Molecular Topography of Intact Phytochrome Probed by Hydrogen–Tritium Exchange Measurements†

Tae-Ryong Hahn, Quae Chae,‡ and Pill-Soon Song*

ABSTRACT: In order to deduce the molecular topography of oat phytochrome, the hydrogen–tritium exchange kinetics of intact phytochrome (molecular weight 124 000) have been measured and compared with the data obtained previously with degraded proteins [Hahn, T.-R., & Song, P.-S. (1982) *Biochemistry 21*, 1394–1399]. In comparison with the large (molecular weight 118 000) and small (molecular weight 60 000) phytochromes, the intact phytochrome revealed a smaller difference in the number of exchangeable protons between the red-absorbing form of phytochrome (Pr) (368 protons per molecule) and the far-red-absorbing form of phytochrome (Pfr) (406). In addition, the intact Pr exchanged 22 more protons than the large Pr (346), whereas the intact Pfr exchanged 36 less protons than the large Pfr (442), indicating that an additional surface area exposed in the large Pfr is shielded in the intact Pfr. Kinetic analyses suggest that the 6000-dalton peptide segment of intact phytochrome interacts with the apoprotein moiety at or near the chromophore domain and induces a local conformational change of phytochrome. Thus, half-lives of group 2 (intermediately exposed) hydrogens are lengthened (from 2 to 5 h) in intact phytochrome. Unlike the situation in degraded proteins, in which fast-exchangeable (group 1) hydrogens are significantly more exchanged in the Pfr form than in the Pr form, most of the additionally exchanged protons in the intact Pfr are attributable to the intermediate (group 2) kinetic component. These results suggest that a fully exposed, hydrogen-bonded peptide domain (α-helix and/or β-pleated sheet) in the large Pfr is shielded in the intact Pfr and that the extra exchangeable protons in the intact Pfr are mainly due to the partial exposure of the hydrogen-bonded peptide segments upon phototransformation. This is consistent with a conformational change of phytochrome upon phototransformation of Pr to Pfr.

Phytochrome, a blue-green pigment protein, plays a central role in various photomorphogenic responses in higher plants. The photoreceptor protein exists in two photoreversible forms; red-absorbing (Pr)[1] and far-red-absorbing (Pfr) forms. The latter produced from the former by red light is believed to be the physiologically active form (Pratt, 1979; Schmidt & Mohr, 1982; Kendrick & Frankland, 1983).

Since the first successful isolation of phytochrome (Butler et al., 1959), diverse approaches have been made in order to understand the molecular mechanism of the phototransformation of phytochrome. Several aspects of the molecular differences between the Pr and Pfr forms of phytochrome have been determined, and possible mechanisms of the phototransformation of phytochrome have been proposed [see recent reviews by Pratt (1982) and Song (1984)]. However, a recent examination of the phytochrome molecule indicates that most of the purified phytochromes over the last 2 decades are derived from partially degraded products of the intact molecule (Kerscher & Nowitzki, 1982; Vierstra & Quail, 1982). During extraction and purification, the native phytochrome of apparent molecular mass 124 kilodaltons (kDa) from oat can readily be degraded to 118 kDa (further to 60 kDa) by endogenous proteases [Vierstra & Quail, 1982; see review by Song (1984)]. Several physical and chemical properties are altered upon limited proteolysis of the intact molecule (Vierstra & Quail, 1983a,b; T.-R. Hahn et al., unpublished results; Song, 1984). Thus, it is necessary to reexamine the properties of intact phytochrome with the methods used for the degraded proteins.

The hydrogen–tritium exchange method has been used extensively to probe structural changes of proteins and nucleic acids [Englander, 1968; Liem et al., 1980; see Englander & Englander (1978) and references cited therein]. This method is particularly useful to detect conformational changes of proteins, because no critical alteration of the protein structure is induced by the method itself. The structural differences between the degraded Pr and Pfr forms of phytochrome have been determined by the method of Hahn & Song (1982). Results showed that the Pfr molecules exchange 70 and 96 more protons than the Pr in both small (60 kDa) and large (118 kDa) phytochromes, respectively, suggesting that the phototransformation of phytochrome is accompanied by an additional exposure of surface area in the Pfr form.

In this study, we discuss the protein structure of intact phytochrome by employing the hydrogen–tritium exchange method. From the comparison of the present data with our previous H–T exchange data on the degraded phytochromes (Hahn & Song, 1982), we wish to elucidate the molecular topography of intact phytochrome in this paper.

