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Low-Temperature Luminescence Characterization of 124-Kilodalton Phytochrome from *Avena sativa*†

Tai-An Cha, August H. Maki,* and J. Clark Lagarias

ABSTRACT: Phytochrome which is predominantly of 124-kDa molecular mass has been investigated by low-temperature luminescence and optically detected magnetic resonance (ODMR) spectroscopy. In addition to characteristic phosphorescence spectra of Tyr and Trp, we find a previously unreported phosphorescence spectrum from a short-lived triplet state having λ_{max} 465 nm. This spectrum (chromophore X) apparently originates from covalent modifications of the protein. The intensity of the chromophore X emission decreases with increasing concentration of a sulfhydryl reagent used in the extraction procedure. Small amounts of these UV-absorbing covalent modifications are not as readily detected in the absorption spectrum of in vitro phytochrome although their effect on structural and physical properties of phytochrome may be significant. Triplet-singlet energy transfer to the tetrapyrrole is confirmed to originate from both Trp and chromophore X in the red light absorbing form of phytochrome. This conclusion is based partially on the observation of Trp and chromophore X ODMR signals while monitoring the delayed fluorescence of the tetrapyrrole. Although no delayed fluorescence was observed from the far-red light absorbing form of phytochrome, enhanced triplet-singlet energy transfer is suggested by the observed increase of the phosphorescence decay rates in this form of phytochrome.

Phytochrome, a protein containing a thioether-linked linear tetrapyrrole chromophore, is responsible for a wide variety of photomorphogenic responses in plants. A phenomenon unique to this chromoprotein is its ability to undergo photoreversible transformation between two forms. Upon absorption of red light (660 nm), the physiologically inactive red-absorbing form of phytochrome (P_r)[1] is converted to the physiologically active far-red-absorbing form (P_{fr}). P_{fr} is converted back to P_r upon absorption of far red light (730 nm). Although extensive studies have been made on the phytochrome-mediated phys-

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[1] Abbreviations: CD, circular dichroism; EG, ethylene glycol; kDa, kilodalton; ME, 2-mercaptoethanol; ODMR, optical detection of (triplet-state) magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; P_r, red light absorbing form of phytochrome; P_{fr}, far-red light absorbing form of phytochrome; SAR, specific absorbance ratio; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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iological responses (Correll et al., 1977), the molecular mechanism of this phototransformation is not well understood. Previous studies on the properties of phytochrome have not led to a clear picture of phytochrome function on a molecular level. The lack of a clear biochemical and biophysical understanding of phytochrome is in part due to the difficulty in purifying the undegraded chromoprotein from plant extracts. These problems are discussed in a recent critical review by Pratt (1982).

Recent immunological evidence has shown that native phytochrome in vivo in both etiolated oat seedlings (Vierstra & Quail, 1982) and etiolated rye seedlings (Kerscher & Nowitzki, 1982) is a single molecular species that exhibits a monomer molecular weight of 124 kDa. In vitro, however, highly purified oat phytochrome has been shown to be a mixture of predominantly 118/114-kDa species which appear to arise from limited proteolysis during extraction (Vierstra & Quail, 1982). For this reason, Lagarias and co-workers have recently developed a new purification scheme which reduces limited proteolysis during the protein isolation. This methodology has led to the isolation of a phytochrome species exhibiting predominantly a monomer molecular weight of 124000 on discontinuous gradient $NaDodSO_4$–polyacrylamide gel electrophoresis. Furthermore this highly purified phytochrome preparation exhibits several distinct physiochemical characteristics when compared with those reported earlier for more-degraded, 118/114-kDa phytochrome preparations. These properties include (1) a larger molecular weight, (2) a blocked amino terminus, (3) a significantly reduced amount of dark reversion, (4) a shift of absorption maxima, (5) a larger apparent P_{fr}/P_r photoequilibrium ratio, and (6) a new CD band in the blue spectral region (J. C. Litts, J. M. Kelly, and J. C. Lagarias, unpublished results). Consequently, these findings suggest that the highly proteolytically labile regions of 124-kDa phytochrome are significant structural and functional domains of the native protein. Therefore, previously reported spectroscopic data on 118/114-kDa phytochrome preparations need reexamination, and conclusions drawn need reconsideration.

