Fluorescence Lifetimes and Relative Quantum Yields of 124-Kilodalton Oat Phytochrome in H_2O and D_2O Solutions

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ABSTRACT: The lifetimes and relative quantum yields of the three fluorescence components of the P_r (red-absorbing) form of 124-kDa oat phytochrome in H_2O and D_2O solutions have been determined by single-photon-timing techniques. Lifetime and quantum yield of the main component, which is the shortest lived (about 40 ps at low concentration) and reflects the photochromic properties of P_r, are not affected by D_2O. The medium-lifetime component (about 180 ps at low concentration), which is attributable to a photochromic source behaving comparable to that responsible for the main component, is not affected either. Only the least occurring and longest lived component (about 1 ns), which is nonphotochromic, i.e., unaffected by red irradiation, markedly increases in lifetime (to about 1.4 ns). Thus, its relative contribution to the total fluorescence yield increases in D_2O. The spectra of the individual fluorescence components, obtained by a global analysis of the decay traces from 124-kDa P_r in H_2O solution at different observation wavelengths, differ only slightly in wavelength. We conclude that the primary photoreaction in the P_r transformation to P_{fr} (far-red-absorbing form of phytochrome) of the main phytochrome component exhibiting the shortest lived fluorescence does not involve a proton transfer as has been suggested in the literature (instead, the most likely process is $Z \rightarrow E$ isomerization of the 15,16 double bond).

Phytochrome is a plant chromoprotein that acts as a photoreceptor for numerous photomorphogenic responses [see Braslavsky (1984) and Lagarias (1985) for recent reviews]. It occurs in two forms, P_r and P_{fr},[1] which are thermally stable but photochemically interconvertible (photochromic or so-called "photoreversible" forms). Apart from a configurational *E,Z* isomerism about one of the exocyclic C=C double bonds of the chromophore (Rüdiger, 1983; Thümmler & Rüdiger, 1983), nothing is known yet of the molecular aspects of the $P_r \rightleftarrows P_{fr}$ transformations. Song and co-workers (Sarkar & Song, 1981; Moon et al., 1985) in studies of phytochrome in tris(hydroxymethyl)aminomethane–D_2O solution by stationary absorption and fluorescence spectroscopy have reported deuterium isotope effects on the fluorescence quantum yield and lifetime of P_r phytochrome. The results were tentatively interpreted to arise from a proton-transfer process as the primary photochemical reaction initiating the transformation of P_r. However, our own picosecond-resolved fluorescence studies using the single-photon-timing technique[2] have more recently shown (Holzwarth et al., 1984) that the fluorescence decay of 124-kDa phytochrome[3] is non single exponential (see also last paragraph under Conclusions). It comprises a main-decay component (1; 90–92% amplitude) with a lifetime of 48 ± 3 ps at 275 K and two longer-lived components with lifetimes of 190 ± 30 ps (2; 7–9%) and 1.04 ± 0.06 ns (3; 1%). As a consequence of their much longer lifetimes, the fluorescence of components 2 and 3 accounts for about 30% of the total integrated fluorescence of the phytochrome sample. Thus, information on the primary excited-state processes of the main component 1 of P_r cannot be obtained in a simple way from the stationary fluorescence of phytochrome preparations.

It is pertinent in this context to reiterate that all phytochrome preparations are likely to be heterogeneous with respect to the fluorescence decay of P_r. [This does not necessarily mean that the observed simultaneous formation of two or three primary photoproducts, I^i_{700}, from P_r (Aramendía et al., 1987; Braslavsky et al., 1980; Cordonnier et al., 1981; Furuya, 1983; Linschitz et al., 1966; Ruzsicska et al., 1985) has its origin in the heterogeneity of the sample, i.e., all I^i_{700}'s may just as well arise from the main component 1.] This heterogeneity has been revealed only in picosecond time-resolved fluorescence measurements (Holzwarth et al., 1984; Wendler et al., 1984). All other methods employed so far in photophysical investigations give results for the mixture. We shall therefore refer to "composite" P_r where the data so require.

