Nature of Phototransformation of Phytochrome As Probed by Intrinsic Tryptophan Residues†

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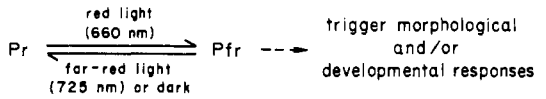
ABSTRACT: The phototransformation of the photomorphogenic photoreceptor phytochrome was probed by the intrinsic luminescence of the tryptophan (Trp) residues. The red light absorbing form of phytochrome (Pr) showed a decreased tryptophan phosphorescence intensity, compared to that of the far-red light absorbing form of phytochrome (Pfr), and a delayed fluorescence from the chromophore upon excitation of the tryptophan residues with 290-nm light. The tryptophan phosphorescence in both Pr and Pfr showed decreased lifetimes (0.29 and 1.84 s, respectively) compared to that of the free tryptophan (6.00 s). In addition, the decay kinetics of the delayed fluorescence in Pr showed a short-lifetime component (0.24 s), which is similar to the tryptophan phosphorescence lifetime value. This is due to an efficient triplet–singlet (^{3}Trp–^{1}Pr) energy transfer in the Pr form. The increases in the tryptophan phosphorescence quantum yield and lifetime in the Pfr form have been interpreted on the basis of chromophore reorientation on the protein surface as a result of the Pr → Pfr phototransformation. The Stern–Volmer quenching of the tryptophan fluorescence by potassium iodide suggests a substantial exposure of the tryptophan residues in the Pfr form, compared to those in the Pr form. A modified Stern–Volmer plot of the quenching data further confirms preferential exposure of the tryptophan residues in the Pfr form (46% "exposed" tryptophan residues in the Pr form as compared to 72% in the Pfr form). These results provide strong support for the hydrophobic model of Pfr [Hahn, T. R., & Song, P. S. (1981) *Biochemistry 20*, 2602–2609].

Phytochrome, the ubiquitous plant pigment protein that triggers plant photomorphogenesis by absorbing red light, exists in two different photoreversible forms, namely, Pr[1] and Pfr. Red light triggers photomorphogenic responses in plants by phytochrome according to the scheme [for a recent review, see Pratt (1982) and Rüdiger (1980)]



A number of previous studies have reported the varying reactivities of different amino acid residues of the phytochrome protein moiety on phototransformation from the Pr form to the Pfr form (Butler et al., 1964; Roux & Hillman, 1969; Hunt & Pratt, 1981). It has also been noted by different laboratories that there are no significant differences in the CD spectra of phytochrome within the spectral region from 200 to 225 nm (Tobin & Briggs, 1973; Song et al., 1979; Hunt & Pratt, 1981). In addition, no differences were revealed with immunochemical methods (Rice & Briggs, 1973; Cundiff & Pratt, 1975). On the basis of spectroscopic analysis, Song et al. (1979) concluded that there are no major conformational differences of the apoprotein between the Pr and Pfr forms, and a model for the Pr → Pfr transformation was proposed (Song et al., 1979; Hahn & Song, 1981; Sarkar & Song, 1981a). In essence, the tetrapyrrole chromophore, which is tightly attached to the phytochrome apoprotein, covers a "hydrophobic surface" in the Pr form. Upon phototransformation of Pr, the chromophore reorients itself on the protein surface, thereby exposing the "covered hydrophobic surface" in the Pfr form. However, the chromophore configurations and/or protein conformations of the Pr and Pfr forms remain similar, i.e., no gross photoisomerism or conformational changes, respectively (Song et al., 1979; Song & Chae, 1979).

By use of 8-anilinonaphthalene-1-sulfonate (ANS) as a hydrophobic probe, it has been recently shown that the hydrophobic surface is significantly exposed in the Pfr form (Hahn & Song, 1981). The Pfr form also has a higher affinity of binding toward hydrophobic chromatographic materials than does the Pr form (Yamamoto & Smith, 1981; Smith, 1981). In addition to this, it has been shown that the chromophore becomes more accessible to $KMnO_4$ oxidation and borohydride reduction in the Pfr form as compared to the Pr form (Hahn et al., 1980; Song, 1982). It has also been observed by Song et al. (1979) that at least one tryptophan residue is at or near the chromophore binding crevice. In this paper we present data that contribute to the understanding of a crucial feature of the hydrophobic model of the Pfr form, namely, the reorientation of the chromophore, as probed by the fluorescence and phosphorescence of the tryptophan residues in phytochrome molecules (Pr and Pfr). The proposition that the chromophore reorientation in Pr → Pfr phototransformation changes the efficiency of the energy transfer from the tryptophan residue(s) to the chromophore will be described herein.