The phosphorescence of phytochrome has been reported previously (Sarkar & Song, 1982); the purity and the molecular weight of their samples, however, were not clearly addressed. In this report, we present data on the intrinsic low-temperature luminescence characteristics of two differently prepared 124-kDa phytochrome preparations from etiolated oat seedlings. Optical detection of magnetic resonance (ODMR) spectroscopy (Maki & Zuclich, 1975; Kwiram, 1982) has also been employed in connection with phosphorescence and tetrapyrrole-delayed fluorescence to identify different chromophores involved in the emission. We arrive at conclusions which are at variance with those reached by Sarkar & Song (1982). In addition to the normal protein-emissive chromophores, tyrosine and tryptophan, new emissive chromophoric species, which become covalently associated with phytochrome during its isolation from oats, have been identified spectroscopically. We also show that these new emissive species, which may arise from phenolic modifications frequently encountered during protein purification from plants (Anderson, 1968; Loomis, 1974), are not always easily detected by absorption measurements on phytochrome.

Materials and Methods

Phytochrome from etiolated oat seedlings (*Avena sativa* L. cv Garry) was extracted and purified by two methods. Phytochrome prepared by the first method exhibited >95% purity and was shown to contain only 124-kDa (~70%) and 118-kDa (~30%) species of the chromoprotein when examined by $NaDodSO_4$–polyacrylamide gel electrophoresis (J. C. Litts, J. M. Kelly, and J. C. Lagarias, unpublished results). This phytochrome sample, which will be referred to as 124-kDa phytochrome, was prepared by utilizing hydrophobic chromatography, hydroxylapatite chromatography, and poly(ethylene glycol) fractionation and exhibited a SAR value[2] of 0.94 in 50 mM Tris-HCl buffer pH 7.8, which contained 25% (v/v) EG, 100 mM $(NH_4)_2SO_4$, and 1 mM EDTA. A second phytochrome sample, which showed a significantly increased absorbance near 320 nm but exhibited similar $NaDodSO_4$–polyacrylamide gel electrophoresis characteristics with the first sample, was prepared by utilizing the method of Litts (1980) with the following modifications: (1) the extraction buffer contained 7.2 mM ME and 3 mM PMSF, (2) all buffers used after the initial $(NH_4)_2SO_4$ fractionation contained only 3 mM PMSF and no ME, (3) a linear gradient from 0 to 300 mM NaCl in 50 mM Tris–phosphate buffer, pH 7.5, was utilized to elute phytochrome from the DEAE-Sepharose (CL-6B) column, and (4) the final phytochrome product was dialyzed into 50 mM Tris-phosphate buffer, pH 7.8, containing 25% (v/v) EG, 0.1 mM EDTA, and 14 mM ME just prior to spectroscopic measurements. The phytochrome prepared in this way exhibited a SAR value of 0.70. This second sample will be referred to as "covalently modified" 124-kDa phytochrome owing to the increased absorbance in the near-UV spectral region. In addition to these oat phytochrome samples, 124-kDa phytochrome from etiolated rye seedlings, purified with blue agarose affinity chromatographic procedures (Smith & Daniels, 1981), was kindly provided by Dr. W. O. Smith, Jr., for comparative purposes. Before phosphorescence measurements, the rye phytochrome was dialyzed into 25 mM Tris-HCl buffer, pH 7.8, containing 25% (v/v) EG. Phytochrome concentrations for these studies have been based on the extinction coefficient for the red absorption band of P_r ($\epsilon_{668} = 1.21 \times 10^5$ L mol^{-1} cm^{-1}) which was determined by quantitative amino acid analysis (J. C. Litts, J. M. Kelly, and J. C. Lagarias, unpublished results).

For the low-temperature luminescence and ODMR measurements, 10 μL of the phytochrome preparations at concentrations between 7 and 11 μM were transferred to 1 mm i.d. quartz tubes and immersed in liquid nitrogen (ca. 77 K) or liquid helium (ca. 4.2 K). The liquid helium was further pumped down to reach 1.1 K temperature for slow-passage ODMR measurements. The 25% (v/v) EG present in the buffer served as a cryogenic solvent for these low-temperature luminescence measurements.