It was imperative from the above to reinvestigate the problem of a possible deuterium isotope effect on the P_r fluorescence by the single-photon-timing technique, which discriminates between the individual emission components. The measurements were performed at a low phytochrome concentration in view of the turbidity developing in D_2O buffer, in particular at lower temperatures (e.g., 275 K) and higher concentrations [see also Moon et al. (1985)] (for a comment

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[1] Abbreviations: KPB, potassium phosphate buffer containing K_2HPO_4 and KH_2PO_4, pH 7.8 at 4 °C; P_r and P_{fr}, red- and far-red-absorbing forms of phytochrome, respectively; PBEG/H_2O, 0.1 M KPB/25% v/v ethylene glycol/1 mM sodium ethylenediaminetetraacetate in H_2O; PBEG/D_2O, 0.1 M KPB/25% v/v ethylene glycol-d_6/1 mM sodium ethylenediaminetetraacetate in D_2O; SAR, specific absorbance ratio for P_r, A_{666}/A_{280}; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRFR, spectral ratio A_{729}/A_{672} of phytochrome ($P_r + P_{fr}$) after saturating red-light irradiation.

[2] The single-photon-timing (or time-correlated single-photon-counting) technique [for a review, see Wild et al. (1977)] used in our studies allows for simultaneous resolution of up to four picosecond and nanosecond components of a composite fluorescence [cf. G. W. Suter, A. R. Holzwarth, P. Klein-Bölting, E. Bittersmann, and W. Stempfle (unpublished results)].

[3] The properties of our 124-kDa phytochrome preparations are in agreement with those described by Vierstra and Quail (1982) and subsequent literature as "native" phytochrome.

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on results at higher concentrations, see Results and Discussion).

Materials and Methods

Phytochrome

Chromatographic Materials and Analytical Reagents. Hydroxylapatite, Bio-Gel HTP, and Affi-Gel Blue gel (100–200 mesh) were from Bio-Rad Laboratories, München; Sepharose CL-6B was from Pharmacia, Freiburg; K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, KCl, and ethylene glycol were from Merck, Darmstadt; sodium ethylenediaminetetraacetate, Na_2SO_3, phenylmethanesulfonyl fluoride, 50% aqueous poly(ethylenimine), and flavin mononucleotide were from Sigma, Taufkirchen; tris(hydroxymethyl)aminomethane was from Serva, Heidelberg; (±)-dithiothreitol was from Boehringer, Mannheim; the aqueous calibration buffers were a phosphate solution (pH 7.00 ± 0.01 at 25 °C) from Radiometer, Copenhagen, and a borate–hydrochloric acid solution (pH 8.00 ± 0.02 at 20 °C) from Merck. The water used was tridistilled. The proteins for SDS–PAGE calibration were from Bio-Rad and Boehringer, and those for fast protein liquid and gel exclusion chromatography (thyroglobulin, 669 kDa, 85 Å; ferritin, 440 kDa, 61 Å; catalase, 232 kDa, 52.2 Å; aldolase, 158 kDa, 48.1 Å; bovine serum albumin, 67 kDa, 35.5 Å; ovalbumin, 45 kDa, 30.5 Å) were from Pharmacia.

For fast protein liquid chromatography, a MicroPak TSK 3000SW column (7.5 × 300 mm) in conjunction with a 75 × 7.5 mm prefilter from Varian was employed. The chromatographic system was home-built, employing a 112 Beckman pump and two 150 B Altex detectors at 280 and >600 nm, respectively (both companies in San Ramõn, CA). The flow rate was 0.2 mL/min at a pressure of 8 bar. The buffer was 100 mM KPB and 2 mM sodium ethylenediaminetetraacetate (pH 7.8 at 25 °C). The column void volume was determined with Dextran Blue from Pharmacia. All buffers were filtered through Millipore 0.2-μm acetylcellulose filters prior to use.

The plant tissue was collected by a procedure that was similar to that of Litts et al. (1983), with the following modifications: oat seedlings (600 mL) of *Avena Sativa* L. cv. Garry (from Stamford Seed Co., Buffalo) were sown with 1.7 L of water and 3 L of vermiculite on a 60 × 40 × 10 cm plastic tray under white light, stored in darkness at 4 °C for 24 h, and then grown in the dark for 96 h (25 °C, >95% relative humidity). The tissue was harvested (3–4-cm shoots tips) under green safe light and used either immediately or after freezing with liquid N_2 and storage at −75 °C. Typical yields of fresh tissue were 200 g per tray.

The irradiations and the spectroscopic measurements were performed with a microprocessor-controlled PE 356 spectrophotometer equipped with a Perkeo (Zeiss) slide projector (250-W tungsten–iodine lamp); 660-nm line interference (Schott, full-width half-maximum 15 nm) and cut-off filters for $\lambda > 715$ nm were used for red and far-red irradiations, respectively.

Discontinuous SDS–PAGE was performed according to Laemmli (1970) on 7–15% linear polyacrylamide gels (240 × 170 × 1.5 mm) and stained with Coomassie Blue.