Materials and Methods

Phytochrome was isolated from dark-grown oat seedlings (*Avena sativa L.*) by using the affinity procedure of Smith & Daniels (1981) as modified by Song et al. (1981). Seedling tissue extracts were chromatographed on a Brushite column to obtain a crude phytochrome preparation. Phytochrome was then fractionated with 30% ammonium sulfate, redissolved in 0.1 M potassium phosphate buffer containing 14 mM 2-

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[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonate; EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; Pfr, far-red light absorbing form of phytochrome; P_0, excitation polarization; Pr, red light absorbing form of phytochrome; Trp, tryptophan; Tyr, tyrosyl; CD, circular dichroism; FMN, flavin mononucleotide; NMR, nuclear magnetic resonance.

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mercaptoethanol, and chromatographed on an Affi-Gel Blue column (Bio-Rad) equilibrated with the same buffer, followed by washing with 0.1 M potassium phosphate buffer, pH 7.8, containing 0.5 M KCl and 14 mM mercaptoethanol. Phytochrome was then eluted with 10 mM FMN, in the starting buffer. After ammonium sulfate fractionation, it was then applied to a Bio-Gel A0.5-m column equilibrated with 0.1 M sodium phosphate buffer, pH 7.8, containing 50 mM KCl and 0.1 mM EDTA. Phytochrome, thus obtained, was fractionated and then resuspended in 0.1 M sodium phosphate buffer, pH 7.8, for all the phosphorescence studies. Fluorescence lifetime measurements and quenching studies were done without changing the buffer. Phytochrome preparations of specific absorbance ratio ~0.83 (A_{660}/A_{280}) were exclusively used for these studies. Affi-Gel and Bio-Gel A0.5-m columns were purchased from Bio-Rad. Potassium iodide for the quenching studies was obtained from MCB and used without further purification. Sodium thiosulfate was purchased from Fisher Scientific. Guanidine hydrochloride (Gdn·HCl) was purchased from Mallinkrodt and was recrystallized from methanol before use. All the other chemicals were purchased from Sigma Chemical Co.

Absorption spectra were recorded on a Cary 118C spectrophotometer. Phosphorescence spectra were recorded on an Aminco-Bowman spectrofluorometer with a phosphoroscope attachment. Phosphorescence lifetimes were measured with a phosphorescence lifetime attachment for the Aminco-Bowman spectrofluorometer. Phosphorescence decay curves were recorded on a Tektronix oscilliscope (564B). Quartz tubes of 3-mm diameter were used for phosphorescence measurements.

Fluorescence quenching measurements were made on a Perkin-Elmer MPF-3 spectrofluorometer at ice-bath temperature (~279 K) as a function of KI (small aliquots of 5 M KI containing about 10^{-4} M $Na_2S_2O_3$ to prevent I_3^- formation were added to 2.0 mL of a sample solution in a fluorometric cuvette having an optical density of 0.1 or less at the exciting wavelength, 290 nm). After each addition of KI, the solution was gently agitated, and the fluorescence intensity was measured.

Fluorescence lifetimes were measured on an SLM 480 phase-modulation spectrofluorometer as described elsewhere (Fugate & Song, 1976). Since the fluorescence lifetime of the tryptophan residue is short, only the 30-MHz phase lifetimes were used for fluorescence quenching studies by lifetime measurements. The fluorescence excitation polarizations were measured on an Aminco-Bowman spectrofluorometer, and the polarization degrees (P_0) were calculated according to Azumi & McGlynn (1962).

Results

Figure 1 shows the Trp phosphorescence spectra (λ_{ex} ~ 290 nm, where Trp absorbs nearly exclusively) of Pr, Pfr, and denatured phytochrome in phosphate buffer, pH 7.8, containing 0.5% dextrose (1% in the case of the Gdn·HCl-denatured phytochrome) to reduce snow formation of the phytochrome solution at 77 K.

The relative phosphorescence quantum yields, Φ_p, are also listed in Table I, relative to the Φ_p = 0.17 of free tryptophan in the pH range of 2–12 at 77 K (Bishai et al., 1967). It can be seen that the phosphorescence itensities of both Pr and Pfr are significantly lower than that of either free tryptophan or Gdn·HCl-denatured Pr; furthermore, Φ_p(Pfr) is somewhat greater than Φ_p(Pr) (Table I and Figure 1).