Saturating illumination of phytochrome solutions was accomplished by using a slide projector lamp (Sylvania CZA, 500 W, 120 V) equipped with a red interference filter (660 nm, 10-nm band-pass; Ditric Optics) or a far-red plastic filter (FRS 700 Plexiglas, Rohm and Haas dye no. 58015, 0.125 in. thick) prior to freezing. All sample manipulations and spectroscopic measurements were performed under a dim fluorescence lamp equipped with one blue (no. 2424) and one green (no. 2092) Plexiglas filter.

The apparatus for measurements of 77 K phosphorescence spectra and lifetimes, 4.2 K fluorescence spectra, and slow-passage ODMR spectra at ca. 1.1 K have been described elsewhere (Maki & Co, 1976; Cha & Maki, 1982). The luminescence was passed through a McPherson, Inc., Model

[2] A criterion for purity frequently employed for phytochrome is the SAR (specific absorbance ratio) which is defined as the ratio of the absorbance of P_r at its absorption maximum in the red spectral region (ca. 668 nm) to the absorbance in the UV spectral region (280 nm) which corresponds to the apoprotein absorbance (Pratt, 1978).

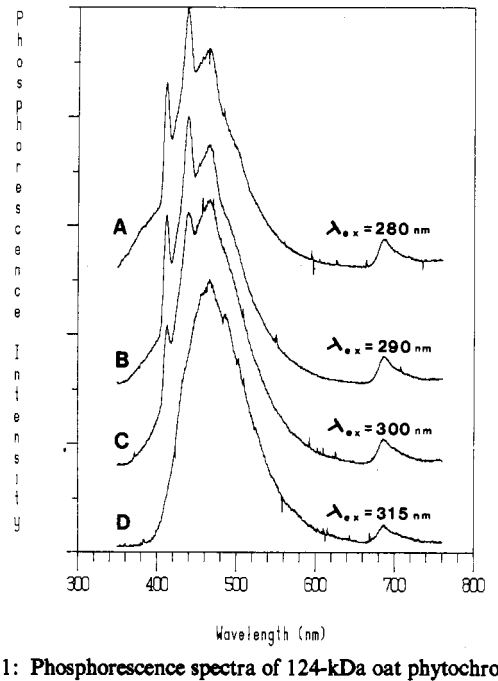

FIGURE 1: Phosphorescence spectra of 124-kDa oat phytochrome (P_r) at 77 K using differing excitation wavelengths. Excitation bandwidth is 16 nm. The phytochrome was dissolved in 50 mM Tris-HCl, 25% (v/v) EG containing 100 mM $(NH_4)_2SO_4$, and 1 mM EDTA, pH 7.8 measured at 4 °C. Sample concentration is 7 μM.

2051 monochromator with 3-nm slits and was detected with a cooled RCA, Inc., Model C31034 photomultiplier which has a relatively constant quantum efficiency out to 860 nm. Luminescence spectra reported are not corrected, however, for wavelength dependence of the grating efficiency or photomultiplier response. Phosphorescence decays were analyzed and deconvoluted by computer by using the procedure described previously (Maki & Co, 1976). The complex nonexponential decays were arbitrarily fit to a maximum of three exponential components. The analysis was considered satisfactory when differences between the theoretical decay curves and the experimental data were less than 5%. Slow-passage ODMR frequencies were obtained by averaging the peak frequencies observed with microwaves swept in both directions at the same scan rate.

Absorption spectra were made on a Hewlett-Packard Model 8450A UV/vis spectrophotometer. The sample compartment was modified for temperature control (at 4 °C) by utilizing a recirculating water–EG bath. Red and far-red actinic irradiations were accomplished with a Sylvania 24-V, 250-W EMM lamp and a red interference filter (660 nm, 10-nm band-pass; Ditric Optics) and a far-red plastic filter (FRS 700 Plexiglas, Rohm and Haas dye no. 58015, 0.125 in. thick).