Materials and Methods

Hydroxylapatite [$Ca_{10}(PO_4)_6(OH)_2$] was made by the method of Siegelman et al. (1965) with 1 mol of KOH to convert 1 mol of brushite to hydroxylapatite. Tritiated water (500 mCi/g) was obtained from New England Nuclear (Boston, MA). Poly(ethylenimine) and $(NH_4)_2SO_4$ (ultra pure, special enzyme grade) were purchased from Eastman Kodak (Rochester, NY) and Schwarz/Mann (Orangeburg, NY), respectively. Affi-gel Blue and Bio-Gel A-1.5 m were

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[1] Abbreviations: Pr, red-absorbing form of phytochrome; Pfr, far-red-absorbing form of phytochrome; kDa, kilodalton; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; EG, ethylene glycol; 2-ME, 2-mercaptoethanol; Tris, tris(hydroxymethyl)aminomethane; FMN, flavin mononucleotide; ANS, 8-anilinonaphthalene-1-sulfonate; TNM, tetranitromethane.

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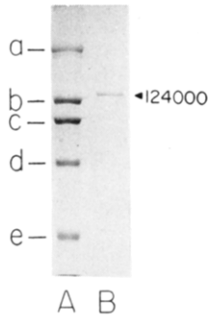

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified intact phytochrome. A slab gel (thickness, 1.5 mm) containing 7.5% acrylamide was prepared by the method of Laemmli (1970) and stained with Coomassie Blue R. Lane A, reference; each band contains 2.5 μg of (a) myosin (M_r 200 000), (b) β-galactosidase (M_r 116 250), (c) phosphorylase (M_r 92 500), (d) bovine serum albumin (M_r 66 200), and (e) ovalbumin (M_r 45 000). Lane B, 1 μg of purified intact phytochrome; the arrow indicates the calibrated molecular weight of phytochrome.

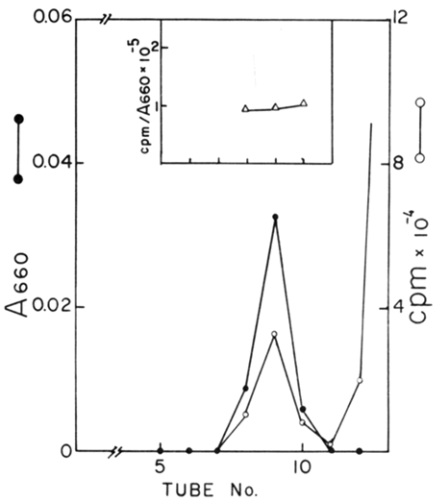

FIGURE 2: Gel filtration profile (Sephadex G-25–150, 1.0 × 12.0 cm) of phytochrome (Pr) incubated in tritiated water (10 mCi/mL) at 278 K for 1 h. A 0.25-mL aliquot was applied to the column and eluted with 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM EDTA and 14 mM 2-ME at a flow rate of 2.5 mL/min. A 0.1-mL aliquot of each fraction (0.5 mL/tube) was counted for radioactivity. The inset shows the ratio of counts per minute to absorbance over the peak region.

obtained from Bio-Rad Laboratories (Richmond, CA). Other chemicals including FMN and Sephadex G-25–150 were from Sigma Chemical Co. (St. Louis, MO). Deionized, redistilled water was used for all buffers for phytochrome purification and sample preparations.

The undegraded, intact phytochrome (molecular weight 124 000 as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis) (Figure 1) was purified from etiolated oat seedlings (*Avena sativa* L., CV Garry), as described by Vierstra & Quail (1983a). In essence, 2 kg of fresh tissue was extracted with 1.5 L of 100 mM Tris buffer, pH 8.3, containing 140 mM $(NH_4)_2SO_4$, 10 mM EDTA, 20 mM $NaHSO_4$, 4 mM phenylmethanesulfonyl fluoride (PMSF), and 50% (v/v) ethylene glycol (EG). The tissue was illuminated for 10 min at 277 K under a gold fluorescent lamp (approximately 8000 lm) before extraction. Extracts were precipitated with 30 mL of 10% poly(ethylenimine), and the supernatant was used for 25% $(NH_4)_2SO_4$ fractionation. The phytochrome pellet obtained was dissolved in 300 mL of 50 mM Tris buffer, pH 7.8, containing 14 mM 2-mercaptoethanol (2-ME), 5 mM EDTA, 2 mM PMSF, and 25% EG and chromatographed through a hydroxylapatite column (70 mL). The column was stepwise eluted with potassium phosphate buffer (5, 15, and 60 mM), pH 7.8. After the phytochrome was fractionated with 30% saturated $(NH_4)_2SO_4$, the pellet obtained was resuspended in 10 mM potassium phosphate buffer, pH 7.8, containing 5 mM EDTA, 14 mM 2-ME, and 2 mM PMSF under the far-red light and then chromatographed through an Affi-gel Blue column (100 mL). Phytochrome eluted with 10 mM FMN was further chromatographed on a Bio-Gel A-1.5 m column (2.4 × 48 cm). The final preparation (8 mg) in 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM EDTA, 14 mM 2-ME, and 5% glycerol had a specific absorbance ratio (A_{660}/A_{280}) of 1.0–1.1.