The luminescence spectrum of Pr (λ_{ex} ~ 290 nm) is composed of not only the Trp phosphorescence but also the fluorescence (680 nm) of the chromophore. The latter is absent in both the spectra of Pfr and Gdn·HCl-denatured phytochromes. The chromophore fluorescence with λ_F ~ 680 nm has been recorded previously (Song et al., 1973, 1979; Sarkar & Song, 1981a). Song et al. (1979) also observed that the large molecular weight Pr, but not the small molecular weight Pr, exhibited a sensitized fluorescence upon excitation of the Trp residues (λ_{ex} ~ 280 nm).

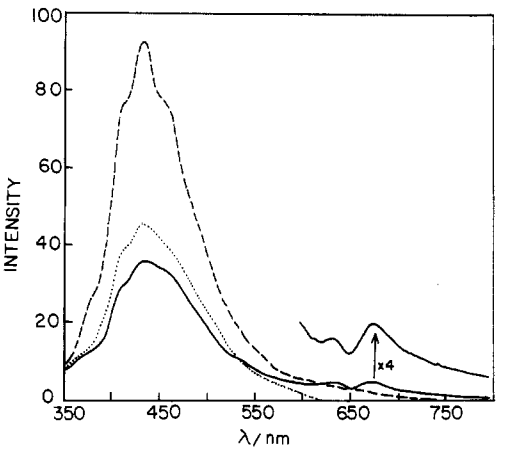

FIGURE 1: Phosphorescence spectra of Pr (solid line), Pfr (dotted line), and denatured Pr (in 6 M guanidine hydrochloride; dashed line) in 0.1 M sodium phosphate buffer, pH 7.8, at 77 K. The solutions contained 0.5% (for Pr and Pfr) and 1.0% (for denatured Pr) dextrose. Excitation wavelength, 290 nm; absorbance at 290 nm, 0.091 for Pr and Pfr and 0.1 for the denatured Pr. All spectra were recorded at the same band-passes (4 nm) and sensitivity, except for the expanded recording of the delayed fluorescence of Pr at 670–680 nm.

Table I: Phosphorescence Lifetime of Tryptophan Residues at 77 K[a]

sample	phosphorescence lifetime (τ_p) (s)	phosphorescence quantum yield (Φ)
Pr	0.29	0.05
Pfr	0.25[b]	0.06
	1.84[b]	
free Trp	6.00	0.17[c]
α-chymotrypsin	5.80	
denatured Pr	5.68[d]	0.12

[a] λ_{ex} = 290 nm; λ_{em} = 440 nm. A 310-nm cutoff filter was used at the emission side. All the samples were prepared in 0.1 M sodium phosphate buffer, pH 7.8. Dextrose (0.5%) was added to reduce snow formation. [b] Two-component lifetimes resolved by the peeling procedure shown in Figure 2. [c] From Bishai et al. (1967). [d] Denatured with 6 M Gdn·HCl; major decay component, 84%; 1% dextrose was added.

Figure 2 shows the oscilloscope tracing of the phosphorescence decays of Trp residues in the Pr and Pfr phytochromes upon excitation with 290-nm light. The phosphorescence lifetimes calculated from the apparent first-order plots of the decay curves are also shown in Table I. The phosphorescence lifetimes of free Trp and Trp in α-chymotrypsin were found to be 6.0 and 5.8 s, respectively (Table I), as compared to the literature values of 5.8 s for free Trp (Bishai et al., 1967) and 5.2 s for Trp in chymotrypsin (Galley & Stryer, 1969).

The delayed fluorescence of the chromophore (λ_{ex} ~ 290 nm; Figure 1) decays exponentially (Figure 2), with a lifetime of 0.24 s. The direct fluorescence of Pr (λ_{ex} ~ 660 nm) at 77 K has a lifetime less than 1 ns (Song et al., 1979). Since the delayed fluorescence (λ_{ex} ~ 290 nm) exhibits a long decay time, the delayed fluorescence arises from an energy transfer from the phosphorescent ^{3}Trp residue(s) to the Pr chromo-

