Short Communication

Photochemistry of 124 Kilodalton Avena Phytochrome In Vitro

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ABSTRACT

The photochemical properties of purified 124 kilodalton (kD) Avena cv Garry phytochrome are examined and compared with those of the proteolytically degraded 118/114 kD species. The proportion of the chromoprotein in the far red absorbing form, Pfr, following saturating red irradiation is 0.86 for 124 kD phytochrome, substantially higher than the values of 0.79 determined here and 0.75 reported in the literature for 118/114 kD preparations. The ratio of the quantum yields for Pr to Pfr phototransformation and for Pfr to Pr phototransformation (φPr/φPfr) is 1.76 for the 124 kD molecule and 0.98 for the 118/114 kD species. Based on extinction coefficients determined using the Lowry assay as a measure of protein weight, the individual phototransformation quantum yields for 124 kD phytochrome are 0.17 for Pr → Pfr (φPr) and 0.10 for Pfr → Pr (φPfr). Comparison of these quantum yields with those of the 118/114 kD species (where φPr = φPfr = −0.11) indicates that proteolytic degradation of the 124 kD molecule to the 118/114 kD species significantly affects only φPfr. Therefore, the lower proportion of Pfr at photoequilibrium observed for 118/114 kD preparations is explained mainly in terms of a reduced efficiency of Pr → Pfr phototransformation. The absolute Pfr absorbance spectrum for 124 kD phytochrome obtained by correcting the measured spectrum for residual Pr exhibits a maximum at 730 nm and differs from previous absolute Pfr spectra for both ‘120’ kD and 60 kD phytochrome in that it lacks a shoulder in the red region of the spectrum.

Phytochrome can exist in two photoconvertible forms, Pr and Pfr, with Pfr generally considered to be the physiologically active form (9, 13, 15). A common experimental approach in phytochrome physiology involves attempts to correlate quantitatively the magnitude of a particular response with the spectrophotometrically measured level of Pfr. Because the absorbance of Pr and Pfr overlap in the red spectral region, saturating red light irradiation establishes a photoequilibrium mixture containing both Pr and Pfr. Therefore, when difference spectroscopy is used to determine the absolute amount of Pr and/or the ratio of Pr and Pfr produced by a specific irradiation, the proportion of total phytochrome present as Pfr following saturating red irradiation ([Pfr]Pr=565) must be known. As stated previously (1, 2), [Pfr]Pr=565 may be determined only for phytochrome in vitro in the absence of other pigments absorbing in the red and far red spectral regions and the results then extrapolated to experiments involving crude extracts or intact tissue (for examples, see Refs. 3, 4, 9, 10, 15). For this reason, it is important that such in vitro measurements be made on undegraded, undenatured phytochrome. The [Pfr]Pr=565 for Avena phytochrome was first calculated to be 0.81 for the proteolytically degraded 60 kD species (2) and later reported to be 0.75 for the ‘120’ kD species at that time considered to be undegraded (11, 12). However, recent observations have demonstrated that the 118 and 114 kD chromoproteins that comprise 120 kD Avena phytochrome preparations are artifically derived by limited proteolysis during purification from a larger 124 kD molecule (17). Similar proteolytic degradation also occurs with rye, corn, pea, and zucchini phytochrome (Vierstra, Cordonnier, Pratt, and Quail, unpublished; 6). Because this proteolysis alters the spectral properties of phytochrome (18), previous [Pfr]Pr=565 determinations made using the 120 kD species are not likely to represent those for phytochrome in vivo. Comparison of the absorbance spectra of purified 124 kD preparations with those of 120 kD preparations reported in the literature (11, 19) suggests that the [Pfr]Pr=565 for 124 kD phytochrome is higher than 0.75.

In this paper, the [Pfr]Pr=565 and quantum yields for phototransformation (φPr, φPfr) for purified 124 kD phytochrome are presented and compared with those for the 118/114 kD species.