For the Stokes radius determination fast protein liquid and gel exclusion chromatography were calibrated with known molecular weight proteins, and phytochrome was assumed to be globular.

Phytochrome Purification. For preparative chromatography an automated fraction collector FC-300, Frac-CC from Pharmacia, coupled with a Uvicord III 2089 dual-wavelength (280 and >600 nm) single-channel detector from LKB, Stockholm, was used.

The purification procedure was basically that of Vierstra and Quail (1983) with the following modifications. The oat tissue was used in the nonirradiated form. The KPB extraction buffer contained, in addition, (±)-dithiothreitol (15 mM). A total of 25 mL (instead of 10 mL) of a solution of 10% v/v poly(ethylenimine)/L of extract was added. The phytochrome was precipitated with $(NH_4)_2SO_4$ and resuspended in about 200 mL of a buffer that contained 10 mM (±)-dithiothreitol (instead of 14 mM 2-mercaptoethanol). No conductivity measurements were performed. The three hydroxylapatite chromatography elution buffers contained 10 mM (±)-dithiothreitol (instead of 14 mM 2-mercaptoethanol). The third buffer was 100 mM (instead of 20 mM) in KPB. The phytochrome fraction was then precipitated (as also in all subsequent precipitations) with a solution containing 3.3 M $(NH_4)_2SO_4$ and 100 mM KPB [instead of varying amounts of 3.3 M $(NH_4)_2SO_4$ and 50 mM tris(hydroxymethyl)-aminomethane]. A total of 0.75 mL of this solution/mL of phytochrome solution was used. The Affi-Gel Blue gel chromatography buffers contained (±)-dithiothreitol (5 mM) instead of 2-mercaptoethanol (14 mM), and the last two buffers were 500 mM (instead of 250 mM) in KCl. The gel exclusion chromatography column (Sepharose 6B, 2.6 × 65 cm) was equilibrated with a buffer consisting of 100 mM KPB, 2 mM sodium ethylenediaminetetraacetate, and 2 mM (±)-dithiothreitol (instead of 5 mM sodium ethylenediaminetetraacetate and 14 mM 2-mercaptoethanol). The final phytochrome preparations were resuspended in PBEG/H_2O (air-saturated solutions; for the composition of the PBEG buffers see, footnote 1) and either used directly or stored at −75 °C after freezing in liquid N_2. Samples for measurements in PBEG/H_2O buffer were prepared by diluting the parent solution with the same buffer to the desired concentration, followed by centrifugation. For the preparation of the D_2O solutions, P_r was precipitated with 40% $(NH_4)_2SO_4$ and centrifuged and the supernatant discarded. The pellet was washed twice with D_2O and then resuspended in PBEG/D_2O.

Protein determinations were carried out following the procedure described by Vierstra and Quail (1983) and references cited therein.

pH and pD Determinations (with P. F. Aramendia). These measurements should have been carried out by electrode standardizations with calibration buffers in the same solvents as those of the P_r samples. Since no standard buffers have been defined as yet in these solvent mixtures (Mussini et al., 1985), the glass electrode was calibrated with the phosphate and borate–hydrochloric acid calibration buffer solutions (see Chromatographic Materials and Analytical Reagents). The pH of the PBEG/H_2O buffer solution was 7.8 at 278 K. The pD of PBEG/D_2O was obtained by adding 0.41 pH unit (molar units at 298 K; Covington et al., 1968) to the value measured, without correction for the 25% ethylene glycol added in view of the small correction required for up to 33% of methanol and ethanol (<0.1 pH unit at pH around 7.5; Bates et al., 1963). For the appropriate acid strength of the PBEG/D_2O solution, the required concentration of deuteriophosphoric acid (pK_a = 7.88 at 275 K; Christensen et al., 1976) was calculated by assuming validity of the Debye–Hückel expression for the activity coefficients in deuteriated solutions. The phytochrome D_2O solutions were sufficiently stable when the deuteron activity equaled the proton activity in H_2O solution (Moon et al., 1985).

Irradiation and Fluorescence Measurements

Absorption spectra were measured before and after the fluorescence experiments. For the fluorescence measurements,

the phytochrome samples were handled under dim green light. All irradiations and fluorescence measurements were performed at 275 ± 1 and 293 ± 1 K. For the irradiations, line interference filters (Schott) with λ_{max} = 660 or 735 nm (both with full-width half-maxima of 15 nm) were inserted into the collimated beam from a 250-W tungsten–iodine lamp of a slide projector. Table II lists the stationary fluorescence data for the P_r form under various conditions (see below for details), and Table III gives the time-resolved data.

