Tertiary Structure of Phytochrome Probed by Quasi-Elastic Light Scattering and Rotational Relaxation Time Measurements†

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ABSTRACT: Quasi-elastic light scattering and rotational relaxation time measurements were used to examine whether or not there is a significant gross conformational difference between the Pr and Pfr forms of large (118 kdalton) phytochrome. In quasi-elastic light scattering studies, the observed diffusion coefficients (D) for Pr and Pfr and, hence, their Stokes radii (R_h) were found to be nearly indistinguishable ($D_{20,w} = 2.7 \times 10^{-7}$ cm^2/s; $R_h = 81$ and 82 Å for large Pr and Pfr, respectively). The Stokes radii for small Pr and Pfr (60 kdalton) were also found to be similar (50 and 49 Å, respectively). A preliminary study with intact phytochrome (124 kdalton) also showed no significant differences between the Pr and Pfr diffusion coefficients (1.76×10^{-7} and 1.82×10^{-7} cm^2/s, respectively) in 10 mM potassium phosphate buffer, pH 7.8, containing 5% glycerol. Rotational relaxation time measurements with ANS as the fluorescence probe could not distinguish between the Pr and Pfr forms (rotational relaxation times are 103.5 and 104.1 ns, respectively) of large phytochrome. Lower values for the observed rotational relaxation times of large phytochrome labeled with fluorescamine as the fluorescence probe seemed to be due to the conformational flexibility of the protein domain to which the probe is covalently linked and/or due to the local rotational freedom of the probe.

The ubiquitous plant photoreceptor for red light mediated photomorphogenesis, phytochrome, converts from the biologically inactive Pr[1] form to the biologically active Pfr form after red light irradiation. The Pfr form can revert to the Pr form either by absorbing far-red light or in the dark as shown in the following scheme:

$$\mathrm{Pr} \underset{730\ \text{nm or dark}}{\overset{660\ \text{nm}}{\rightleftharpoons}} \mathrm{Pfr}$$

Red light irradiation seems to cause no drastic changes in the conformation/configuration of the chromophore of the Pr form upon phototransformation (Song et al., 1979; Song & Chae, 1979). The secondary structure of the large phytochrome ($M_r \sim 118\,000$)[2] also is essentially identical, as measured by CD spectroscopy (Tobin & Briggs, 1973; Song et al., 1979; Hunt & Pratt, 1980). In addition, attempts to differentiate between the Pr and Pfr forms of large, degraded phytochrome by immunochemical methods were negative (Pratt, 1973; Rice & Briggs, 1973; Cundiff & Pratt, 1975). Gel filtration and electrofocusing methods also could not detect any differences in the behavior of the Pr and Pfr forms (Rice & Briggs, 1973; Briggs & Rice, 1972).

On the basis of differential reactivities of Pr and Pfr toward certain chemicals, protein conformational changes upon phototransformation were suggested (Pratt, 1982). However, a large protein conformational change may be ruled out on the basis of these limited accessibility studies (Song et al., 1979; Smith, 1981). Exposure of a new surface area (hydrophobic in nature) on the Pfr protein has been suggested (Hahn & Song, 1981; Smith, 1981; Yamamoto & Smith., 1981). A mechanism for phototransformation due to the reorientation of the chromophore, thereby uncovering the binding site surface area, has also been proposed (Song et al., 1979; Hahn & Song, 1980; Sarkar & Song, 1981; Song, 1983). Support for the chromophore reorientation has been provided by Sundqvist & Björn (1983), who showed that there are linear dichroic differences between the transition dipoles of the longest wavelength absorbing bands of Pr and Pfr. Thus, it is critical to examine whether or not there are any significant tertiary structural differences between these forms. We have used two very conformationally sensitive techniques, quasi-elastic scattering (Berne & Pecora, 1976; Bloomfield & Kim, 1978; Chu, 1974; Bloomfield, 1981) and rotational relaxation time measurements (Weber, 1953), to explore the problem. In this paper, we report the results obtained on the basis of the above-mentioned techniques on the gross conformational differences and/or shapes of the Pr and Pfr forms of phytochrome.

Materials and Methods

Phytochrome Isolation and Purification. Large phytochrome (M_r 118 000) was isolated from dark-grown etiolated oat tissues (*Avena sativa L.*, Garry oats, Whitney-Dickinson Seeds, Inc., Buffalo, NY) as described earlier (Song et al., 1981; Sarkar & Song, 1982). Final preparations of the large molecular weight phytochrome for quasi-elastic light scattering studies included either 0.1 M sodium phosphate buffer, pH 7.8, containing 50 mM KCl or 0.05 M sodium phosphate buffer, pH 7.8, after fractionation with ammonium sulfate and followed by passage through a small G-75 column (1.5 × 20 cm) equilibrated with the same buffer to remove the residual ammonium sulfate. Undegraded intact phytochrome was prepared by the method of Vierstra & Quail (1983a), and the final solution was in 0.1 M potassium phosphate buffer, pH 7.8, containing 5% glycerol. For the small molecular weight phytochrome (M_r 60 000), purified large molecular weight phytochrome was treated with 1 μg of trypsin/mg of phyto-

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Trp, tryptophan; Tyr, tyrosine; CD, circular dichroism; ANS, 8-anilinonaphthalene-1-sulfonate; Pr, red light absorbing form of phytochrome; Pfr, far-red light absorbing form of phytochrome; SAR, specific absorbance ratio (A_{660}/A_{280}); SDS, sodium dodecyl sulfate.