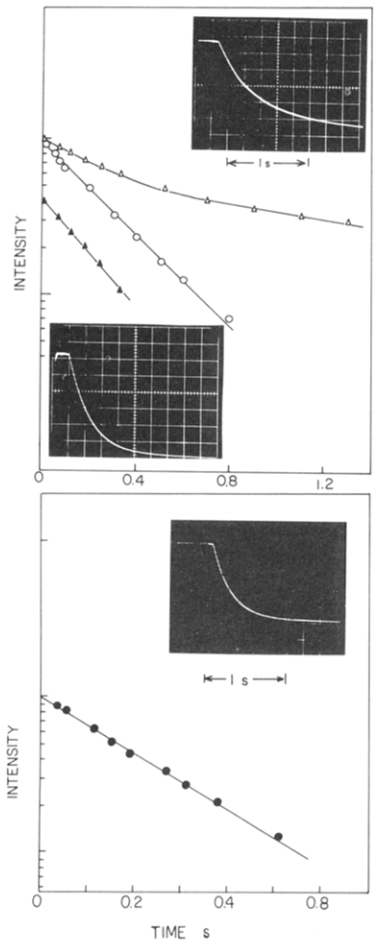

FIGURE 2: (Top panel) Phosphorescence decays of tryptophan in the Pr (open circles) and Pfr (open triangles) forms of phytochrome. The corresponding oscilloscope tracings are shown as insets. Correlation coefficients > 0.99. Excitation wavelength, 290 nm (band-pass, 4 nm); emission wavelength, 440 nm (band-pass, 6 nm). Other conditions were the same as for Figure 1. The shorter lifetime component (solid triangles) from the Pfr phosphorescence decay was resolved by the peeling procedure as described previously (Hahn & Song, 1981). (Lower panel) Decay of the delayed fluorescence of Pr at 77 K. Emission wavelength, 685 nm (with an additional 640-nm cutoff filter). Excitation wavelength, 290 nm (band-pass, 4 nm). Other conditions were the same as for Figure 1. Correlation coefficient > 0.99.

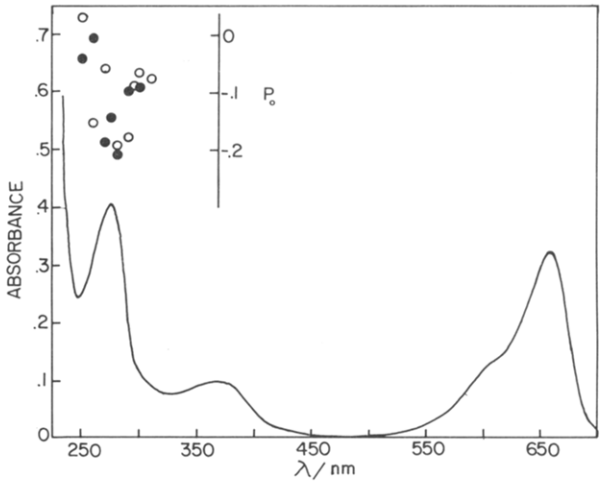

FIGURE 3: Excitation polarization (P_0) of the delayed emission of phytochrome (Pr) in 0.1 M sodium phosphate buffer, pH 7.8, containing 5% dextrose to minimize snow formation at 77 K. Emission wavelengths: 670 (solid circles) and 675 nm (open circles). The absorption spectrum is also shown. Excitation and emission band-passes, 4 and 6 nm, respectively.

Table II: Tryptophan Fluorescence Lifetimes (τ_F) of Phytochrome (293 K) [a]

phyto-chrome	30 MHz phase (ns)	30 MHz modu-lation (ns)	10 MHz phase (ns)	10 MHz modu-lation (ns)	component analysis [b] τ_1 (ns)	component analysis [b] τ_2 (ns)
Pr	3.46	3.82	3.36	3.99	3.57 (~100)	[c]
Pfr	3.33	4.07	3.76	3.83	5.29 (52)	1.76 (48)

[a] In 0.1 M sodium phosphate buffer, pH 7.80, containing 50 mM KCl and 0.1 mM EDTA. Samples were excited at 290 nm (OD = 0.05) with a 310-nm cutoff filter (long pass) on the emission side. [b] Based on a two-component analysis by means of the classical de Prony method described by Weber (1980). Values in parentheses are in percent. [c] A negative root of −7.98 (2%) from the analysis is meaningless and discarded.

phore. The lack of fluorescence (or insensitivity of the detector system) from the Pfr chromophore makes it difficult to ascertain whether or not the ^{3}Trp → chromophore energy transfer takes place in the Pfr phytochrome as well. However, the fact that the Trp phosphorescence lifetime and Φ_p values are higher in Pfr than in Pr suggests that the energy transfer is significantly suppressed in the Pfr molecule.

To deduce the average orientations of the energy-donating Trp residue(s) relative to the chromophore transition dipole, we measured the fluorescence excitation polarization over the Trp absorption region with respect to the chromophore emission (Figure 3). It can be seen that the degree of fluorescence polarization over the Trp absorption region is generally negative and is neither of the two theoretical limits ($^1/_2$ and $-^1/_3$).