Fluorescence lifetimes were measured by single-photon timing (see Table IIIA). Excitation (Wendler et al., 1984) and detection systems (Holzwarth et al., 1982, 1983), as well as the analytical procedure and sample flow system (Holzwarth et al., 1984), have been described previously. In addition, after each measurement the sample was first irradiated in the external reservoir with 660 nm until the $P_r \rightleftarrows P_{fr}$ photoequilibrium was established, and then irradiated back to P_r with λ = 735 nm. This was done in order to assure exhaustive cycling since during the decay measurements only a small part of the total sample volume underwent photoconversion. The excitation wavelength was λ^{exc} = 640 nm, and the emission wavelength in general was λ^{em} = 680 nm. The individual fluorescence spectra of the three decay components were calculated from the fluorescence decays recorded at the emission wavelengths 660, 670, 680, 690, 700, and 710 nm. In this case, the spectral amplitudes were corrected for the wavelength-dependent response of the photomultiplier. In all emission experiments, the photoconversion of P_r was below 10%.

The relative amplitude of an individual fluorescence component j (Table IIIB) at a given excitation wavelength, λ^{exc}, is defined by

$$A_j(\lambda^{em}) \triangleq k_{rad}\epsilon_j(\lambda^{exc})c_j i_j^f(\lambda^{em})$$

The function $i_j^f(\lambda^{em})$ is the fluorescence spectrum, k_{rad} the rate constant for fluorescence, and c_j the concentration. The normalized relative yields of emission ϕ_j^N(rel) (Table IIIC) were calculated from the normalized amplitudes A_j^N (Table III, footnote *d*) and lifetimes τ_j (Table IIIA). The normalization accounts for the absorbance of the sample at the excitation wavelength, the time of the measurement, the laser power, and the prompt response of the apparatus. The sum of ϕ_j^N affords a normalized total yield, ϕ_T^N (Table IIIC).

Relative fluorescence quantum yields were determined (i) by use of the lifetimes and normalized amplitudes of the components (see Table III, footnote *d*) in the picosecond-resolved fluorescence measurements and (ii) from stationary fluorescence measurements on a computer-controlled Spex-Fluorolog (Holzwarth et al., 1978; Wendler et al., 1984) in 1-cm cuvettes at λ^{exc} = 640 nm (Table II). The bandwidths were 5 nm in the excitation monochromator and 10 nm in the emission monochromator. The spectra were corrected for wavelength dependence of photomultiplier sensitivity and lamp intensity (Wendler et al., 1984). Rhodamine 101, ϕ_f = 1.0 (Karstens & Kobs, 1980), and cresyl violet, ϕ_f = 0.54 (Magde et al., 1979), in ethanol were used as the standards. Absorbances of sample and reference were matched at the respective excitation wavelength. The areas of the emission spectra were integrated in the range 650–850 nm. No correction was made for the refractive index difference between standard and sample solutions.

Results and Discussion

Phytochrome Isolation and Characterization. The complete purification procedure (see Materials and Methods) and characterization of the 124-kDa oat phytochrome used in this work as well as in our previous photophysical investigation (Holzwarth et al., 1984) and the photochemical investigations [accompanying papers, Aramendía et al. (1987) and Heihoff et al. (1987); previous work, Ruzsicska et al. (1985)] have not yet been described. [For the identification of 124-kDa phytochrome (by SDS–PAGE) as the "native" form of the protein, see Bolton and Quail (1982) and Vierstra and Quail (1982).]

Table I: Purification of Nonirradiated 124-kDa Phytochrome from 2 kg of Etiolated Oat Seedlings[a]

step	ΔA[b,c]	vol (mL)[c]	units[c,d]	yield (%)[c]	average yield from 4 runs (%)
supernatant after PEI[e]	0.03	2250	68	100	100
$(NH_4)_2SO_4$ I	0.29	216	61	90	88
HAC[f] pool	0.57	83	47	70	60
$(NH_4)_2SO_4$ II	1.02[g]	42	43	63	58
AGBC[h] pool	0.46	60	28	40	42
$(NH_4)_2SO_4$ III	1.74[g]	15	26	38	42
GEC[i]	0.23	102	24	35	38
$(NH_4)_2SO_4$ IV	0.99[g]	20	20	30	32

[a] For the results of protein determinations, see Phytochrome Isolation and Characterization. [b] ΔA refers to the difference in absorbance at 665 and 730 nm taken from phytochrome difference spectra (far-red minus red adapted) in 1-cm cuvettes. [c] Data of one representative run. [d] One unit defined as the amount of P_r per milliliter giving ΔA = 1 in 1 cm [see Litts et al. (1983)]. [e] PEI, 50% aqueous poly(ethylenimine). [f] Hydroxylapatite chromatography. [g] $A_{667}(P_r)$ was measured instead of ΔA; $\Delta A = 1.13A_{667}(P_r)$. [h] Affi-Gel Blue gel chromatography. [i] Gel exclusion chromatography.

