1.19	28.6

For rate constants listed, see the text for definition.
*Phosphate buffer, 20 mM, pH 7.8, containing 1 mM $(Na)_4$-EDTA, and 2.8 mM 2-mercaptoethanol, stored at ice water temperature until used.

TABLE II

THE FLUORESCENCE DECAY ANALYSIS OF PHYTOCHROME (Pr) IN TERMS OF THREE COMPONENTS, AS A FUNCTION OF VISCOSITY (η/η_o)

Medium	η/η_o	T°C	τ_1		τ_2		τ_3	
			ps	%	*ps*	%	*ns*	%
Buffer	0.98	21	47	(95)	198	(2.4)	1.058	(2.3)
(0 % glycerol)	1.10	16.5	49	(96)	356	(1.5)	1.096	(2.4)
	1.20	13	51	(95)	186	(2.0)	1.054	(2.8)
	1.47	6	60	(92)	292	(4.4)	1.174	(3.8)
40 % glycerol	3.1	21	50	(88)	260	(5.4)	1.146	(6.2)
	4.7	15.5	49	(86)	221	(7.8)	1.120	(6.6)
	7.2	10.5	61	(85)	279	(7.5)	1.188	(9.3)
	16.1	2	61	(85)	299	(5.8)	1.168	(9.5)
67 % glycerol	19	19	52	(67)	314	(12)	1.225	(21)
	21	15.6	55	(67)	351	(12)	1.242	(21)
	21	15	56	(70)	357	(11)	1.241	(19)
	34	9	54	(74)	328	(11)	1.233	(16)

The two exponential decays observed in this study can be interpreted in terms of the following scheme:

$$\begin{array}{ccccc} {}^1\mathrm{Pr} & \overset{k_1}{\rightleftharpoons} & \mathrm{Pr}' & \xrightarrow{k_2} & \text{Lumi-R?,} \\ k_0 \downarrow & \overset{}{\underset{k_{-1}}{}} & & & \\ \mathrm{Pr} & & & & \end{array}$$

Scheme II

where ^{1}Pr and Pr′ represent the excited singlet state of phytochrome and its primary intermediate, respectively, with the corresponding rate constants, k_0 and k_1/k_{-1}. The intermediate decays further, with rate constant k_2.

With the reasonable assumption for an efficient photoreceptor that k_1 is greater than the absolute difference $(k_0 - k_2)$, the following expressions can be derived:

$$1/\tau_1 = k_1 + k_{-1} \tag{1}$$

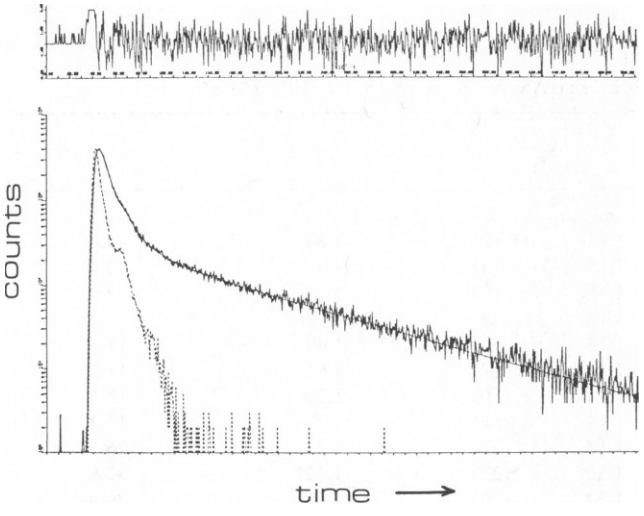

FIGURE 4 Fluorescence decay curve of phytochrome and its best three-component fit (solid line) and the residuals (*upper* panel) for the data shown in Fig. 3 *a*.

$$1/\tau_2 = k_2 + \frac{k_{-1}}{k_1 + k_{-1}} k_o . \tag{2}$$

The relative amplitudes of two lifetime components (τ_1 and τ_2) are given by:

$$\frac{C_2}{C_1} = \frac{k_{-1}}{k_1} . \tag{3}$$

Solving for k_1 and k_{-1}, we obtain the expression

$$k_1 = \frac{(1/\tau_1)}{1 + (C_2/C_1)} \tag{4}$$

$$k_{-1} = \frac{C_2}{C_1} k_1 . \tag{5}$$

Values of the rate constants calculated are listed in Table I. It can be seen that k_1 decreases somewhat with increasing viscosity, whereas k_{-1} increases steeply with increasing viscosity. The fact that the short component lifetime is only slightly viscosity dependent is explicable in terms of Eq. 1 with two rate constants having opposing dependencies on viscosity (see Table I) and with k_1 being much greater than k_{-1}. The viscosity independence of τ_2 is attributable to k_2, which dominantly contributes to the long fluorescence decay component, as can be seen from Eq. 2.

The viscosity dependence of rate constants k_1 and k_{-1} may reflect conformational/configurational isomerization of the Pr chromophore in its primary photoprocess (9, 18). However, k_1 depends only moderately on the medium viscosity, whereas k_{-1} increases strongly with increasing viscosity. It is possible that this differential viscosity dependence of the forward and backward reactions arises from a restricted conformational flexibility of the chromophore in the primary intermediate and/or its proximal amino acid residues under higher viscosity conditions. In less viscous media, the chromophore may assume an equilibrium distribution of conformation within the solvation cage from which the reverse reaction (k_{-1}) proceeds with the reorganization of the solvent cage. In higher viscosity media, this reorganization is less extensive, according to the proposed model. This would explain the differential dependences of k_1 and k_{-1} on viscosity.

Previously, Holzwarth et al. (9) reported fluorescence lifetimes of phytochrome in buffer solution. They analyzed the fluorescence decay curves into three exponential decays. Only the fastest component has been related to the Pr ↔ Pfr primary reaction. They attributed the slower decay components as arising from some impurities dependent upon the method of phytochrome preparation. In the present analyses, we proposed that both the fast and the slow components arose from the reversible ^{1}Pr ↔ Pr′ conversion. The viscosity dependence of k_1 and k_{-1} is consistent with this model.

In the presence of only weak actinic light of 730 nm (via monochromator), which partially converts the Pfr form of phytochrome produced by the exciting picoseconds pulses

(under such conditions, ~50% of Pr was converted to Pfr after a series of pulse excitation experiments; total time of experiment—10 min), the short component lifetime was ~90 ps. The longer lifetime component detected under the photocycling conditions with the weak actinic light of 730 nm (see Fig. 1) is probably due to a new fluorescent intermediate that is detectable in frozen matrix under steady state conditions (22).

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