There are eight Trp residues in large molecular weight phytochrome (Hunt & Pratt, 1980). The fluorescence maximum of phytochrome occurs at a relatively short wavelength of 330 nm, suggesting that the majority of the Trp residues are in hydrophobic environments.

The Trp fluorescence decay of Pr is virtually exponential, whereas that of Pfr is highly heterogeneous (Table II). The relatively long fluorescence lifetime in Pr suggests that all the fluorescent Trp residues are in hydrophobic environments. In the Pfr form, approximately half of the Trp fluorescence emission decays with a short lifetime (1.76 ns), suggesting that some of the Trp residues are accessible to polar environments (including aqueous medium).

To ascertain the degree of exposure of the Trp residues, we analyzed the fluorescence quenching of the "exposed" Trp residues by KI by the Stern–Volmer kinetics. The Stern–Volmer quenching results are shown in Figure 4 (K_Q = 0.82 ± 0.07 M^{-1} and $\bar{r}$ = 0.997 for Pr, and K_Q = 1.13 ± 0.09 M^{-1} and $\bar{r}$ = 0.998 for Pfr). The fluorescence quenching is shown to be dynamic, rather than static, since the fluorescence lifetime decrease follows the Stern–Volmer kinetics (Figure 4, inset), as in the case of fluorescence quenching. It can be seen that the Trp fluorescence of Pfr is quenched 1.5 times more efficiently than that of Pr (K_Q = 0.72 M^{-1} and $\bar{r}$ = 0.993 for Pr, and K_Q = 1.11 M^{-1} and $\bar{r}$ = 0.995 for Pfr). This suggests that there are more exposed Trp residues in Pfr than in Pr.

A quantitative estimate of the differential exposures of the Trp residues in phytochrome was determined from the modified Stern–Volmer kinetics (Lehrer, 1971) with the equation

$$F_0/\Delta F = 1/f_{acc} + 1/(f_{acc}K_Q[KI])$$

where F_0 is the fluorescence intensity in the absence of KI, ΔF represents the decrease in fluorescence intensity as a

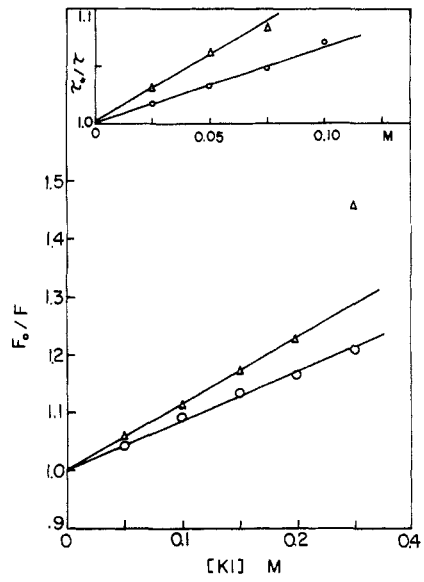

FIGURE 4: Stern–Volmer quenching plots of the tryptophan fluorescence and lifetime (top inset) of Pr (open circles; absorbance, ~0.1 at 290 nm) and Pfr (open triangles; absorbance, ~0.1 at 290 nm) in 0.1 M sodium phosphate buffer, pH 7.8, containing 50 mM KCl and 0.1 mM EDTA at 293 K as a function of KI concentration (ca. 0.1 mM $S_2O_3^{2-}$ was added). Excitation wavelength, 290 nm; emission wavelength, 330 nm. F_0 and F are the Trp fluorescence intensities in the absence and presence of KI. τ_0 and τ are the Trp fluorescence lifetimes in the absence and presence of KI. A 310-nm cutoff filter (long pass) was used on the emission side. Correlation coefficients > 0.99.

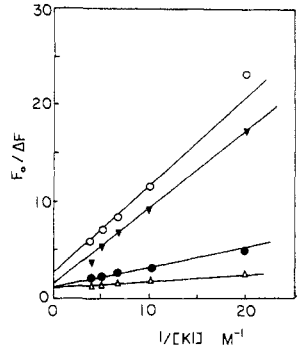

FIGURE 5: Modified Stern–Volmer quenching plots of the Trp fluorescence of Pr (open circles), Pfr (solid triangles), denatured Pr (solid circles), and free tryptophan (open triangles) in 0.1 M phosphate buffer, pH 7.8, containing 50 mM KCl and 0.1 mM EDTA at 293 K. Excitation wavelength, 290 nm; emission wavelength, 330 nm; absorbances at 290 nm, 0.1. Correlation coefficients > 0.98.

function of KI concentration, and f_{acc} stands for the fraction of exposed or accessible Trp residues.