[2] Notations used for the different forms of oat phytochrome are as follows: intact phytochrome, M_r 124 000 phytochrome; large phytochrome, M_r 118 000 phytochrome resulting from proteolysis of the intact protein; small phytochrome, M_r 60 000 phytochrome resulting from proteolysis of large phytochrome.

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chrome and was incubated at 4 °C for about 15 h. This was then followed by treatment with trypsin inhibitor (1 μg of trypsin inhibitor/mg of protein) for 30 min, centrifugation, and application to an Affi-Gel blue column (Bio-Rad) equilibrated with 0.1 M sodium phosphate buffer, pH 7.8, containing 0.5 M KCl. Elution of small phytochrome from this column was done with 0.1 M sodium phosphate buffer, pH 7.8, containing 0.5 M KCl after it was washed with about 3 column volumes of equilibration buffer. Pooled fractions were collected, fractionated with ammonium sulfate, and then passed through a Bio-Gel A 1.5-m column equilibrated with 0.1 M sodium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 50 mM KCl. Phytochrome preparations of SAR values ≥ 0.84, 0.84, and 1.18 for intact, large, and small phytochrome, respectively, were used for these studies. The molecular weights of the intact, large, and small phytochromes used in these studies were found to be about 124 000 (on 5% polyacrylamide), 118 000, and 60 000 (on 7.5% polyacrylamide), respectively, by gel electrophoresis following the method of Laemmli (1970). All preparations were found to be more than 95% pure on SDS–polyacrylamide gel electrophoresis.

Analytical gel chromatography was run with large phytochrome on a Bio-Gel A 1.5-m column (1.5 × 110 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7.83, at 4 °C. The flow rate of the column was maintained at 15 mL/h. The gel was calibrated with blue dextran ferritin (M_r 540 000), catalase (M_r 240 000), hemoglobin (M_r 68 000), and sperm whale metmyoglobin (M_r 20 000). Large phytochrome, with $A_{660} \sim 0.14$ in 0.05 M sodium phosphate buffer, pH 7.83, was applied to this column for molecular weight determination. Phytochrome concentration calculations were based on the extinction coefficients $\epsilon_{660} \sim 7.0 \times 10^4$ cm^{-1} M^{-1} for large (Tobin & Briggs, 1973) and $\epsilon_{666} \sim 7.3 \times 10^4$ cm^{-1} M^{-1} for intact phytochrome (Vierstra & Quail, 1983b).

Quasi-Elastic Light Scattering. Quasi-elastic light scattering studies were performed as described by Amis et al. (1981). In a typical measurement, phytochrome solutions were filtered through a 0.05-μm pore polycarbonate membrane filter (Nuclepore) just before use. Photon counting autocorrelation spectroscopy was carried out with an argon ion laser (Lexel, Model 75-.2) and a 64-channel correlator (Model 7025, Malvern Instruments, Ltd., Malvern, Worcestershire, England) interfaced to an Apple II microcomputer system (Apple Computer Inc., Cupertino, CA). Data analysis using a nonlinear regression routine to fit the observed correlation function to a single exponential and a second-order cumulant expansion was performed on a Harris/7 computer.

The normalized autocorrelation function, $g^2(\tau)$, for quasi-elastic light scattering from a solution of identical, freely diffusing particles is given by (Berne & Pecora, 1976; Koppel, 1972)

$$g^2(\tau) = 1 + Be^{-2\Gamma\tau}$$

where B is an instrument constant and Γ is the relaxation rate. The relaxation rate, Γ, is related to the diffusion coefficient, D, by

$$\Gamma = \kappa^2 D$$

where κ is the scattering vector. κ is a known experimental quantity and is given by

$$\kappa = \frac{4\pi n}{\lambda_0} \sin \frac{\theta}{2}$$

where λ_0 is the vacuum wavelength of the incident light, n is the refractive index of the solvent, and θ is the scattering angle. The measured translational diffusion coefficient can be interpreted in terms of Stokes radius, R_h, defined by the Stokes–Einstein equation:

$$R_h = kT/(6\pi\eta D)$$

where η is viscosity in poise. Since D is inversely proportional to R_h, the quasi-elastic light scattering technique is a sensitive probe of conformational transitions.

Rotational Relaxation Time Measurements. The rotational relaxation time of a molecule can be related to its fluorescence polarization by Perrin's equation (Perrin, 1926):

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau_F}{\rho_h}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{RT\tau_F}{V\eta}\right)$$

where ρ_h is the harmonic mean of the rotational relaxation time of the molecule and τ_F is the mean radiative fluorescence lifetime of the fluorophore. P, the polarization degree, is defined as

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$$

where $I_\parallel$ and $I_\perp$ are the intensities of the components of the fluorescent light polarized parallel and perpendicular to the direction of polarization of the incident radiation and P_0 is the fluorescence polarization in the absence of molecular Brownian rotation. ρ_h is defined as (for a rigid sphere)

$$\rho_h = \frac{3\eta V}{RT} = \rho_0$$

where η is the viscosity (in poises) of the medium, V is the molar volume, R is the gas constant, and T is the absolute temperature. The ratio ρ_h/ρ_0 gives an estimate of the degree of deviation from the spherical, globular shape.