Results and Discussion

Figure 1 shows the phosphorescence spectra of the 124-kDa phytochrome preparation (P_r) at 77 K excited at several wavelengths. The main features of the phosphorescence spectra of the phytochrome excited below 300 nm (Figure 1A,B) resemble Trp emission; both the 0,0 band and the vibronic structures are well resolved. In addition to the typical Trp phosphorescence, the shoulder below 400 nm is attributed to Tyr emission, and the peak at 684 nm is assigned to the delayed fluorescence of the tetrapyrrole chromophore of the phytochrome (see later discussion).

The Trp 0,0 band is located at 411 nm, indicating the hydrophobic environments of the emitting Trps (Purkey & Galley, 1970; Maki & Zuclich, 1975; Hershberger et al., 1980). Since there are 8–10 Trp residues in phytochrome

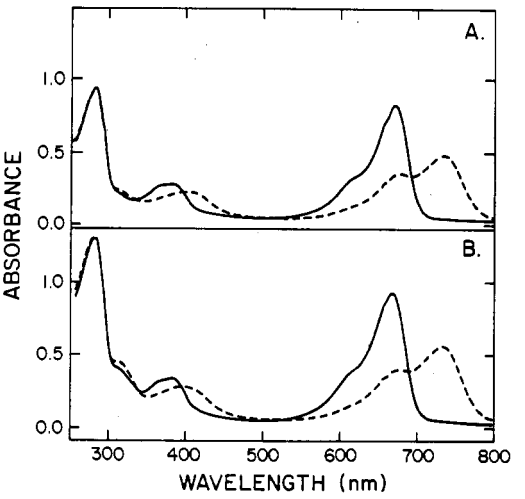

FIGURE 2: Absorption spectra of phytochrome. (A) 124-kDa oat phytochrome dissolved in 50 mM Tris-HCl–25% (v/v) EG buffer containing 100 mM $(NH_4)_2SO_4$ and 1 mM EDTA, pH 7.8 at 4 °C. (—) P_r; (---) P_{fr}. Sample concentration is 7 μM. (B) Covalently modified 124-kDa oat phytochrome dissolved in 50 mM Tris–phosphate–25% (v/v) EG buffer containing 0.1 mM EDTA and 14 mM ME, pH 7.8 at 4 °C. (—) P_r; (---) P_{fr}. Sample concentration is 11 μM.

samples which have been measured to date (Hunt & Pratt, 1980; Roux et al., 1982; J. L. Litts, J. M. Kelly, and J. C. Lagarias, unpublished results), the occurrence of only one Trp 0,0 band suggests that the local environments of the Trp sites which contribute to the emission are quite similar (Hershberger & Maki, 1980). One unusual feature is observed, however; phosphorescence in the region near 465 nm is intense and increases progressively as the excitation wavelength is shifted to the red. Meanwhile, the structured phosphorescence spectrum of Trp loses intensity. This finding suggests that a yet unidentified chromophore which will be referred to as chromophore X in the following text, contributes to the structureless emission peaking near 465 nm. The direct evidence for the presence of chromophore X phosphorescence is provided by observing the emission when the excitation wavelength is moved completely away from the region where the aromatic amino acid residues normally absorb. As shown in Figure 1D, a structureless new emission centered near 465 nm appears when phytochrome is excited at 315 nm.

The absorption spectra of 124-kDa P_r and P_{fr} phytochrome species are shown in Figure 2A. Although this phytochrome sample exhibits no obvious absorption maxima in the region between 300 and 350 nm, when excited in this region, the protein emits a unique phosphorescence which does not originate from normal aromatic amino acid residues (Figure 1D). A likely source of this emission involves phenolic modifications of the protein, which result from covalent attachment of quinone species to amino acid side chains, particularly lysine or cysteine. Such modifications frequently occur during plant protein extraction (Anderson, 1968; Loomis, 1974). The use of sulfhydryl reagents such as ME minimize these modifications by removing the quinone species through nucleophilic and/or reductive pathways. When the amount of ME in the crude extraction buffer is reduced, a significant increase in such modifications is observed. As shown in Figure 2B, this covalently modified phytochrome product exhibits enhanced absorbance at 320 nm. It is interesting to note that this modification does not otherwise change the absorption characteristics of phytochrome. The species responsible for the absorption at 320 nm is not chromatographically separable from the protein by gel filtration under denaturing conditions