The gel permeation chromatography developed by Englander (1963, 1968) was employed to follow both the exchange-in and exchange-out rates of hydrogen–tritium exchange of the intact phytochrome, as described previously (Hahn & Song, 1982). In exchange-in experiments, 1.5 mL of phytochrome (A_{660} ~0.2) in 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM EDTA, 14 mM 2-ME, and 5% glycerol was incubated with 30 μL of tritiated water (specific activity 500 mCi/g). Aliquots (0.25 mL) were taken from the incubation mixture at different time intervals and applied to a Sephadex G-25 column (0.5 × 12.0 cm). The column was eluted with the same buffer without glycerol at a flow rate of 2.5 mL/min. The collected fractions (0.5 mL/tube) were measured for absorbance and radioactivity (0.1 mL). Figure 2 shows a typical elution profile of the gel filtration chromatography, following the above procedure. The ratios of the radioactivity to the absorbance are shown in the inset of Figure 2. As the values of each of three fractions were essentially constant, the protein incorporation with tritium is well separated from the incubated medium. The averages of the constant ratios for two to three fractions were used for the calculation of the number of exchanged hydrogens per molecule.

The kinetics of exchange out were measured by both one-column and two-column methods as described previously (Hahn & Song, 1982). The one-column method was used for initial exchange-out kinetics within 30 min, and the two-column procedure was employed for the exchange-out measurements of longer incubation times. For one-column separation, the incubation mixture, which was prepared in the same manner as the exchange-in experiment and incubated for 3 days at 278 K, was chromatographed on a Sephadex G-25 column (0.5 × 12 cm). During elution, the column operation was stopped, and at any given incubation time, the flow was resumed. During incubation, the tritium bound to the protein was exchanged out to the medium in the column. When phytochrome was incubated for longer than 30 min, the chromatographic resolution deteriorated mainly due to the diffusion of the background tritium into the protein. For longer than 30-min incubation, the two-column procedure was used. The incubation sample (1.5 mL for 3 days) was first separated by a Sephadex G-25 column (2.5 × 10 cm) at a flow rate of 8 mL/min. The collected fractions (2 mL/tube) were immediately measured for absorbance and radioactivity. An aliquot (0.5 mL) taken from the pool of the major fractions of the first column at different incubation times was then passed through a second column (0.5 × 12 cm) to remove the exchanged-out tritium during incubation. A typical elution profile is shown in Figure 3.

All experiments were performed in a cold chamber (278 K) under safe green light. Irradiation of phytochrome for the phototransformation was carried out with a Bausch & Lomb microscope illuminator combined with red (Oriel c572-6600,

Table I: Half-Life ($t_{1/2}$) and Number of Exchanged Hydrogens per Molecule for Each Kinetic Component, Evaluated from the Exchange-Out Curves of Phytochrome

	group 1		group 2		group 3		total
phytochrome	$t_{1/2}$ (min)	^{3}H/molecule	$t_{1/2}$ (h)	^{3}H/molecule	$t_{1/2}$ (h)	^{3}H/molecule	^{3}H/molecule
Pr	6.2	59 ± 3	2.0	75 ± 5	88	34 ± 2	168
60-kDa Pfr[a]	7.9	90 ± 3	2.5	108 ± 6	79	40 ± 3	238
Δ^3H		31		33		6	70
Pr	6.0	118 ± 3	1.6	148 ± 9	62	80 ± 6	346
~118-kDa Pfr[a]	7.3	181 ± 9	1.5	180 ± 10	54	81 ± 7	442
Δ^3H		63		32		1	96
Pr	6.3	159 ± 3	5.0	127 ± 7	54	82 ± 2	368
124-kDa Pfr	7.2	163 ± 4	4.7	160 ± 6	53	83 ± 3	406
Δ^3H		4		33		1	38

[a] Data from Hahn & Song (1982).