The hydroxylapatite chromatography peak for phytochrome possessed a shoulder, which is in contrast to the report by Vierstra and Quail (1983) but is reminiscent of the two phytochrome fractions observed by Litts et al. (1983). Although we used the total protein pool in the subsequent purification steps, only one phytochrome fraction was eluted from the Affi-Gel Blue gel and gel exclusion chromatography columns.

Table I summarizes the yields at each step of one representative procedure. An additional column represents average yields from four parallel purification runs. The increase in the KPB and KCl concentrations with respect to those used previously (Viestra & Quail, 1983) improved the elution efficiencies of the hydroxylapatite and Affi-Gel Blue gel chromatography, respectively (=phytochrome yield).

The absorption of the phytochrome used in all our studies was fully photoreversible in PBEG/H_2O, i.e., the P_{fr} absorption was fully bleached and the P_r absorption fully recovered on irradiation at 735 nm. It gave a single band at 124 kDa on 7–15% gradient SDS–PAGE [shown in Holzwarth et al. (1984) and Ruzsicska et al. (1985)], and the fast protein liquid chromatography profile exhibited one peak both at 280 and at >600 nm. Neither method indicated any contamination by other protein fractions of up to 500 kDa. An SRFR value of 1.35 and the λ_{max} position for P_{fr} (after irradiation at 660 nm: 729 nm) indicate a good spectral quality of the samples, at least in the red region of the spectrum. The SAR values, which generally were between 0.7 and 0.8, are lower than those (0.9–1.0) reported by Litts et al. (1983) and Vierstra and Quail (1983). However, taking into account the finding by single-photon timing that in our phytochrome samples >90% of the chromoprotein is fully photoreversible P_r (Holzwarth et al., 1984),[4] the SAR criterion appears to be an inadequate measure of the chromoprotein purity in spectroscopic investigations that

[4] Absorption spectroscopy is much less sensitive than single-photon timing in monitoring the P_r–P_{fr} photoreversibility since the absorptions of the three components are strongly overlapping.

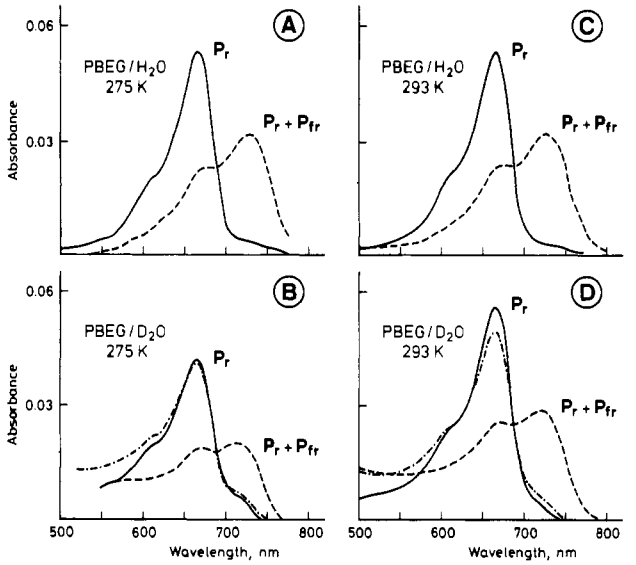

FIGURE 1: Absorption spectra of 124-kDa phytochrome at different temperatures in H_2O and D_2O buffers: (A and C) upon irradiation at 730 nm [P_r (—)] and 660 nm [$P_r + P_{fr}$ (– –)] both before and after the fluorescence measurements; (B and D) upon irradiation at 730 nm before [P_r (—)] and after the fluorescence measurements [P_r (–·–)] and upon subsequent irradiation at 660 nm [$P_r + P_{fr}$ (– –)] (four cycles).