The modified Stern–Volmer plots of the fluorescence quenching data are shown in Figure 5. The f_{acc} values calculated from the intercepts of the plots in Figure 5 are 0.45 and 0.72 for Pr and Pfr, respectively. These results confirm the conclusion derived from the data in Figure 4 that more Trp residues are exposed in Pfr than in Pr. As expected, the f_{acc} values for the denatured phytochrome and free Trp are 0.99 and 0.98, respectively, indicating that the amino acid is fully accessible to the fluorescence quenching by iodide (Figure 5).

Discussion

Energy transfer from the excited state of Trp to the chromophore upon excitation of Pr at 290 nm is evidenced by the fact that the chromophore fluorescence (Figure 1) decays as slowly as a delayed emission (Figure 2). The absorption of exciting light at 290 nm is almost exclusively due to Trp residues (Song & Chae, 1979). The delayed fluorescence lifetime (0.24 s; Figure 2) agrees with the phosphorescence lifetime of Trp in the Pr form (0.29 s; Table I), indicating that the delayed fluorescence shown in Figure 1 arises from the ^{3}Trp → ^{1}Pr (chromophore) energy transfer. It should be noted that the Trp phosphorescence is polarized out of the indole plane (Song & Kurtin, 1969), whereas the Pr transition dipoles coupled to the Trp phosphorescence emission dipole are in-plane polarized (Song & Chae, 1979).

The triplet–singlet energy transfer being discussed here can be characterized as a long-range energy transfer, as predicted by Förster's theory (Förster, 1959; Ermolaev, 1959). The critical energy transfer distance, R_0, at which the probability of energy transfer is 50%, can be calculated from the spectral overlap between the donor (Trp) phosphorescence and the acceptor (chromophore) absorption bands with the equation

$$R_0 = \frac{(8.8 \times 10^{-25})\kappa^2\Phi_{Trp}}{n^4}\int_0^\infty I_P(\tilde{\nu})_{Trp}\epsilon(\tilde{\nu})_{chrom}\frac{d\nu}{\tilde{\nu}^4}$$

where κ^2 is the orientation factor squared (assumed to be $^2/_3$ for a random distribution of the donor and acceptor transition dipoles), n is refractive index, and Φ_{Trp} is the free Trp phosphorescence quantum yield. The spectral overlap integral between the Trp emission (I_P) and chromophore absorption (ϵ) bands has been calculated by using the Δ-function method (Dirks et al., 1980; Yoon et al., 1981).

The values of R_0 for the ^{1}Trp → ^{1}Pr and ^{1}Trp → ^{1}Pfr singlet–singlet transfers are ca. 28 Å (Song et al., 1979). The values for the ^{3}Trp → ^{1}Pr($B_{x,y}$) and ^{3}Trp → ^{1}Pr(Q_x) triplet–singlet transfer processes are 31.3 and 36.3 Å, respectively. The values for the ^{3}Trp → ^{1}Pfr($B_{x,y}$) and ^{3}Trp → ^{1}Pfr(Q_x) pairs are 31.9 and 35.8 Å, respectively. The two sets of values for R_0 for Pr and Pfr, respectively, were calculated since the Trp phosphorescence band overlaps with both near-UV ($B_{x,y}$) and visible (Q_x) absorption bands of the phytochrome chromophore. Assuming that the orientations of the donor and acceptor dipoles are randomly distributed or remain invariant upon Pr → Pfr phototransformation, one can predict from the calculated values of R_0 that the probability of energy transfer is essentially identical for both Pr and Pfr. Thus, if the Pr → Pfr phototransformation does not alter κ^2 significantly, i.e., κ^2(Trp–Pr) ≃ κ^2(Trp–Pfr), one would not expect a major difference in the probability of energy transfer in Pr and Pfr. On the other hand, if the chromophore reorients or relocates from its binding crevice upon phototransformation as required in the proposed hydrophobic model for Pfr (Song et al., 1979; Hahn & Song, 1981), the probabilities of energy transfer in Pr and Pfr are expected to be different. The latter was found to be the case.