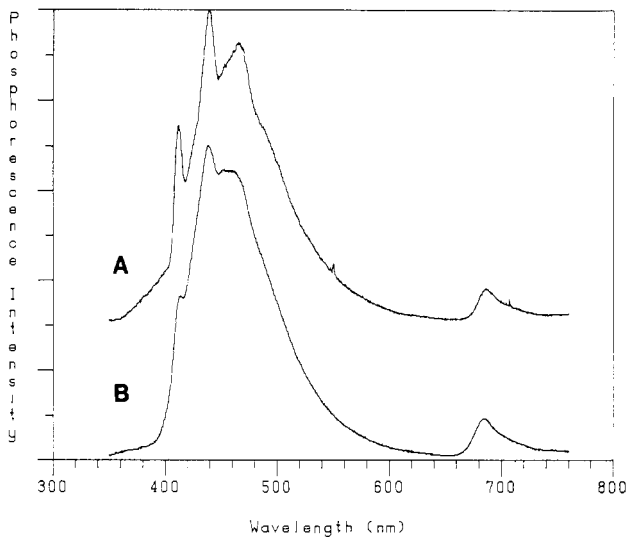

FIGURE 3: Phosphorescence spectra of phytochrome (P_r). Excitation is at 290 nm with 16-nm excitation bandwidth. (A) 124-kDa oat phytochrome (see legend to Figure 2A). (B) Covalently modified 124-kDa oat phytochrome (see legend to Figure 2B).

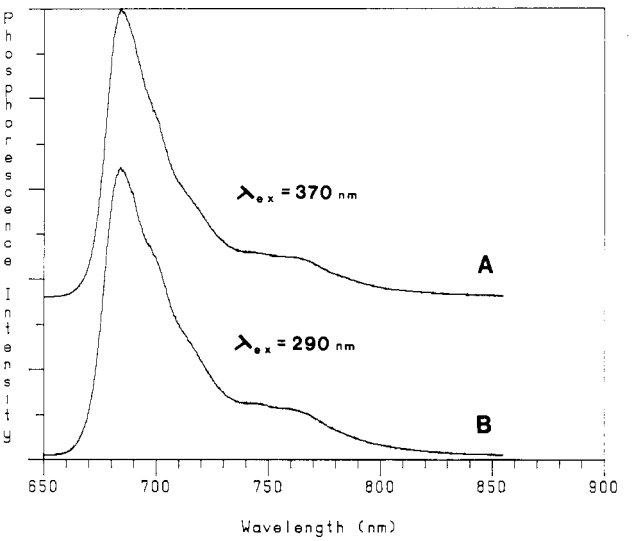

FIGURE 4: Fluorescence spectra of 124-kDa phytochrome (P_r) at 4.2 K. (A) Direct chromophore excitation at 370 nm (16-nm bandwidth). (B) Indirect excitation in protein band at 290 nm (16-nm bandwidth).

which indicates a covalent association with the protein (unpublished results). On the other hand, its contribution to the phosphorescence is enormous. Consistent with this interpretation are the observations of Hunt & Pratt (1980), who found that phytochrome isolated in the absence of ME contained four fewer reactive cysteines per monomer than the protein extracted in the presence of ME. In parts A and B of Figure 3, respectively, the phosphorescence spectra of 124-kDa phytochrome (P_r) and covalently modified phytochrome (P_r) are compared. As shown in Figure 3B, the emission at 465 nm originating from this near-UV absorbing species distorts the structure of the Trp phosphorescence spectrum to a great extent even when excited at 290 nm. Furthermore, the Trp 0,0 band is barely resolved in this spectrum. When this covalently modified phytochrome is excited at 315 nm, the unstructured phosphorescence centered at ca. 465 nm is obtained as well.

Sarkar & Song (1982) reported a phosphorescence spectrum of oat phytochrome purified by a protocol adapted from that of Smith & Daniels (1981). In that study, the reported phosphorescence spectrum resembles our emission spectrum observed from covalently modified phytochrome (Figure 3B). Although no distinct peak occurs at 320 nm in their reported absorption spectrum, the absorbance level throughout the near-UV region is significantly higher than that of the 124-kDa phytochrome species investigated in the present study.