MATERIALS AND METHODS

Phytochrome Preparations. The 124 kD phytochrome was purified from etiolated Avena sativa cv Garry seedlings using the modified Affi-Gel Blue purification protocol of Vierstra and Quail (19). Briefly, this procedure includes: homogenization of the tissue with the phytochrome as Pfr in a buffer containing 50% ethylene glycol and 4 mM phenylmethylsulfonylfluoride, followed by polyethyleneimine and ammonium sulfate precipitation, hydroxypatite chromatography, Affi-Gel Blue affinity chromatography, and gel filtration. All 124 kD preparations used had A665/A260 ratios of 0.92 or greater, Pfr maxima at 730 nm and spectral change ratios (ΔA730/ΔMfr) of 1.06 to 1.08. These preparations were determined by SDS-PAGE (7) to contain exclusively the 124 kD species.

Phytochrome preparations containing the degraded 118/114 kD species (as determined by SDS-PAGE) were prepared by immunoaffinity chromatography as described by Hunt and Pratt (5). These preparations had A665/A260 ratios between 0.79 and 0.83, Pfr absorbance maxima at 724 nm, and spectral change ratios between 1.16 and 1.28. For all experiments, phytochrome prepared by both procedures was dissolved in 100 mM K-phosphate, 5 mM Na4EDTA, and 14 mM 2-mercaptoethanol (pH 7.8). The A of Pr at 665 nm and of Pfr at 750 nm was maintained between 0.1 and 0.2.

Photochemical Measurements. Phytochrome was phototransformed from Pr to Pfr and Pfr to Pr by irradiating the sample at 2°C in a 1-cm pathlength cuvette. Actinic red or far red light was provided by a slide projector with either a 665 nm (10 nm half band width) or 730 nm (9 nm half band width) interference filter (Baird Atomic, PTR Optics, Waltham, MA). Fluence rates were attenuated with neutral density filters, and times of irradiation
were controlled by a camera shutter. The actinic fluence rates were measured with a YSI Radiometer and corrected for internal filtering within the sample as described by Pratt (11). IR light (λ > 800 nm) comprised less than 1% of the total fluence. The corrected actinic fluence rates were between 2.8 and 3.2 × 10⁻¹⁰ mol cm⁻² s⁻¹ for both 665 and 730 nm light. The initial rates of phototransformation were determined from absorbance spectra of the phytochrome recorded with a Perkin Elmer model 557 spectrophotometer after various times of irradiation. The initial rates of phototransformation in either direction were linear for at least the first 10% of total phototransformation. The values for [Pfr]₂⁰⁸₀, φ/Pfr, φr, and φfr were determined using the equations derived by Butler et al. (2) and used subsequently by Pratt (11). The 124 kD phytochrome preparations gave first-order kinetics in both directions for phototransformations of at least 80% indicating that the equations described by Butler et al. (2) are applicable to the 124 kD species.

**Protein Determinations.** Total protein was determined according to the method of Lowry et al. (8) using BSA as a standard.

**RESULTS**

Butler et al. (2) first recognized that a portion of the shoulder at 673 nm in the absorbance spectrum obtained after saturating red irradiation results from residual Pr remaining at photoequilibrium, and they derived equations to estimate this contribution of Pr. The height of this shoulder relative to the height of the Pfr far red maximum is lower for 124 kD than for 118/114 kD phytochrome preparations (Fig. 1). One possible reason for this observation is that 124 kD phytochrome has a higher [Pfr]₂⁰⁸₀ than its degraded counterpart. This suggestion is directly confirmed using the equations derived by Butler et al. (2) to calculate φ/Pfr and [Pfr]₂⁰⁸₀ from the initial rates of phototransformation (Table I). Both φ/Pfr and [Pfr]₂⁰⁸₀ for 124 kD phytochrome (1.76 and 0.862, respectively) are substantially higher than those determined here for the 118/114 kD species (0.98 and 0.791, respectively).

The absolute absorbance spectrum for the Pfr form of 124 kD phytochrome has been calculated from the spectrum obtained at photoequilibrium in red light assuming a [Pfr]₂⁰⁸₀ of 0.86 (Fig. 2). The residual 14% Pr was subtracted from the experimental curve and the resulting curve multiplied by 1/0.86 to produce the spectrum that would have resulted if the photoconversion of Pr to Pfr were complete (i.e. [Pfr]₂⁰⁸₀ = 1.0). The ratio of the absolute Pfr A at 730 nm to the Pr A at 666 nm is 0.68 (Fig. 2). This value can be used to calculate the Pfr extinction coefficient at 730 nm once the Pr extinction coefficient at 666 nm is known.