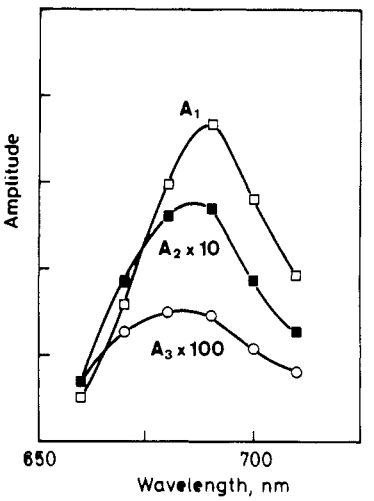

FIGURE 2: Time-resolved corrected emission spectra of fluorescence decay components 1–3 of 124-kDa P_r phytochrome in PBEG/H_2O: 275 K, λ^{exc} = 640 nm, and A_{665} = 0.22 cm^{-1}.

employ visible excitation and assess the photophysical and photochemical heterogeneity (It is, of course, an adequate basis to judge the purity in terms of noncolored proteins).

The degree of protein purification was determined by protein determination after each step of the isolation procedure. Characteristic data, obtained from one run, were as follows: after supernatant PEI (for abbreviations see Table I), 64 units/17 100 mg of total protein; $(NH_4)_2SO_4$ precipitation I, 49 units/1900 mg, purification degree 6.7; AGBC pool, 27 units/58 mg, purification degree 126; $(NH_4)_2SO_4$ precipitation IV, 35 units/29 mg, purification degree 214.

Fast protein liquid and gel exclusion chromatography gave very similar values for the Stokes radius in PBEG/H_2O solution [70 Å for P_r and 73 Å for $P_r + P_{fr}$ after irradiation at 660 nm; estimated error ±10%; the values for phytochrome in the absence of ethylene glycol tended to be lower (B. P. Ruzsicska, T. Arai, W. Schlamann, S. E. Braslavsky, and K. Schaffner, unpublished results].[5]

The phytochrome solutions used in this work had SARs of 0.68 in the case of the PBEG/H_2O sample and 0.50 in the case of the PBEG/D_2O sample of P_r (each after irradiation at 730 nm) and an SRFR of 1.38 ± 0.03 in the case of all H_2O and D_2O samples of $P_r + P_{fr}$ (each after irradiation at 660 nm) (see Figure 1). While in H_2O the phytochrome samples were photoreversible irrespective of the number of cycles, the recovery was incomplete in PBEG/D_2O at 275 K (see Figure 1B,D). At the same time, light scattering of the D_2O solutions increased somewhat during the emission measurements while the H_2O samples were not noticeably affected (see Figure 1).

Time-Resolved Fluorescence Spectra of Phytochrome Components. Figure 2 shows the individual spectra for the relative amplitudes of the fluorescence decay components 1–3 in PBEG/H_2O at a higher concentration (A_{665} = 0.22) and 275 K. The data were evaluated by a global analysis program

Table II: Stationary Fluorescence Quantum Yields of 124-kDa Phytochrome[a]

A_{665} (cm^{-1})	buffer	ϕ 275 K	ϕ 293 K
0.10	PBEG/H_2O[b,c]	(4.4 ± 0.8) × 10^{-3}	
0.06	PBEG/H_2O[c,d]	(3.7 ± 0.4) × 10^{-3}	(3.7 ± 0.4) × 10^{-3}
0.06	PBEG/D_2O[d]	(3.4 ± 0.2) × 10^{-3}	(3.1 ± 0.4) × 10^{-3}

[a] Technical values, uncorrected for emission contributions from the medium- and long-lived components. [b] Holzwarth et al., 1984. [c] Note that the quantum yield measurements in H_2O have been performed with different phytochrome preparations that contained somewhat different amounts of medium- and long-lived fluorescence components. [d] Data from a single phytochrome preparation that has also been used for the time-resolved measurements.

that enables the simultaneous calculation of decay amplitudes and lifetimes determined under various conditions (in this case with varying λ^{em} at the same λ^{exc} = 640 nm) and with the assumption that the lifetimes of the different components do not change with wavelength (G. W. Suter, A. R. Holzwarth, P. Klein-Bölting, E. Bittersmann, and W. Stempfle, unpublished results). The lifetimes thus obtained are τ_1 = 44 ± 4, τ_2 = 163 ± 15, and τ_3 = 900 ± 50 ps, in good agreement with the individual analyses given in Table IIIA and with those previously obtained by Holzwarth et al. (1984).