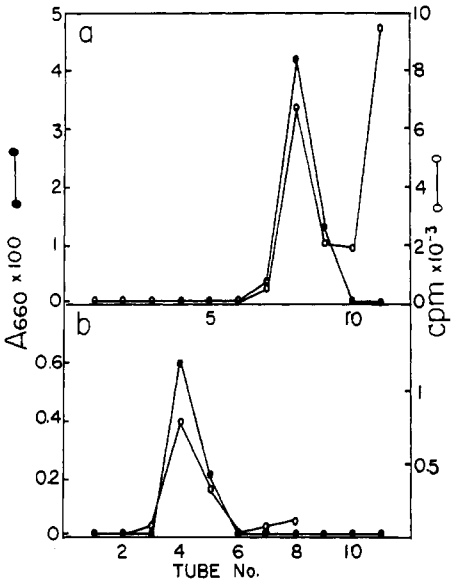

FIGURE 3: Two-column method for tritium exchange-out measurements for intact Pr. (a) Gel filtration profile of the G-25–150 column (2.5 × 10.0 cm). Pr (1.5 mL) incubated in tritiated water (10 mCi/mL) at 278 K for 3 days was applied to the column and eluted with potassium phosphate buffer, pH 7.8, containing 5 mM EDTA and 14 mM 2-ME, at a flow rate of 8.0 mL/min. The collected fractions (2.0 mL/tube) were measured for the absorbance and radioactivity. (b) Gel filtration profile of the second G-25–150 column (1.0 × 12.0 cm) for Pr, 2 h of exchange-out. A 0.5-mL aliquot was taken from the pooled fraction of the first column and passed through a second column at a flow rate of 2.5 mL/min. A 1.0-mL sample of the effluent was collected per tube.

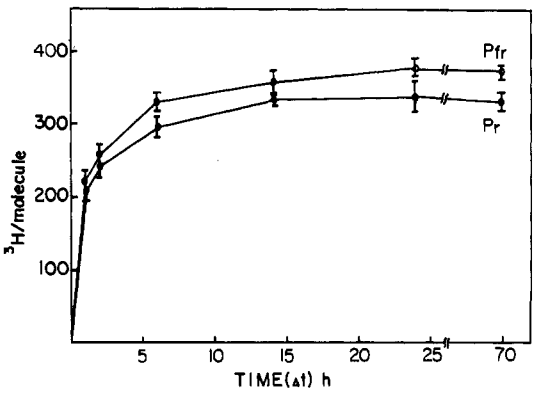

FIGURE 4: Tritium exchange-in curves for intact phytochrome at 278 K in 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM EDTA, 14 mM mercaptoethanol, and 5% glycerol. The number of tritiums exchanged per molecule of phytochrome (^{3}H/molecule) was calculated by using eq 1.

7.5 W/m^2) and far-red (Ealing 26-4457, 1.6 kW/m^2) filters. The absorbance was measured with a Cary 118C spectrophotometer. The concentrations of Pr and Pfr were calculated from the reported molar extinction coefficient for Pr (Vierstra & Quail, 1983b) and the measured Pfr spectrum, respectively.[2] During incubation, spectra for the Pr and Pfr samples were routinely recorded, and no detectable degradation of phytochrome or dark reversion of Pfr was observed. The radioactivity was counted in a xylene-based scintillation fluid containing 0.3% 2,5-diphenyloxazole, 0.02% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 25% Triton X-114 with a Beckman L200 liquid scintillation counter. The number of exchanged hydrogens was calculated as follows:

$$^3\text{H per molecule} = 111(C/C_0)(\epsilon_p/A) \qquad (1)$$

where C_0 and C are the radioactivities of the incubation mixture and the tritium-incorporated protein, respectively, and ϵ_p and A are the molar extinction coefficient and the absorbance of the protein, respectively. The numerical constant of 111 is the atom concentration of hydrogen in water. A mean point was obtained from the measurements of three independent chromatographic runs for both exchange-in and exchange-out experiments.[3]

Results

Hydrogen–tritium exchange experiments run on degraded phytochrome indicated that the Pfr form exchanges more protons than the Pr form by 96 and 70 protons for the large (118 kDa) and small (60 kDa) degraded phytochromes, respectively (Hahn & Song, 1982). The present data for the intact phytochrome (124 kDa) also show that the number of exchanged protons is greater for Pfr than for Pr (Figure 4). However, the difference in the number of exchanged hydrogens per molecule between the intact Pr and Pfr forms is markedly lower (by 38 protons) than that between the degraded Pr and Pfr molecules at equilibrium (Figure 4, Table I).