Perrin's plots $(1/P - 1/3)$ vs. T/η are useful in the determination of ρ_h (Weber, 1953) and can be obtained by measuring polarization degrees as a function of temperature or by varying the viscosity of the medium under isothermal conditions (e.g., by addition of sucrose or glycerol). Extrapolation of the plot gives a value of $1/P_0 - 1/3$, and the rotational relaxation time is calculated from the slope as described by Weber (1953).

Dynamic fluorescence depolarization measurements were made by measuring differential polarized lifetime ($\Delta\tau$) as described earlier (Weber, 1977; Lakowicz et al., 1979; Weber & Mitchell, 1976; Koka & Song, 1977) and using the equation

$$\alpha^2 + \frac{2\alpha}{1 - P_0^2}\left(1 - \left|\frac{P_0}{\tan\Delta}\right|\omega\tau_F\right) + \frac{(1 + \omega^2\tau_F^2)}{1 - P_0^2} = 0$$

where $\alpha = \tau_F m/\rho$, P_0 is the limiting polarization, $m = (3 - P_0)/(1 - P_0^2)$, and ω is the circular frequency (2π times modulation frequency f). The tangent of the differential phase delay, tan Δ, is given by

$$\tan\Delta = 2\pi f\Delta\tau = \omega\Delta\tau$$

where the differential lifetime $\Delta\tau$ is the value measured directly as the difference between parallel and perpendicular decay components. Polarization, τ_F, and $\Delta\tau$ were measured by using an SLM phase-modulation spectrofluorometer as described earlier (Fugate & Song, 1976; Lakowicz et al., 1979; Koka & Song, 1977).

Fluorescence Probe Conjugation. Fluorescamine (Fluram, Roche) was dissolved in spectroscopic grade acetone, and an