In order to study further the generality of these spectral features, a rye phytochrome sample was examined. The rye phytochrome was purified to homogeneity by the blue agarose affinity chromatographic methods (Smith & Daniels, 1981), and it shows similar absorption and $NaDodSO_4$–polyacrylamide gel electrophoresis characteristics with the 124-kDa oat phytochrome examined in the present study (W. O. Smith, Jr., personal communication). When the 124-kDa rye phytochrome sample was excited at 315 nm, a similar emission spectrum as observed in the 124-kDa oat phytochrome sample was obtained, indicating the presence of a species similar to chromophore X. On the basis of these findings, it appears likely that covalent modifications of phytochrome may occur in all the currently employed purification methods. Hence, the presence of these species and their influence on the structural and physical properties of in vitro phytochrome preparations need to be carefully evaluated.

Owing to the significant absorption of phytochrome in the near-UV spectral region, the presence of a slight covalent modification of the protein may not be manifested by a distinct absorption peak near 320 nm. On the other hand, the characteristic emission maximum at 465 nm appears to be more sensitive in detecting low levels of such modifications. In order to ascertain whether the chromophore responsible for this 465-nm emission is an intrinsic property of phytochrome or is entirely an artifact of the isolation from plant tissue, alternative purification protocols are currently under investigation.

Song and co-workers have reported the direct and protein-sensitized fluorescence from the tetrapyrrole chromophore of phytochrome (Song et al., 1973, 1979; Sarkar & Song, 1981, 1982). The variation in reported fluorescence maxima observed in these reports indicates the sensitivity of the emission to sample integrity. The typical fluorescence spectra of 124-kDa phytochrome (P_f) are shown in Figure 4. The fluorescence maximum at 684 nm is invariant among several phytochrome samples which we have studied. Direct excitation of the tetrapyrrole chromophore at 370 nm (Figure 4A) and the excitation of the protein band at 290 nm (Figure 4B) give identical spectra, indicating that an efficient singlet–singlet energy-transfer process is in operation. An identical emission band with much reduced intensity is observed with lengthened lifetimes when the phosphorescence is recorded (Figure 1), indicating that a triplet–singlet energy-transfer process also is operative. In particular, the participation of chromophore X in this triplet–singlet energy-transfer process is evident from the observation that the delayed fluorescence appears with 315-nm excitation where Trp and Tyr do not absorb (Figure 1D). More evidence bearing on triplet–singlet energy transfer is provided by lifetime analyses and ODMR measurements (see below).

The phosphorescence spectrum of 124-kDa phytochrome in the P_{fr} form is quite similar to that of the P_r form. No delayed fluorescence from P_{fr} is observed. There is, however, a very weak delayed fluorescence with a maximum at 684 nm which indicates the presence of the residual P_r form in the photoequilibrium state. In addition, no immediate fluorescence except that expected from the residual P_r is observed. Since our evidence suggests that P_{fr} has a vanishing fluorescence quantum yield, the lack of delayed fluorescence from P_{fr} does not necessarily indicate that triplet–singlet energy-transfer

Table I: Phosphorescence and Delayed Fluorescence Decays of 124-kDa Oat Phytochrome at 77 K[a]

λ_{exc} (nm)	λ_{obsd} (nm)	P_r		P_{fr}	
		α (%)[b]	τ (s)	α (%)[b]	τ (s)
290	411	70	5.1	45	4.8
		30	1.2	42	1.5
				13	0.20
290	684	65	4.8		
		20	1.3		
		15	0.14		
315	465	12	1.1		
		21	0.17		
		67	0.04		
315	684	15	0.18		
		43	0.04		
		42	0.01		

[a] Phytochrome is dissolved in buffer as described under Materials and Methods. The deconvolution procedures for the lifetime analysis are described previously (Maki & Co., 1976). [b] Preexponential factor of lifetime component. Samples were excited for a period of about 5 times the longest lifetime component, so preexponential factors reflect photoequilibrium conditions for all emitting species.

processes are suppressed in P_{fr} as suggested by Sarkar & Song (1982). These authors report that the lifetime of the protein phosphorescence is longer in P_{fr} than in P_r. In contrast, our lifetime analysis (see below) indicates that a larger fraction of the phosphorescence intensity monitored at the Trp 0,0 band has shorter lifetimes in P_{fr} than in P_r. This result suggests that enhanced quenching of the Trp triplet state occurs in P_{fr} relative to P_r.