One absorbance unit at 666 nm of purified 124 kD phytochrome (A₆₆₆/₆₆₆ = 0.97) contains 1.7 mg protein as determined by the Lowry assay. Based on this value and a mol wt of 124,000, the extinction coefficient for Pr at 666 nm is 7.3 × 10⁻⁹ cm² mol⁻¹ and for Pfr at 730 nm is 4.9 × 10⁻⁹ cm² mol⁻¹ assuming a [Pfr]₂⁰⁸₀ of 0.86. The extinction coefficients for the 118/114 kD preparations (A₆₆₆/₆₆₆ = 0.80) determined similarly are 7.5 × 10⁻⁹ cm² mol⁻¹ for Pr and 4.7 × 10⁻⁹ cm² mol⁻¹ for Pfr assuming a [Pfr]₂⁰⁸₀ of 0.79 and an average mol wt of 116,000. This average mol wt was used because we have observed by SDS-PAGE that 120 kD preparations routinely contain equimolar amounts of the 118 and 114 kD chromopeptides.

Using the above extinction coefficients, the absolute quantum yields for phototransformation (φ/Pfr, φfr) were calculated as described by Butler (1). The φ/Pfr determined for 124 kD phytochrome is significantly higher than the value for the 118/114 kD species whereas the φfr for the two preparations are similar (Table I). Recently, a Pr extinction coefficient based on the Lowry assay of 8.6 × 10⁻⁹ cm² mol⁻¹ (recalculated here to correspond to an average mol wt of 116,000) has been reported (14) for highly purified 120 kD phytochrome (A₆₆₆/₆₆₆ = 0.87). If this value is employed, the φ/Pfr and φfr for 118/114 kD phytochrome are 0.10 and 0.11, respectively.

**DISCUSSION**

The 124 kD phytochrome from *Avena* has substantially higher values for the φ/Pfr ratio and for [Pfr]₂⁰⁸₀ than obtained for the
Table 1. Photochemical Characteristics of 124 and 118/114 kD Avena Phytochrome

| Phytochrome Preparation | \[\text{\text{Pfr}}]^{\text{[0]}} \times 10^4 | \text{\text{Pfr}} | \text{\text{Pfr}} | \text{\text{Pfr}} |
|--------------------------|-----------------|--------|--------|
| 124 kD \text{[a]}       | 1.76 ± 0.10     | 0.862  | 0.17   |
| 118/114 kD \text{[b]}    | 0.98 ± 0.02     | 0.791  | 0.11   |

* Average of four independent determinations ± SD.
* Average of three independent determinations ± SD.

Partially degraded 118/114 kD species (Table I; Ref. 11). Because the 124 kD species is apparently undergraded and has spectral properties similar to those of phytochrome in vivo (17, 18), the photochemical properties of this molecule should more closely represent the actual properties in vivo. However, inasmuch as these measurements cannot be made for phytochrome in vivo, the possibility always exists that the values reported here may slightly differ from those in vivo (1, 2).

Difference spectra (Pr−Pfr) for rye, corn, pea, and zucchini phytochrome measured in vivo or after the tissue was homogenized under conditions that preclude phytochrome photochemistry (19) are similar to those obtained for 124 kD Avena phytochrome (i.e. difference minima at 730 nm and spectral change ratios near unity [Vierstra and Quail, unpublished]). Although not conclusive, this similarity suggests that the higher [Pfr] \text{[a]} observed here for Avena phytochrome may be a general characteristic of undergraded phytochrome. Yamamoto and Smith (20) have also reported similar high values for 0.75 and [Pfr] \text{[b]} (1.53 and 0.84, respectively) for rye phytochrome. Although the mol wt of the phytochrome in their preparations was not well characterized, the spectral properties suggest that the polypeptide was not extensively degraded (see 18). On the other hand, because rye phytochrome is also susceptible to the same limited proteolytic degradation during purification as observed with Avena (Vierstra, Cordonnier, Pratt, and Quail, unpublished; 6), its photochemical properties may require reexamination after the molecule has been isolated under conditions that preclude this degradation.