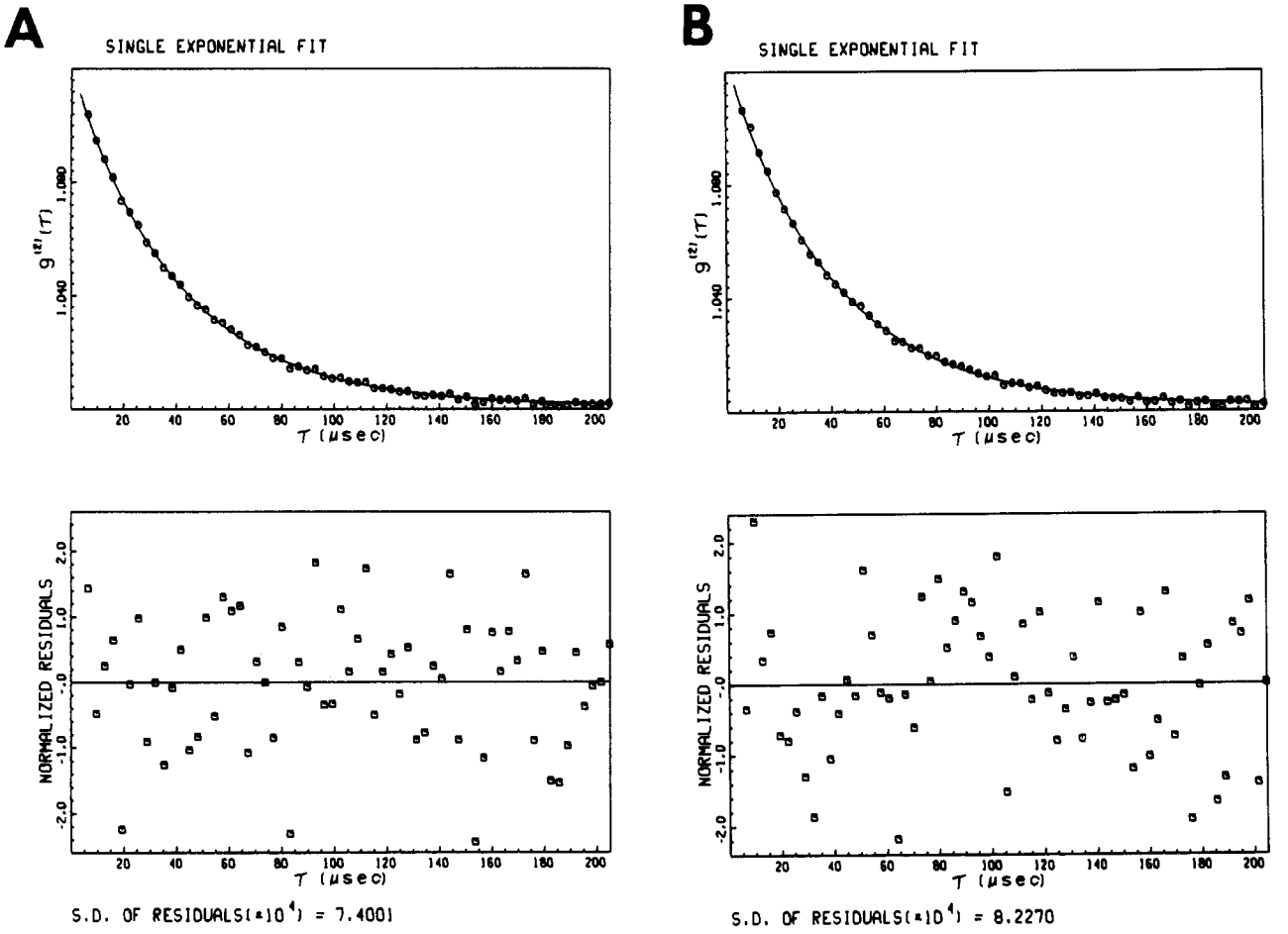

FIGURE 1: Normalized autocorrelation function $g^2(\tau)$ of (A) Pr and (B) Pfr forms at a concentration of 0.2 mg/mL and at an 80° scattering angle. The measurement was carried out in 0.05 M NaP_i buffer, pH 7.8, at 287 K. In the lower portions of these plots, normalized residuals were plotted in units of the standard deviation of residuals of the observed relative to the fitted values of $g^2(\tau)$ at each τ.

appropriate amount of it was mixed with the phytochrome solution to make a 1:3 binding ratio of phytochrome and fluorescamine (Undenfriend et al., 1972). The final concentration of acetone in the mixture was less than 0.2%. Since both free fluorescamine and its hydrolyzed product are nonfluorescent (Udenfriend et al., 1972), the mixture was used for polarization measurement without any further treatment.

An appropriate amount of purified ANS, 8-anilinonaphthalene-1-sulfonate, (Hahn & Song, 1981), was added to the Pr form (final molar ratio was 1:10) in 0.05 M NaP_i buffer, pH 7.8, containing 10% glycerol and incubated overnight at 4 °C. The same solution was used for rotational relaxation time measurements on both Pr and Pfr proteins without any further treatment.

Results

Figure 1 displays the normalized autocorrelation function, $g^2(\tau)$, of Pr and Pfr at a concentration of about 0.2 mg/mL at 80° scattering angle with 488.0 nm light. The solid curves in the upper portions of Figure 1 represent single exponential fits according to

$$g^2(\tau) = 1 + Be^{-2\Gamma\tau}$$

and in the lower portions, normalized residuals are plotted in units of the standard deviation of residuals of the observed relative to the fitted values of $g^2(\tau)$ at each τ. Evidently, both indicate a good fit to the model function.

Calculated relaxation rates, Γ, were obtained with different scattering angles for both Pr and Pfr, and the results were plotted against κ^2. Figure 2 shows the Γ vs. κ^2 plots of Pr and Pfr, respectively, according to the equation $\Gamma = D\kappa^2$, where D is the translational diffusion coefficient. A good linear least-squares fit is indicated in these plots, and slopes of the straight lines in both the plots yielded an identical diffusion coefficient, $D = 2.70 \times 10^{-7}$ cm^2 s^{-1}. Diffusion coefficients of Pr and Pfr were further examined, and the results are

Table I: Diffusion Coefficients and Stokes Radii of Large and Small Phytochromes As Obtained from Quasi-Elastic Light Scattering Studies[a]

	sample	$D(\times 10^{-7}$ cm^2/s)	Stokes radius (Å)
	Large Phytochrome		
I	Pr	2.67 ± 0.13	80
	↓ 660 nm		
	Pfr	2.65 ± 0.12	81
II	Pfr	2.66 ± 0.12	81
	↓ 730 nm		
	Pr	2.65 ± 0.05	81
III	Pr	2.70 ± 0.20	79
	↓ 660 nm		
	Pfr	2.57 ± 0.17	83
	↓ 730 nm		
	Pr	2.67 ± 0.09	80
	Small Phytochrome		
IV	Pr	3.58 ± 0.50	50
	↓ 660 nm		
	Pfr	3.70 ± 0.28	49

[a] Conditions of measurements are the same as described in Figure 2 (large phytochrome). Arrows indicate photoconversion of the same sample (e.g., Pr or Pfr photoconverted to Pfr or Pr) for the next measurement.