The relatively small spectral shifts between the three components confirm our previous conclusion (Holzwarth et al., 1984; Wendler et al., 1984) that components 2 and 3, which indiscriminately had been called "impurities" for want of sufficient data, contain the same or a very similar chromophore as the main component 1.

D_2O Effect on P_r Fluorescence Decay Components. The influence of D_2O on the fluorescence was studied at two temperatures, 275 and 293 K, with a P_r concentration corresponding to A_{665} = 0.06. The lifetimes of component 1 in H_2O and D_2O are essentially the same (Table IIIA), irrespective of temperature. Those of component 2 do not show a significant variation either. Only component 3 in general exhibits a considerably longer lifetime in D_2O (Table IIIA), which results in a larger relative contribution to the total fluorescence yield (Table IIIC).

Parallel measurements were also performed at a higher concentration (A_{665} = 0.22). The lifetime results are the same (see lifetime data in the preceding paragraph) despite the fact that in the PBEG/D_2O buffer the turbidity was now much greater than in the more dilute sample [Moon et al. (1985)

[5] While the values for the Stokes radii were readily reproducible in the various determinations of our phytochrome preparations, the literature data on this dimension are for varying types of solutions and therefore difficult to correlate [cf. Lagarias and Mercurio (1985), Sarkar et al. (1984), and Song (1985)].

Table III: Fluorescence Lifetimes, Relative Amplitudes, and Normalized Relative Quantum Yields of 124-kDa Phytochrome in PBEG/H_2O and PBEG/D_2O at A_{665} = 0.06[a]

(A) Lifetimes (ps) [$I(t) = \sum_{j=1}^{3} A_j \exp(-t/\tau_j)$][b]

	in PBEG–H_2O			in PBEG–D_2O		
temp (K)	τ_1	τ_2	τ_3	τ_1	τ_2	τ_3
275	40 ± 4	197 ± 10	1110 ± 60	41 (41) ± 4	210 (210 ± 10	1460 (1540) ± 60
293	34 ± 4	170 ± 10	1040 ± 60	32 (31) ± 4	180 (160) ± 10	1450 (1440) ± 60

(B) Relative Amplitudes (%)[b]

	in PBEG–H_2O			in PBEG–D_2O		
temp (K)	A_1	A_2	A_3	A_1	A_2	A_3
275	93.0 ± 0.7	6.2 ± 0.6	0.8 ± 0.2	93 (94) ± 0.7	6.1 (5.6) ± 0.4	0.8 (0.8) ± 0.2
293	93.7 ± 0.7	5.8 ± 0.6	0.5 ± 0.2	94 (94) ± 0.7	5.4 (5.8) ± 0.4	0.7 (0.6) ± 0.2

(C) Normalized Relative Quantum Yields [$\phi_j^n = A_j^N \tau_j$][c-e] and Normalized Relative Total Quantum Yield [$\phi_T^N = \sum_{i=1}^{3} \phi_j^N$]

	in PBEG–H_2O				in PBEG–D_2O			
temp (K)	ϕ_1^N	ϕ_2^N	ϕ_3^N	ϕ_T^N	ϕ_1^N	ϕ_2^N	ϕ_3^N	ϕ_T^N
275	0.65 ± 0.05	0.20 ± 0.02	0.15 ± 0.01	1	0.58 (0.62) (0.56) ± 0.05	0.19 (0.19) (0.19) ± 0.02	0.19 (0.20) (0.16) ± 0.01	0.96 (1.01) (0.91) ± 0.07
293	0.67 ± 0.05	0.20 ± 0.02	0.12 ± 0.01	1	0.58 (0.61) (0.55) ± 0.05	0.18 (0.20) (0.18) ± 0.02	0.20 (0.19) (0.18) ± 0.01	0.96 (1.00) (0.91) ± 0.07

[a] Average values of four to five cycles; experimental errors are standard deviations. [b] In parentheses, first cycle after dissolution of the phytochrome in PBEG/D_2O. [c] In parentheses, first and last cycles after dissolution of the phytochrome in PBEG/D_2O. [d] A_j^N is the amplitude from the data fit: $A_j^N = A_j(S/A_{640})t_m p$. S is the total counts accumulated in the prompt response of the apparatus, A_{640} is the sample absorbance at λ^{exc} = 640 nm, t_m is the total time of the measurement, and p is the average laser power. [e] Normalized to ϕ_T^N in PBEG/H_2O at the same temperature.

have already noticed a tendency toward greater turbidity of relatively concentrated solutions].