Table I lists the lifetimes of phytochrome at 77 K obtained by monitoring the Trp 0,0-band phosphorescence and the phosphorescence from chromophore X and by monitoring the delayed fluorescence peak of the tetrapyrrole chromophore at 684 nm. Nonexponential decays were observed in all cases. The phosphorescence decays of P_r, when the Trp 0,0 band is monitored with 290 nm excitation, deconvolute into a long component (5.1 s) representing Trp and a short component (1.2 s) representing Tyr as expected. The phosphorescence intensity from the X chromophore under these conditions contributes too insignificant a fraction to be analyzed. The phosphorescence lifetimes of chromophore X were determined by direct excitation at 315 nm. Its major component possesses a very short lifetime of ca. 40 ms.

The delayed fluorescence originating from the triplet–singlet energy-transfer route will possess lifetimes similar to the lifetimes of the donor (Maki & Co, 1976). When chromophore X is excited at 315 nm, the lifetimes of the delayed fluorescence are comparable to and even shorter than those obtained from chromophore X phosphorescence. This finding provides direct evidence for the participation of chromophore X in the triplet–singlet energy-transfer process. The lifetimes of the delayed fluorescence by using 290 nm excitation deconvolute into three components. The dominant fraction of the intensity (65%) emits with a lifetime of 4.8 s which is too long to be accounted for by Tyr or chromophore X. Therefore, the energy transfer from Trp to the tetrapyrrole chromophore is confirmed by this major lifetime component. The possibility of triplet–singlet energy transfer from Tyr to tetrapyrrole chromophore is suggested by the observation of an intermediate lifetime component (1.3 s) in the delayed fluorescence. This lifetime is too long to be accounted for by energy transfer from chromophore X, but the possibility of an efficient transfer process from Trp with a lifetime reduction to this value cannot be ruled out. These phosphorescence and delayed fluorescence lifetime analyses of P_r provide unambiguous evidence that at least Trp and chromophore X participate in the triplet–singlet energy-transfer processes, resulting in the appearance of delayed fluorescence from the tetrapyrrole chromophore. A significant portion of the energy transfer may be due to trivial processes, however, since Förster transfer processes (Förster, 1965) are expected to reduce the normal lifetime of the donor. We find that a large fraction of the delayed fluorescence decays with normal donor triplet lifetimes.

Sarkar & Song (1982) reported only a single lifetime component for the P_r phosphorescence (290 ms) and the delayed fluorescence (240 ms) and interpreted their data as the result of triplet–singlet energy transfer from Trp to the tetrapyrrole chromophore. A reduction of the Trp triplet state lifetime from ca. 5 s to ca. 300 ms by a triplet–singlet Förster energy-transfer mechanism would result in the quenching of all but about 5% of the normal phosphorescence intensity (Maki & Co, 1976). The actual phosphorescence quenching reported by Sarkar & Song (1982) is approximately 60% when a native P_r preparation is compared with the denatured species. This is inconsistent with their reported reduction of phosphorescence lifetime which would require quenching by about 95%. In fact, their reported lifetime corresponds roughly to the average lifetime of all the emitting species (Trp, Tyr, and chromophore X) obtained in our analysis. Therefore, any interpretation based on a single component lifetime requires critical reevaluation.