The exchange-in kinetics for intact phytochrome show essentially the same patterns as the previous results for the degraded phytochromes (Hahn & Song, 1982); a rapid initial exchange is followed by an intermediate rate exchange as the

[2] A value of 73 000 M^{-1} cm^{-1} at 665 nm was used for comparison with previous results (Hahn & Song, 1982), in which we used 70 000 M^{-1} cm^{-1} at 665 nm for large Pr (Tobin & Briggs, 1973). If higher reported extinction coefficients are used, the number of tritiums exchanged per molecule of phytochrome calculated by eq 1 will be changed. For example, if values of 118 000 M^{-1} cm^{-1} at 665 nm for large Pr (Brandlmeier et al., 1981) and 121 000 M^{-1} cm^{-1} at 668 nm for intact Pr (Cha et al., 1983) are employed, the number of exchanged tritiums calculated by eq 1 will be increased by 69% and 66%, respectively.

[3] Exchangeable hydrogens for large phytochrome (118 kDa) were redetermined along with intact phytochrome, with values in agreement with the previously published data (Hahn & Song, 1982) within experimental errors.

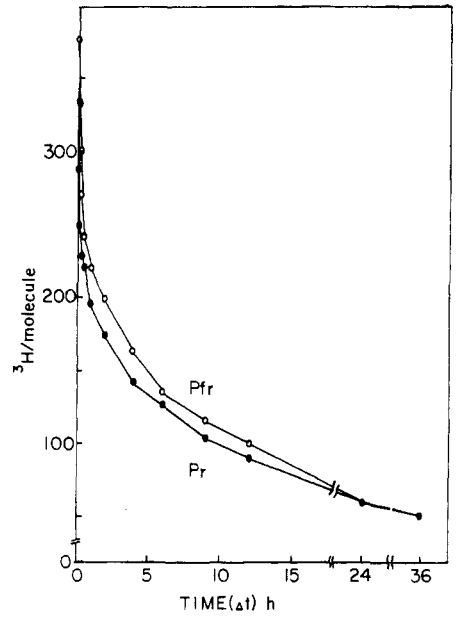

FIGURE 5: Tritium exchange-out curves of intact phytochrome at 278 K against 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM EDTA and 14 mM mercaptoethanol. The number of tritiums exchanged per phytochrome molecule (^{3}H/molecule) was calculated by using eq 1.

system approaches equilibrium. At 5-h incubation, the system almost levels off, and after 24-h incubation, no further increase in exchange-in has been observed (Figure 4). In order to analyze the hydrogen–tritium exchange kinetics, kinetic measurements of exchange-out are essential. In contrast to exchange-in, the exchange-out kinetics can be resolved as pseudo first order, as the concentration of water far exceeds that of the bound tritium.

Figure 5 shows the tritium exchange-out kinetic curves for the intact phytochrome in 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM EDTA and 14 mM 2-ME at 278 K. Within 1 h, about half of the exchange-in tritiums are exchanged out, and the kinetic curves follow a gradual decrease and then level off. The exchange-out kinetics should follow simple first order if the environments of the exchangeable hydrogens are homogeneous. Generally, several groups of first-order kinetic components can be resolved in a native protein [see Englander & Englander (1978) and references cited therein]. Hydrogens of an "instantaneous exchange group" exchange too rapidly to measure, as the exchanged-in protons are instantaneously exchanged out before the column operation is completed. Group 1 hydrogens exchange in a relatively fast rate (half-life in minutes), and group 2 has an apparent half-life of several hours. The half-life of group 3 hydrogens usually is several days.

The exchange-out curves for intact phytochrome were resolved by the peeling procedure (Van Liew, 1967), as has been described for the degraded phytochromes (Hahn & Song, 1982). Figure 6 represents a first-order plot for exchange-out after peeling (subtracting) the slowest first-order kinetic component (group 3) from the original plot. The slowest exchange component (Table I) is resolved from a first-order plot of the original data (Figure 5), where the extrapolated intercept and the slope represent the magnitude and the rate constant of the exchange component, respectively. The inset of Figure 6 shows the fast kinetical component (group 1) after peeling an intermediate exchange component (Group 2) from the curves in the main panel of Figure 6. Again the extrapolated intercept is the magnitude of the exchange component, and the slope indicates the rate constant of the exchange component. Results analyzed are shown in Table I.