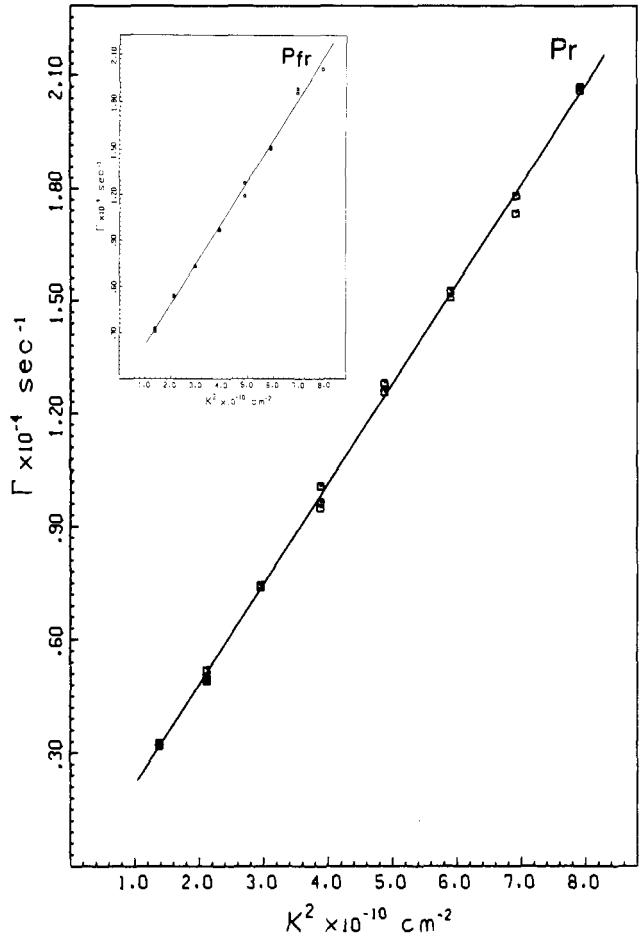

FIGURE 2: Relaxation rates, Γ vs. κ^2, as a function of different scattering angles for Pr in 0.05 M NaP_i buffer, pH 7.8, at 287 K. The concentration of phytochrome was 0.2 mg/mL. (Inset) Pfr.

summarized in Table I. During these measurements (≤30 s for each scattering measurement), phytochrome solutions were continuously irradiated either with red light for the Pfr form or with far-red light for the Pr form. The Ar laser itself did not cause conversions (<10%) of either form of phytochrome. Clearly, phototransformation followed by photoreversion and vice versa did not reveal any differences in diffusion coefficients between the Pr and Pfr forms.

Attempts to measure the diffusion coefficient at higher phytochrome concentrations (e.g., 1 mg/mL, 0.8 mg/mL, etc.) showed time-dependent changes in the value of D (data not shown). This aggregation phenomenon seemed to be more localized within the scattering volume, since a shaking of the cell brought the value of D close to the original value. Self-aggregation of large phytochrome has been reported previously on the basis of gel exclusion chromatographic studies (Pratt, 1973; Rice & Briggs, 1973; Grombein & Rüdiger, 1976). Lowering of ionic strength further to 15 mM sodium phosphate buffer does not alter the observed diffusion coefficients of large Pr and Pfr at a 0.2 mg/mL concentration. However, upon an increase of the ionic strength to 150 mM, while the concentration was kept the same, the Pr diffusion coefficient measured at a 90° scattering angle decreases to a much smaller extent with time than does the in situ photoconverted Pfr diffusion coefficient (results not shown). Similar trials with small phytochrome at concentrations of about 1 mg/mL in 0.1 M sodium phosphate buffer, pH 7.8, containing 50 mM KCl, do not show any time-dependent aggregation behavior with an incident wavelength of λ_0 = 514.5 nm of the Ar ion laser. It is interesting to point out here that Gardner et al.

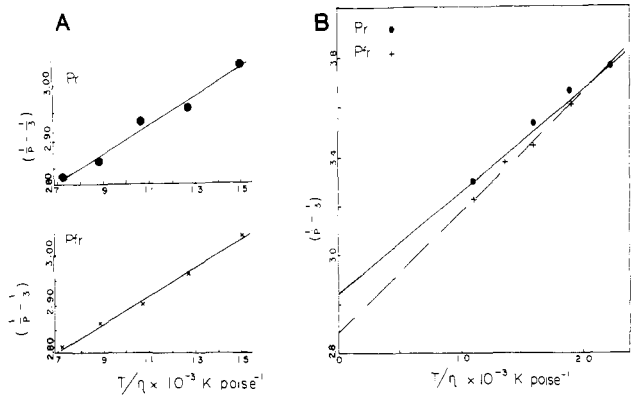

FIGURE 3: (A) Perrin's plots of phytochrome labeled with fluorescamine (1:3 ratio) in 0.05 M NaP_i buffer, pH 7.8, containing 20% sucrose by varying the temperature: (top panel) Pr; (lower panel) Pfr. λ_{ex} = 380 nm; a cut-off filter (CS3-72, ~450 nm cut-off) was used at the emission side. (B) Perrin's plots of phytochrome labeled with ANS (1:10 ratio) in 0.05 M NaP_i buffer, pH 7.8, containing 10% glycerol.

(1971) observed no aggregation of small phytochrome under various experimental conditions. The observed diffusion coefficients of Pr and Pfr were found to be the same within experimental errors (Table I). Large phytochrome also showed time-dependent aggregation behavior at λ_0 = 514.5 nm. Preliminary studies indicated that in both 0.1 and 0.05 M potassium phosphate buffer, pH 7.8, containing 5% glycerol and at 11.3 °C, intact phytochrome appears to form large aggregates even at a concentration of about 0.1 mg/mL. A time-dependent decrease in diffusion coefficients of both intact Pr and Pfr forms may also be observed. However, upon a lowering of the molarity of the buffer to 10 mM (containing 5% glycerol), the time-dependent aggregation phenomenon disappears. Under these conditions the diffusion coefficients of intact Pr and Pfr are roughly (1.76 ± 0.25) × 10^{-7} and (1.82 ± 0.62) × 10^{-7} cm^2/s, respectively.