No delayed emission from the Pfr chromophore was detectable (Figure 1). The Trp phosphorescence lifetime of Pfr is markedly longer than that of Pr, vide infra (Figure 2 and Table I). These results clearly indicate that the probability of energy transfer is lower with Pfr, as compared with Pr. It has been shown that triplet–singlet energy transfer shortens the phosphorescence lifetime of Trp in the chymotrypsin–proflavin complex (Galley & Stryer, 1969). Since the theoretical values of R_0 for the ^{3}Trp → ^{1}Pr and ^{3}Trp → ^{1}Pfr transfers are virtually identical, the lower efficiency or lack of energy transfer in the Pfr form, relative to the Pr form, can be best explained in terms of the chromophore and/or Trp reorientations, which result in a decrease in the orientation factor κ. However, we place more importance on the chromophore reorientation than on the Trp reorientation since the

phototransformation-induced reorientation of Trp (if any) is likely to follow a chemical or steric relaxation of the chromophore with respect to its binding site and since a gross conformational change of the apoprotein does not seem to be involved in the Pr → Pfr phototransformation (Tobin & Briggs, 1973; Song et al., 1979; Song, 1982; Hahn & Song, 1981). Also, the 360-MHz NMR spectra of Pr and Pfr show virtually identical peptide backbones (Song et al., 1982).

The angle ($\Delta\theta$) between the donor and the acceptor dipoles can be calculated from the excitation polarization with respect to the delayed emission in Pr by using the Levshin equation. For excitation at 270–300 nm, where Trp, Tyr, and chromophore absorb to varying degrees, the following expression describes the excitation polarization (P_0) with respect to the delayed emission from the Pr chromophore:

$$\langle P_0(\lambda)\rangle = \sum_{i=1}^{8} f_i(\lambda)P_{\mathrm{Trp},i} + \sum_{j=1}^{22} f_j(\lambda)P_{\mathrm{Tyr},j} + f_{\mathrm{Pr}}(\lambda)P_{\mathrm{Pr}}$$

where $f_i(\lambda)$, $f_j(\lambda)$, and $f_{\mathrm{Pr}}(\lambda)$ are the fractions of the donor excitations contributing to the delayed emission by Trp, Tyr, and chromophore absorptions, respectively. The last term can be neglected since the chromophore absorbs negligibly compared to the aromatic amino acid residues in the UV region (Song & Chae, 1979; Brandlmeier et al., 1981). We have not been able to establish the efficiency of the energy transfer (if any) from Tyr to the chromophore. For all practical purposes, we can safely assume that Tyr residues are not substantially excited at $\lambda > 280$ nm (cf. Figure 1). The above equation can then be simplified to the two-term expression

$$P_0(\lambda > 280\ \mathrm{nm}) \simeq \sum_{i=1}^{8} f_i P_{\mathrm{Trp},i} \simeq f_1 P_{\mathrm{Trp},1} + f_2 P_{\mathrm{Trp},2}$$

where f_1 and f_2 represent fractional polarization contributions of the fast and slow energy transfers from Trp residues, respectively (cf. Figure 2). Since the delayed fluorescence lifetime (0.24 s) agrees with the Trp phosphorescence lifetime (0.29 s) of the Pr form, i.e., $f_1 \gg f_2$ (energy transfer from Trp residues not in the vicinity of the chromophore and not in an optimal orientation is negligible), the average angle between the major energy donor Trp and the chromophore dipoles ($\Delta\theta$) can be calculated by a one-term Levshin equation:

$$P_0 = [3\cos^2(\Delta\theta) - 1]/[\cos^2(\Delta\theta) + 3]$$

Results are $\Delta\theta = 69°$ at 285 nm and 70° at 290 nm. These values suggest that the ^1L_b transition dipole (in-plane) of Trp is nearly perpendicular to the chromophore Q_y transition dipole (in-plane), so that the ^3L_a transition dipole of the former is favorably oriented (parallel for optimum orientation) for energy transfer.

Since the ^{3}Trp → Pfr energy transfer is negligible or less than efficient in Pfr as compared to that in Pr (cf. Table I),[2] it can be concluded that the Pr → Pfr transformation disturbs the orientational condition necessary for the ^{3}Trp → ^1Q_x energy transfer in Pfr. This explanation is consistent with the proposed model for the Pr → Pfr phototransformation in which the chromophore reorients with respect to its binding crevice.

The suggestion that the chromophore orientations of the Pr and Pfr forms of phytochrome are distinctly different is further supported by the fact that phytochrome-bound FMN transfers its excitation energy preferentially to Pr and not to Pfr via the singlet → singlet mechanism, although the R_0 values are virtually identical (25.8 and 22.2 Å for the ^{1}FMN → ^{1}Pr and ^{1}FMN → ^{1}Pfr transfers, respectively) (Sarkar & Song, 1981b, 1982).