Group 1 protons exchange with half-lives of 6–7 min. Groups 2 and 3 show markedly slower exchange rates, with half-lives of 5 and 50 h, respectively. As can be seen in Table I, the half-lives of groups 1 and 3 in the intact phytochrome are essentially identical with those in the degraded phytochromes, whereas group 2 hydrogens exchange significantly slower in the intact protein ($t_{1/2} \sim 5$ h) than in the degraded proteins ($t_{1/2} \sim 2$ h).

As shown in the exchange-in data (Figure 4), exchange-out measurements also show that the intact Pfr exchanges more protons (by 38) than the Pr (Table I). Interestingly, the intact Pr exchanges 22 more protons than the large Pr (a 6% increase), while the intact Pfr exchanges 32 less protons than the large Pfr (a 7% decrease). In addition, most of the additionally exchangeable protons in the intact Pfr are attributable to group 2. These results suggest that a large surface area exposed in the large Pfr is substantially shielded in the intact Pfr. In the degraded phytochromes, more than half of the additionally exchanged protons in Pfr are of the fast-exchanging component (group 1).

Discussion

The difference in the total number of exchangeable protons per molecule between the Pr and Pfr forms is smaller with the intact (by 38 protons) than with the large phytochrome (by 96 protons) (Table I), indicating that the additional surface area exposed in the large Pfr is substantially shielded in the intact Pfr. This result is consistent with 8-anilinonaphthalene-1-sulfonate (ANS) and tetranitromethane (TNM) experiments of phytochromes. ANS binds to the large Pfr with higher affinity than the corresponding Pr (Hahn & Song, 1981), but it shows no significant difference between the intact Pr and Pfr forms (T.-R. Hahn, P. H. Quail, H. K. Sarkar, P.-S. Song, and R. D. Vierstra, unpublished results), suggesting that the ANS binding site exposed in the large Pfr is significantly protected in the intact Pfr. Proteolytic degradation of the intact phytochrome to the large phytochrome increases TNM oxidation of the Pfr chromophore by 6-fold without changing the oxidation rate of the Pr chromophore (T.-R. Hahn et al., unpublished results). Thus, the chromophore of the intact Pfr is also substantially shielded.

Of 1773 theoretically exchangeable hydrogens calculated from the known amino acid composition of the intact phytochrome (Vierstra & Quail, 1983a), aside from unmeasurably fast exchangeable protons, only 368 (21%) and 406 (23%) hydrogens are actually exchanged in the intact Pr and Pfr, respectively. In large phytochrome, the observed values were 346 (21%) for Pr and 442 (26%) for Pfr out of 1681 theoretical values (Hahn & Song, 1982). As less than half of the total exchangeable hydrogens are actually measurable at the defined pH (7.8) and temperature (278 K) [see Englander & Englander (1978) and references cited therein], the phytochrome molecules appear to possess a compact structure.

The total exchanged protons are increased by 22 on going from the large Pr to the intact Pr. However, the ratio of the observed exchanged protons to the theoretical values remains constant (21%) (vide supra). This result suggests that the difference in the number of exchanged protons between the large and intact Pr's is due to the different molecular sizes, and thus no gross changes in the tertiary structure are involved. On the other hand, the intact Pfr exchanges 36 less protons than the large Pfr, and the ratio of the observed to the calculated exchangeable protons is decreased by 3% on going from 118- to 124-kDa protein. The result suggests that in contrast to the Pr forms, the additional 6-kDa peptide segment in intact

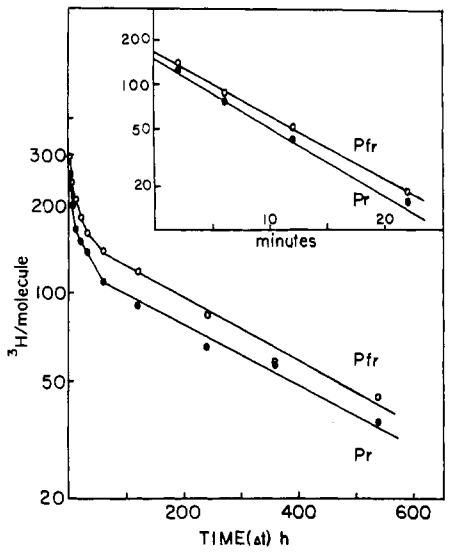

FIGURE 6: Peeling plots for groups 1 and 2 exchangeable tritiums (exchange-out) after subtracting the group 3 component from the first-order plot of exchange-out curves from Figure 1. The inset shows the peeling plots after subtracting the group 2 kinetic components from the plots shown in the main panel.

phytochrome induces an apparent conformation change or shields part of the peptide chains in the intact Pfr.