Figure 3 shows the Perrin's plots, $(1/P - 1/3)$ vs. T/η, for both large Pr and Pfr labeled with fluorescamine or ANS. These plots were obtained by varying the temperature of the respective solutions from 273 to 298 K. Attempts to vary the viscosity by the addition of different amounts of either sucrose or gycerol isothermally caused a visual precipitation of the protein at higher sucrose or glycerol concentrations. In addition, since the time-dependent aggregation phenomenon is observed for large phytochrome at higher concentrations by quasi-elastic light scattering, the concentration of phytochrome for the rotational relaxation measurements was kept near 0.2 mg/mL. The plots in Figure 3 show a good linear relationship.

The rotational relaxation times were calculated from these plots as follows:

$$\beta = \frac{\text{slope}}{\text{intercept}} = \frac{R\tau_F}{V}$$

$$\rho_h = \frac{3V\eta}{RT} = \frac{3\eta\tau_F}{\beta T}$$

Table II summarizes the fluorescence lifetimes of large Pr and Pfr labeled with fluorescamine and ANS, respectively. Lifetimes were measured in the presence of polarizers with the emission polarizer making an angle of 54.7° to the excitation polarizer to remove the effect of rotational Brownian motion on the observed lifetime (Spencer & Weber, 1970).

The observed rotational relaxation times are listed in Table III. Since the ratio ρ_h/ρ_0 for fluorescamine-labeled large phytochromes (both Pr and Pfr) was found to be less than 1, indicating the possibility of local rotation of the fluorophore

Table II: Lifetime of Phytochrome Labeled with Fluorescamine and ANS, Respectively[a]

sample	modulation frequency and mode (ns)				component analysis[b]			
	30 MHz		10 MHz					
	modulation	phase	modulation	phase	τ_1 (ns)	α_1 (%)	τ_2 (ns)	α_2 (%)
fluorescamine								
Pr	6.67	6.32	7.80	6.77	6.17	94.6	59.78	5.4
Pfr	7.08	5.76	7.64	6.04	6.48	~100		
ANS								
Pr	7.10	2.33	10.72	5.74	14.36	51.0	0.80	49.0
Pfr	5.75	2.52	10.28	5.03	17.82	65.6	1.73	34.4

[a] Fluorescamine-labeled phytochromes were in 0.05 M NaP_i buffer, pH 7.8, containing 20% sucrose, whereas ANS-labeled phytochromes were in the same buffer containing 10% glycerol. λ_{ex} = 380 nm, and a cut-off filter, CS3-71, was used at the emission side. [b] Lifetime component analysis was carried out on the basis of the method described by Weber (1981). α_1 and α_2 are the percent amplitude of τ_1 and τ_2 components, respectively.

Table III: Rotational Relaxation Times of Pr and Pfr Labeled with Fluorescamine (in 0.05 M NaP_i Buffer, pH 7.8, Containing 20% Sucrose) and ANS (in 0.05 M NaP_i Buffer, pH 7.8, Containing 10% Glycerol), Respectively[a]

sample	probe	rotational relaxation time at 293 K in H_2O (ns)		ratio of obsd/calcd
		obsd (ρ_h)	calcd (ρ_0)	(ρ_h/ρ_0)
Pr	fluorescamine	65.20	105.72	0.62
Pfr	fluorescamine	68.46	105.72	0.65
Pr	ANS	103.47	105.72	0.98
Pfr	ANS	104.10	105.72	0.99

[a] The values at 293 K in water are reported after correction for the respective viscosities as follows: $\rho_h^{obsd}/\rho_{H_2O}{}^{20\ °C} = \eta/\eta_{H_2O}{}^{20\ °C}$, where η is the viscosity of the solution and $\eta_{H_2O}{}^{20\ °C}$ is the viscosity of water at 293 K. λ_{ex} = 380 nm (fluorescamine); λ_{ex} = 380 nm (ANS); a CS3-71 cutoff filter was used on the emission side.

Table IV: Parameters and Rotational Relaxation Times from Observed tan Δ of Phytochrome Labeled with Fluorescamine As Measured by the Dynamic Depolarization Method[a]

	Pr	Pfr
P_0	0.336	0.340
τ_F (ns)	6.17	6.48
$\Delta\tau_F$ (10 MHz) (ns)	0.416	0.648
$\Delta\tau_F$ (30 MHz) (ns)	0.282	0.455

	f (MHz)	ρ (ns)
Pr	10	126.43, 2.09
	30	98.81, 1.31
Pfr	10	76.42, 3.77
	30	57.65, 2.34

[a] The phytochrome/fluorescamine ratio was 1:3, and the buffer used was 0.05 M NaP_i, pH 7.8, containing 20% sucrose.

and/or conformational flexibility of the amino group(s) linked to fluorescamine, dynamic depolarization studies were also carried out (Mantulin & Weber, 1977; Weber, 1977; Lakowicz et al., 1979). The results are listed in Table IV.