Fluorescence lifetimes are determined by the rate constants of the three processes that an excited state can undergo: k_d for radiationless decay, k_{rad} for fluorescence, and k_{react} for photoreaction, i.e., $\tau_{measd} = 1/(k_d + k_{rad} + k_{react})$. Since the absolute quantum yield of the fluorescence of P_r is quite low (Table II), the fluorescence lifetimes τ_1 and τ_2 are predominantly determined by the rate constants of the photochemical and radiationless decay processes. Therefore, any effect of D_2O vs. H_2O on k_d and k_{react} should also cause a change in τ_{measd}.

The decays via both the radiationless deactivation and reaction channels occur with relatively large quantum yields, approximating those measured for composite P_r: $\phi_{react} \geq 0.5$ (Heihoff et al., 1987);[6] $\phi_d \approx 1 - \phi_{react}$ (ϕ_d is the quantum yield of radiationless decay). We do not observe any significant change by D_2O on $k_d + k_{react}$ of component 1, which is the excited state of functional P_r (Wendler et al., 1984; Holzwarth et al., 1984). We conclude, therefore, that *the primary photochemical step of the $P_r \rightarrow P_{fr}$ transformation is not subject to a measurable deuterium isotope effect.*

The normalized total yields of emission, ϕ_T^N, from the low-concentration samples in D_2O and in H_2O (Table IIIC) are not significantly different either. This lack of a D/H isotope effect on the emission quantum yield is confirmed by the results of our stationary fluorescence measurements (Table II).

The contribution to the total fluorescence quantum yield by component 3, which alone exhibits important lifetime and quantum yield changes with D_2O (Table IIIA,C), can be held responsible for the observation by Moon et al. (1985) of an *apparent* deuterium isotope effect on the P_r emission under experimental conditions not discriminating between the three decay components.

Conclusions

One may now consider the similarity in photochromicity and H_2O/D_2O response of the components 1 and 2, which comprise over 97% of the composite P_r fluorescence decay amplitude. Since their respective relative amplitudes and lifetimes are very similar for all phytochrome sizes (Holzwarth et al., 1984; Wendler et al., 1984), the presence of these two components could be an inherent property of the pigment. It is too early now to speculate about the molecular differences between the two species. By the same criteria—lack of photochromicity and insensitivity of the primary photoreaction to the H_2O/D_2O change—component 3 still qualifies as an "impurity" as defined by Holzwarth et al. (1984).

The lack of a measurable isotope effect on the lifetimes of components 1 and 2 could, in principle, arise from equal effects of opposite signs by D_2O on radiationless deactivation (k_d) and the primary photoreaction (k_{react}), which would result in a constant value of τ_{measd}. Such a coincidence cannot be excluded at the present (neither can we exclude a photochemical proton transfer if the proton transferred should not be exchangeable by deuteron even when subjected to several photochromic cycles). It is perhaps more likely, however, that the lack of an isotope effect indicates that neither any proton transfers are involved in the primary photoreactions of these components nor any effects of N–D substitution or bridging on the deactivation processes occur. In the absence of proton transfer in the primary photoreaction of P_r, there remains $Z \rightarrow E$ isomerization of the 15,16 double bond as the most probable alternative, since a P_{fr} chromopeptide fragment has been shown by Rüdiger (1983) and Thümmler and Rüdiger (1983) to possess the 15,16*E* configuration.

This interpretation is in accord with our recent proposal [Ruzsicska et al., 1985; see also, Aramendía et al. (1987)] that the primary photoreaction of P_r (to I_{700}^i), as well as the subsequent ground-state processes leading from the photoproducts I_{700}^i to the secondary intermediates I_{bl}^i, occurs within the chromophore without interference by the protein. The possibility that a tautomeric change occurs at a subsequent stage of the pathway to P_{fr} is presently under investigation.

After submission of our manuscript a paper (Song et al., 1986) has come to our attention in which viscosity effects on the time-resolved fluorescence decay of P_r are reported to be better fitted with three than with two components. Yet their interpretation is arbitrarily based on the two-component model.

[6] Note that $\phi_{react} \geq 0.5$ equals the quantum yield of the photoreaction of P_r to the set of I_{700}^i intermediates only [cf. Heihoff et al. (1987)] and that the quantum yield for the overall $P_r \rightarrow P_{fr}$ transformation is only 0.15 (Holzwarth et al., 1984; Kelly & Lagarias, 1985) owing to partial thermal reversion of the intermediates to starting material.

Our own investigation demonstrates that a three-component analysis is crucial for a correct evaluation of the results of both studies, which appear to be compatible although the interpretation is not.

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