Most of the exchangeable hydrogens in the exposed functional groups and random-coil domains of the peptide backbone are not measurable (Englander, 1963; Englander & Englander, 1978). Thus, it has been assumed that the exchangeable hydrogens measured represent hydrogen-bonded protons in the α-helical and/or β-pleated sheet domains. As has been suggested previously (Hahn & Song, 1982), group 1 and group 2 hydrogens are assigned to fully and intermediately exposed hydrogen-bonded protons, respectively. Group 3 is hydrogens in the folded peptide domains sterically blocked from direct contact with water. As can be seen in Table I, group 3 hydrogens exchange extremely slowly with half-lives of more than 2 days. Thus, a relatively small number of group 3 hydrogens are actually observed, and essentially identical values can be seen in different spectral (Pr vs. Pfr) and molecular (118 vs. 124 kD) forms.

Group 1 exchangeable hydrogens show essentially identical half-lives regardless of the different spectral forms and molecular weights of phytochrome. On the other hand, the half-lives of the group 2 exchangeable hydrogens of intact phytochrome are significantly longer than those of the degraded proteins. Furthermore, the additionally exchangeable protons in the intact Pfr relative to the degraded proteins are confined in the group 2 kinetic components. In the degraded phytochromes, significant numbers of the additionally exchanged protons in Pfr are attributed to the group 1, fast-exchangeable hydrogens. These results suggest that a fully exposed peptide domain with a hydrogen-bonded secondary structure (α-helix and/or β-pleated sheet) in the large Pfr is now shielded in the intact Pfr. Results also suggest that the 6-kDa peptide segment in the intact phytochrome induces a noticeable change in the protein structure and thus causes lengthening of the half-lives of group 2 hydrogens. This conformational change may occur at or near the chromophore domain of the intact phytochrome as deduced from ANS (T.-R. Hahn, P. H. Quail, H. K. Sarkar, P.-S. Song, and R. D. Vierstra, unpublished results) and TNM results (T.-R. Hahn et al., unpublished results) and may be responsible for the spectral shift and the structure of the chromophore. As reflected by the changes in the visible region of the absorption spectrum (Vierstra & Quail, 1983) and the CD spectra of intact Pfr, the 6-kDa peptide appears to interact with the intact Pfr chromophore. The peptide is apparently exposed and accessible to proteolytic attack in the Pr form; thus, it causes almost no effect on the Pr spectra, suggesting that the Pr chromophore is buried within the binding pocket for both large and intact phytochrome (Hahn & Song, 1981; Song et al., 1979; Song, 1984; T.-R. Hahn et al., unpublished results).

It should be noted here that the phototransformation of phytochrome may be accompanied by changes in the secondary structure of the intact phytochrome, as judged from the far-UV region of the CD spectra ($\sim$5% increase in the CD signal at $\sim$200–250 nm for the intact Pfr) (T.-R. Hahn, P. H. Quail, H. K. Sarkar, P.-S. Song, and R. D. Vierstra, unpublished results). There are no significant changes in the far-UV CD spectra upon phototransformation of the large phytochromes (Tobin & Briggs, 1973; Song et al., 1979). Thus, it may be assumed that the additionally exchanged protons confined within the intermediate (group 2) kinetic component in the intact Pfr molecule are possibly due to an increase in the folded α-helical and/or β-pleated sheet structure upon Pr $\rightarrow$ Pfr phototransformation.

In conclusion, the 6-kDa peptide segment interacts with the 118-kDa protein moiety at or near the chromophore domain in intact phytochrome. Furthermore, an exposed α-helical and/or β-pleated sheet domain in the large Pfr is now shielded in the intact Pfr by the 6-kDa peptide and/or its induced conformational change, as can be deduced from the observation that an increase in only the group 2 (intermediate) exchangeable hydrogens occurs in the intact Pfr upon phototransformation from the Pr form.