Discussion

In quasi-elastic light scattering, conformational sensitivity arises from the inverse proportionality of the diffusion coefficient to the Stokes radius (Stokes–Einstein relationship), whereas in rotational relaxation time the measured quantity, ρ_h, is directly proportional to the hydrated volume and, thus, to the r_h^3 (hydrated radius) of the molecule.

Plots of Γ vs. κ^2 for the large Pr and Pfr forms of phytochrome are virtually superimposable (Figure 2), and the Stokes radii and diffusion coefficients of Pr and Pfr for both large and small molecular weight phytochromes as determined from the quasi-elastic light scattering measurements are essentially the same within experimental errors (Table I). Preliminary results also revealed few differences between the diffusion coefficients of intact Pr and Pfr. Similarly, the rotational relaxation times of Pr and Pfr labeled with ANS are also found to be the same. The ratio of observed relaxation time and calculated relaxation time on the basis of monomer molecular weight is almost unity. Since this ratio determines the deviation of the molecule under study from the rigid spherical model, it could suggest that phytochrome in solution behaves as a near spherical globular protein. These observed rotational relaxation times of large Pr and Pfr lead to their hydrated radii of 32.16 and 32.24 Å, respectively. Although these values are quite close to the calculated hydrated radius of large phytochrome (37.5 Å), on the basis of monomer molecular weight, partial specific volume, and a hydration factor of 0.4 g of H_2O/g of phytochrome, they are substantially different from their respective Stokes radii obtained from the light scattering data.

Large phytochrome is believed to exist in solution as a dimeric form [Hunt & Pratt, 1980; for a review, see Rüdiger (1980)]. Under the similar conditions of a light scattering experiment (i.e., 0.05 M sodium phosphate buffer), large phytochrome comes out very close to catalase (M_r 240 000) on a Bio-Gel A 1.5-m column, indicating an almost dimeric molecular weight. However, it is difficult to say with certainty whether the observed Stokes radius is due to a dimeric form or an elongated monomeric form, on the basis of these data. Failure to observe higher rotational relaxation times makes it more difficult to come to such a conclusion. The lower rotational relaxation time of large phytochrome with ANS may be a result of the binding of the probe to a flexible part of the protein.

The lower ratio of the observed and calculated relaxation rates for large phytochrome labeled with fluorescamine ($\rho_h/\rho_0 < 1$) could be due to several factors: (i) lower fluorescence lifetime of the probe (~6 ns); (ii) conformational flexibility of the protein; (iii) local freedom of rotation of the probe about the covalent linkage in the protein conjugate. Differential phase fluorometry, used to examine whether or not this lower value of rotational relaxation time with fluorescamine as the fluorescence probe is due to a local anisotropic rotation, yielded two values for the rotational relaxation time. This is expected from the quadratic dependence of ρ on tan Δ. Clearly, one of these two values of ρ for Pr at each modulation frequency is close to the values obtained for the ANS-labeled phytochrome, and the other values for both Pr and Pfr are very short (2.09 and 1.31 ns for Pr and 3.77 and 2.34 ns for Pfr at modulation frequencies 10 and 30 MHz, respectively). The

Table V: Calculated $(\tan \Delta)_{max}$ and Observed $\tan \Delta$ for Fluorescamine-Labeled Phytochrome in 0.05 M NaP_i Buffer, pH 7.8, Containing 20% Sucrose at 293 K

sample	f (MHz)	calcd $(\tan \Delta)_{max}$	obsd $\tan \Delta$	defect (%)
Pr	10	0.0649	0.0261	59.8
	30	0.1600	0.0538	66.4
Pfr	10	0.0686	0.0407	40.7
	30	0.1671	0.0858	48.7

longer rotational relaxation time of Pfr is shorter than that of the Pr form. This suggests an additional conformational flexibility of the protein moiety linked to the fluorescamine in the Pfr form, which occurs upon phototransformation. It has been shown that the Pr form of phytochrome has two more exposed Lys residues than does the Pfr form (Roux, 1972). It is unlikely that fluorescamine is preferentially attached to these two extra Lys residues, since the linked probe in the Pfr form upon phototransformation from Pr would be expected to decrease the rotational flexibility of the attached fluorophore. If this were the case, the larger values of ρ would not have decreased in the Pfr form. In addition, the values at two different modulation frequencies are not in agreement (Table IV), thus suggesting the occurrence of anisotropic rotations due to the local rotation of the fluorophore, that is attached to a flexible region of the protein.

One can calculate the maximum value of $\tan \Delta$, $(\tan \Delta)_{max}$, by using the following equation:

$$(\tan \Delta)_{max} = \frac{P_0 \omega \tau_F}{1 + [(1 - P_0^2)(1 + \omega^2 \tau_F)]^{1/2}}$$

Failure of the experimentally determined value of $\tan \Delta$ to reach the $(\tan \Delta)_{max}$ is an additional criterion for testing the existence of anisotropic rotations. As can be seen from the Table V, there are tangent defects in both fluorescamine-labeled Pr and Pfr with that particular measurement. However, this should be further examined by measuring the $\tan \Delta$ vs. temperature, since the final test should be a failure to reach the value of $(\tan \Delta)_{max}$ at all temperature ranges used for the measurements (Weber, 1977; Fugate, 1978).