The fluorescence quenching experiments shown in Figures 4 and 5 were performed to determine the extent to which the Trp residue(s) involved in the energy transfer in the Pr form is (are) exposed in the Pfr form. It is possible that the newly generated hydrophobic surface on the Pfr protein (Hahn & Song, 1981) includes one or more Trp residues, which become accessible to the solvent and the heavy atom quencher KI. A preferential quenching of the Trp fluorescence in Pfr is indeed observed in terms of both intensity and lifetime measurements (Figures 4 and 5). It should be noted that the CD spectra of both Pr and Pfr were found to be unperturbed by the presence of KI, indicating that the fluorescence quenching by KI is not due to a KI-induced conformational change. In fact, the data shown in Figure 4 (inset) establish that the quenching is dynamic.

In the foregoing discussion and earlier work (Hahn & Song, 1981), we specifically focused our attention on chromophore reorientation in the phototransformation of phytochrome. Such a chromophore reorientation is analogous to the ligand binding and dissociation processes of proteins. Thus, it is expected that chromophore reorientation entails at least local conformational changes of the peptide segments in the vicinity of the chromophore binding site. It is clear that there are no detectable conformational changes in the secondary structure of the protein (Tobin & Briggs, 1973; Song et al., 1979). Also, the claim that a local conformational change occurs outside the chromophore vicinity upon phytochrome phototransformation (Hunt & Pratt, 1981; Pratt, 1982) is unlikely without chromophore movement.

Therefore, in addition to the data presented here, the following results are consistent with the crucial role of the chromophore reorientation in the phototransformation of phytochrome: (a) 8-anilinonaphthalene-1-sulfonate (ANS) bleaches the Pfr chromophore, as ANS occupies the chromophore hydrophobic crevice, and both the dark reversion and photoreversion of Pfr are inhibited by ANS and their kinetics are modified (Hahn & Song, 1981); (b) the deuterium solvent isotope effect on Pr fluorescence is enhanced only after photocycling Pr → Pfr → Pr in D_2O (Sarkar & Song, 1981a); (c) the 360-MHz NMR signal of Pr at 6.17 ppm in D_2O disappears after photocycling, i.e., chromophore NH and/or tyrosyl OH protons exchange with deuterons only in the Pfr form (Song et al., 1982); (d) a significant difference in the hydrogen–tritium exchange between Pr and Pfr forms of phytochrome arises from the chromophore-carrying domain (60000 daltons) of undegraded phytochrome (Hahn & Song, 1982).

Concluding Remarks. The phytochrome-mediated movement of *Muogeotia* chloroplasts in response to polarized light can be best explained in terms of chromophore reorientation (Haupt & Weisenseel, 1976), thus lending support for the in vivo chromophore reorientation hypothesis. It is also of interest to ascertain whether the preferential triplet energy transfer from a tryptophyl residue(s) to the Pr chromophore plays an important role in the UV-induced morphogenesis of plants. Work is in progress to determine the specificities of tryptophyl singlet vs. triplet energy transfer to the chromophore.

[2] The minor component having a phosphorescence lifetime of 0.25 s (Table I and Figure 2) in Pfr is likely due to the phosphorescence contribution of the photostationary concentration of Pr in the Pfr solution.

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Infrared and Raman Spectra of *S*-Methyl Thioacetate: Toward an Understanding of the Biochemical Reactivity of Esters of Coenzyme A†

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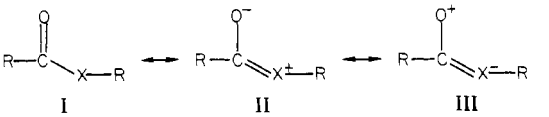
ABSTRACT: The infrared and Raman spectra of *S*-methyl thioacetate and $CD_3C(O)SCH_3$ have been determined. These spectra and those in the literature for *S*-ethyl thioacetate have been assigned. These data together with literature assignments for thioformic acid, thioacetic acid, and *S*-methyl thioformate were used in normal coordinate calculations. The stretching force constant for the carbonyl group of thioesters and thio acids is found to be very similar to that for the carbonyl group of ketones. In addition, the value of the stretching force constant for the C(O)–S bond in thioesters and thio acids indicates that this entity has no double-bond character. The carbonyl group of acetyl coenzyme A is essentially the same as that of a ketone.

The biochemical importance of coenzyme A and its acyl derivatives (Gregory & Lipmann, 1952) has led to a continuing interest in thioesters. These molecules have low carbonyl stretching frequencies, similar to those of amides, but the hydrogen-bond acceptor basicity of the carbonyl oxygen of a thioester is significantly lower than that of an amide (Baker & Harris, 1960).

Baker and Harris proposed an explanation for these properties that invoked resonance structures I–III, in which X is



I II III

sulfur. Structure III implies $(2p–3d)_\pi$ bonding that is im-

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