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Human Blood Platelet Secretion: Optical Multichannel Analyzer Measurements Using Acriflavine as a Release Indicator†

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ABSTRACT: Blood platelets preloaded with the fluorescent amine acriflavine release the trapped fluorophore after stimulation with thrombin or the divalent cation ionophore A23187. Release was detected by an increase in acriflavine fluorescence, which is otherwise strongly quenched in the platelet, by using an optical multichannel analyzer to monitor the spectral and temporal reaction parameters. The secretion of [^{14}C]serotonin and acriflavine is well correlated, suggesting that acriflavine, like serotonin and the closely related fluorescent drugs mepacrine and acridine orange, is accumulated in and released from platelet dense bodies. Acriflavine secretion at 37 °C in the absence of external calcium is characterized by a short delay, followed by a rapid biphasic increase in fluorescence that implies at least a three-stage secretory process. For saturating levels of thrombin the delay was 1.5 s and release was 90% complete within 6-7 s. The delay could not be shortened by prestimulation under conditions that induce shape changes but not release, i.e., with ADP, arachidonic acid, or low levels of thrombin or A23187. Acriflavine secretion induced by A23187 was similar but less effective; the reaction was slower, the yield was smaller, and, in contrast to thrombin, the longer lag period could be significantly shortened by prestimulation.

Blood platelets are activated by agents of diverse structure, responding in a characteristic pattern of shape changes, secretion, and aggregation. The actual mechanism of secretion has not been elucidated in detail, but it is generally assumed that it includes fusion of the organelle and plasma membranes, followed by extrusion of the organelle contents (Skaer, 1981). The best characterized secretory granules of human platelets are the dense bodies, which contain and selectively release amines (mainly serotonin), nucleotides (mainly ATP and ADP), and calcium ions (Pletscher et al., 1971). Virtually all the serotonin in whole blood is contained in the platelet dense bodies and platelets possess a highly efficient system to transport serotonin and other amines across the plasma and granule membranes (Rudnick et al., 1980). This physiological mechanism has been widely used to load dense bodies with radiolabeled serotonin for studying the release reaction. Furthermore, it has been established that fluorescent amines such as mepacrine and acridine orange selectively accumulate in dense bodies, allowing a precise visualization of these granules with microscopic techniques (Skaer, 1981; Lorez et al., 1975; Skaer et al., 1981).

The present report indicates that the fluorescent dye acriflavine is, like other amines, specifically accumulated in platelet dense bodies. The acriflavine fluorescence is efficiently quenched during incorporation of the dye into the platelet, and the apparent quantum yield increases 5-fold or more upon secretion. We have used this system to investigate the rapid release induced by thrombin and the ionophore A23187 in real time, focusing our interest on the common and different features of secretion induced by the two stimuli under physiological conditions.

Experimental Procedures

Preparation of Gel-Filtered Human Blood Platelets. Citrated blood was obtained from the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross within 20 h of collection, and platelet-rich plasma (PRP)[1] was prepared by standard techniques (Bettex-Galland & Lüscher, 1960). All further operations and all experiments (except where noted) were carried out with platelets continuously maintained at 37 °C. The PRP was titrated with acid citrate dextrose (ACD) to pH 6.5, diluted to 1×10^9 platelets/mL with citrate buffer (30 mM sodium citrate, 100 mM NaCl, 4.8 mM glucose, 3 mM KCl, pH 6.5), and incubated for 20 min to obtain platelets with a flat disk shape (Patscheke, 1981). Ten milliliters of this ACD-PRP was applied on a thermostated Sepharose 2B column (35 mL of Sepharose) equilibrated with the elution buffer (147 mM NaCl, 4.8 mM glucose, 3 mM KCl, 3 mM MES/NaOH, pH 6.5). The platelets, collected at a rate of 0.5 mL/min, retained their discoid shape on the column. The pH of the gel-filtered platelets was adjusted to

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[1] Abbreviations: ACD, acid citrate dextrose (NIH formula A: 0.8% citric acid, 2.2% sodium citrate, 2.45% hydrous glucose); ISIT, intensified silicon intensified target; MES, 2-(*N*-morpholino)ethanesulfonic acid; OMA, optical multichannel analyzer; PMS, phenylmethanesulfonyl; PRP, platelet-rich plasma; TES, 2-[[tris(hydroxymethyl)methyl]-2-amino]ethanesulfonic acid.

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