The phosphorescence lifetime analyses of P_r and P_{fr} forms of 124-kDa phytochrome are compared in Table I. There are significant differences in the decay profiles of P_r and P_{fr}. In general, the longest lived (Trp) component is reduced in relatively intensity in P_{fr} relative to P_r, and its lifetime is also reduced. The intermediate component increases in relative intensity, and a new, short-lived (ca. 200 ms) component appears. These changes are consistent with an enhancement of triplet–singlet energy-transfer efficiency in P_{fr} relative to P_r. An enhancement of energy-transfer efficiency from both Tyr and Trp to the tetrapyrrole of P_{fr} is expected on the basis of the spectral shift of the blue absorption band from λ_{max} ~380 nm in P_r to λ_{max} ~415 nm in P_{fr} (Figure 2A). A significantly larger spectral overlap is thus achieved between this absorption band and the dominantly Tyr and Trp phosphorescence spectrum shown in Figure 1B (λ_{exc} 290 nm). Such changes in transfer efficiency are to be expected without any reorientation of the donor or acceptor chromophores. The spectral overlap of the Trp and Tyr phosphorescence spectra with the tetrapyrrole red absorption band of P_r and P_{fr} is relatively minor and can be neglected in considering its contribution to energy transfer. The phosphorescence of chromophore X, however, is considerably red shifted relative to that of Trp and Tyr. Thus, its spectral overlap with both the blue band and red band of the tetrapyrrole must be considered, since significant overlap occurs with both of these bands.

Slow-passage ODMR techniques measure the microwave-induced transitions within the triplet-state manifold. The microwave frequencies measured correspond to the zero-field splittings of sublevels for a particular triplet state. For a triplet–singlet energy-transfer pair, it is possible to detect the magnetic resonance of the triplet donor by monitoring the delayed fluorescence of the energy acceptor, since the microwave-induced transitions which change the sublevel populations of the donor triplets may affect the acceptor fluorescence intensity by means of unequal transfer rates from the individual sublevels (Maki & Co, 1976). Table II lists some of the zero-field ODMR frequencies of 124-kDa phytochrome

Table II: Zero-Field ODMR Frequencies of 124-kDa Oat Phytochrome (P_r) by Monitoring the Phosphorescence and the Delayed Fluorescence[a]

λ_{exc} (nm)	λ_{obsd} (nm)	ν_1 (GHz)	ν_2 (GHz)	ν_3 (GHz)	assignment
315	465	1.4	3.2	4.5	chromophore X
315	684		3.3	4.3	chromophore X
290	411	1.7	2.7		Trp
			3.2	4.6	chromophore X
290	684		2.7		Trp
			3.2		chromophore X

[a] ODMR measurements were done at 1.1 K. The frequencies reported are the average of the peak frequencies observed with microwaves swept in both directions at the same scan rate.

(P_r) obtained by monitoring both the direct phosphorescence and the delayed fluorescence. In the present case, it is rather difficult experimentally to measure the zero-field ODMR frequencies by monitoring the delayed fluorescence, since there is a 1000-fold more intense immediate fluorescence background (Figure 4) which accompanies the delayed fluorescence. Nevertheless, after long accumulations using signal averaging, the ODMR frequencies could be measured, and their assignments are given in Table II.

When 124-kDa phytochrome is excited at 315 nm and the phosphorescence at 465 nm is monitored, three ODMR frequencies are obtained which are centered at ca. 1.4, 3.2, and 4.5 GHz. These frequencies do not correspond to the zero-field ODMR frequencies of either Trp or Tyr (Maki & Zuclich, 1975), supporting the idea that there is a new chromophore X present in addition to the aromatic amino acid residues. The two higher frequencies are also detected when delayed fluorescence is monitored under these conditions of excitation, indicating that chromophore X participates in the energy-transfer process. When 124-kDa phytochrome is excited at 290 nm and the Trp 0,0 band at 411 nm is monitored, two of the transitions corresponding to the chromophore X triplet state are obtained in addition to the two ODMR frequencies normally observed for Trp (Table II). In this case, only the intermediate (ν_2) frequencies of Trp and of chromophore X are detected when delayed fluorescence is monitored. These features, together with the findings in the phosphorescence spectra and the lifetime analysis discussed earlier, provide clear evidence that chromophore X, possibly generated from phenolic modifications, contributes to the phosphorescence and participates along with Trp in triplet–singlet energy transfer to the tetrapyrrole. The possibility that chromophore X type modifications occur as natural constituents of phytochrome in plants cannot be dismissed.

Added in Proof

The work referred to as J. C. Litts, J. M. Kelly, and J. C. Lagarias, unpublished results, has now been accepted for publication [see Litts et al. (1983)].

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Registry No. L-Tyrosine, 60-18-4; L-tryptophan, 73-22-3.

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