The most prominent feature of the absolute Pfr spectrum for 124 kD phytochrome is the absence of a shoulder in the red spectral region. This shoulder is also absent from the absolute Pfr spectrum of our 118/114 kD preparations determined similarly using a [Pfr] \text{[c]} of 0.79 (unpublished). These spectra contrast with previously reported absolute Pfr spectra for 120 and 60 kD phytochrome where in each case such a shoulder was present (2, 12). It is possible that the Pfr form of these earlier phytochrome preparations did indeed contain a Pfr shoulder or that the previous [Pfr] \text{[d]} values used to calculate the absolute Pfr spectra underestimated the contribution of Pr to the absorbance spectrum obtained at photoequilibrium in red light.

The extinction coefficients used here to calculate \(\text{Pfr}\) and \(\text{Pfr}\) are based on a colorimetric estimation of total protein (8) using BSA as a standard. The value of \(7.5 \times 10^4 \text{ cm}^2 \text{ mol}^{-1}\) for the Pr form of 118/114 kD phytochrome agrees well with previous determinations using the Lowry assay for phytochrome preparations of similar purity (14, 16). However, Roux et al. (14) have reported that the Lowry assay significantly overestimates Avena phytochrome protein content and therefore its use would underestimate extinction coefficients. Using quantitative amino acid analysis as a more accurate measure of total protein, they calculated a substantially higher Pr extinction coefficient for the 118/114 kD species (recalculated here to be 9.9 \(\times 10^4 \text{ cm}^2 \text{ mol}^{-1}\), assuming an average mol wt of 116,000). Inasmuch as a similar analysis is not available for 124 kD phytochrome, comparison of quantum yields for the two mol wt preparations requires the use of the less accurate determination of extinction coefficients employed here.

Comparison of the \(\text{Pfr}\) and \(\text{Pfr}\) for the 124 and 118/114 kD preparations indicates that photochemistry of 124 kD phytochrome to 118/114 kD significantly affects only \(\text{Pfr}\). A reduced efficiency for \text{Pr} → \text{Pfr} phototransformation would at least partially explain the lower \([\text{Pfr}]^{\text{[e]}}\) obtained for 118/114 kD phytochrome. Because this photochemistry also shifts the Pfr absorbance spectrum to shorter wavelengths (18), the greater degree of spectral overlap between the Pr and Pfr forms of the degraded species may also contribute to the reduced \([\text{Pfr}]^{\text{[e]}}\). Further photochemistry of 118/114 kD phytochrome to a 60 kD chromoprotein appears to increase \(\text{Pfr}\) and \([\text{Pfr}]^{\text{[f]}}\) to values intermediate between those for 124 and 118/114 kD preparations (2, 11). Both \(\text{Pfr}\) and \(\text{Pfr}\) may be affected by this later cleavage (11).

Several photochemical properties of 118/114 kD phytochrome determined here differ significantly from those previously described. A \([\text{Pfr}]^{\text{[g]}}\) of 0.79 was obtained here versus 0.75 reported by Pratt (11) even though identical values for \(\text{Pfr}\) and \(\text{Pfr}\) were employed. This discrepancy appears to result primarily because the value \(A_{\text{max}}/A_{\text{max}}\) used to calculate \([\text{Pfr}]^{\text{[h]}}\) is different in the phytochrome preparations used by Pratt and ourselves (see Fig. 1 and Ref. 11) and may reflect true differences in \([\text{Pfr}]^{\text{[i]}}\). We have obtained a value of 0.11 using the 118/114 kD species for both \(\text{Pfr}\) and \(\text{Pfr}\) which is in contrast to the value of 0.17 determined previously (11). We do not fully understand the reasons for this later difference although several factors may play a cumulative role. These factors include: the aforementioned use of phytochrome preparations that have markedly dissimilar absorbance spectra at photoequilibrium in red light and may also have had differing degrees of degradation; calculation of \(\text{Pfr}\) and \(\text{Pfr}\) using different values for \([\text{Pfr}]^{\text{[j]}}\) and for extinction coefficients; and the use of different methodologies in each case to determine the initial rates of phototransformation.

The data presented here provide further evidence that the 6 to 10 kD peptide segment(s) proteolytically cleaved from the 124 kD molecule are critical in maintaining the molecular integrity of native phytochrome. Understanding how these segment(s) affect \text{Pr} → \text{Pfr} phototransformation, alter the Pfr absorbance spectrum and stabilize Pfr against dark reversion, (see 19) and why they are preferentially cleaved in the Pr form may provide insights concerning the molecular nature of phytochrome action.

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