In conclusion, both conformationally sensitive studies (quasi-elastic light scattering for intact, large, and small molecular weight forms and rotational relaxation time measurements for large molecular weight forms) were unable to distinguish any gross conformational difference between the Pr and Pfr forms of phytochrome. However, these results could not provide any specific indication regarding molecular shape (e.g., spherical, elongated, etc.) nor the solution form (e.g., monomer or dimer) of large phytochrome. The relative hydrophobic character of phytochromes increases from small to intact phytochrome. Under the experimental conditions chosen for the light scattering measurements of small phytochrome, both large and intact phytochrome form oligomers. Upon a lowering of both the concentration of phytochrome (large or intact) and the ionic strength of the buffer, this aggregation phenomenon disappears. Intact phytochrome aggregates more than does large phytochrome.[3] On the basis of these observations, it can be said that large phytochrome, upon tryptic digestion to small phytochrome, loses the hydrophobic part that is responsible for its aggregation at higher ionic strengths and higher concentrations. The higher aggregation characteristics of intact phytochrome can be attributed to the extra 6-kdalton peptide segment. This 6-kdalton peptide could itself act as a hydrophobic site, or it may cause a conformational change (large vs. intact) to expose a more extensive hydrophobic surface on the intact form as compared to the large form.

Phototransformation of Pr to Pfr was previously proposed to cause chromophore reorientation (Song et al., 1979; Hahn & Song, 1981; Sarkar & Song, 1982; Song, 1983). If so, the results presented in this paper suggest that the chromophore reorientation does not cause major changes in the gross conformation of the protein moiety of phytochrome. In a reversible conformational change, proteins cannot gain or lose significant amounts of volume [e.g., only 0.8% change in D occurs in hemoglobin (Sanders et al., 1981)]. Thus, it is perhaps not surprising that photoreversible phytochrome does not exhibit a large conformational change, especially given the small energy of a red photon.[4]

[3] The degree of ANS fluoescence polarization increases by 0.042 ± 0.004 upon phototransformation (Pr to Pfr) of intact phytochrome in 20 mM KP_i buffer plus 5% glycerol, pH 7.8, 277 K, in the presence of a 20-fold excess of ANS (unpublished results). This increase in polarization degree may indicate a preferential aggregation of the Pfr form and/or additional ANS binding.

[4] We thank one of the referees for pointing this out.

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Use of High-Speed Size-Exclusion Chromatography for the Study of Protein Folding and Stability†

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ABSTRACT: The urea denaturation of sperm whale myoglobin and the thermal denaturation of ribonuclease have been studied by following the associated volume changes by size-exclusion chromatography on a Toya Soda TSK 3000SW gel permeation column. The permeation properties of the gel have been shown to be invariant in the following solvent systems: 0.2 M NaCl; 8.0 M urea–0.2 M NaCl; and 6.0 M guanidinium chloride (GdmCl). A precise measurement of the volume changes associated with solvent-induced protein denaturation is thus practicable. The column was calibrated in the above solvent systems by using 12 well-characterized proteins as standards. In the case of the denaturation of myoglobin by urea, the rate of equilibration of folded and unfolded species is slow on the time scale of the chromatographic experiment, and the two forms are well separated on the column in the transition region. Both the folded and unfolded species are shown to undergo significant swelling in urea. This result suggests that the view of denaturation based solely on the preferential solvation of the unfolded protein is incorrect. The rate of interconversion between folded and unfolded ribonuclease is fast relative to the time scale of the chromatographic experiments performed in this study. This is reflected in the fact that only one peak is observed in the elution profiles of ribonuclease in the transition region. Thermally unfolded ribonuclease has a smaller volume than the unfolded state in urea or GdmCl, suggesting that it has residual structure. The van't Hoff ΔH for the thermal unfolding of ribonuclease calculated from the size-exclusion chromatographic experiments (36 ± 3 kcal/mol) is significantly lower than previously reported values. This suggests that there are contributions to the enthalpy of unfolding which have negligible volume changes.

The elucidation of the folding pathways of globular proteins remains one of the major objectives of contemporary protein chemistry (Anfinsen & Scheraga, 1975; Nemethy & Scheraga, 1977; Schultz, 1977; Jaenicke, 1980). Among the many theoretical and experimental approaches to this problem, denaturation studies continue to play a crucial role (Tanford, 1968, 1970; Privalov, 1979; Schellman & Hawkes, 1980). A thermodynamic analysis of thermal or solvent-induced unfolding processes provides the free energy of stabilization of the native state of globular proteins and a measure of the cooperativity (Privalov, 1979, 1982) of the unfolding process. Many properties can be used to monitor folding–unfolding equilibria, including the large volume changes associated with these processes. Although intrinsic viscosity remains the most theoretically straightforward method for determining such volume changes, gel filtration techniques offer an alternative approach (Ackers, 1970).

More than a decade ago, Tanford and co-workers showed that analytical gel filtration can be used to study proteins, denatured in a variety of solvents (Fish et al., 1970). However, the high resistance to flow, low mechanical strength, and poor chemical resistance of the soft gels based on dextran, polyacrylamide, and agarose, which were the only ones available at that time, made it difficult to apply this approach to denaturation studies requiring high concentrations of denaturants

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