Ultraviolet Resonance Raman Spectra of Pea Intact, Large, and Small Phytochromes: Differences in Molecular Topography of the Red- and Far-Red-Absorbing Forms†

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Received January 28, 1993; Revised Manuscript Received April 19, 1993

ABSTRACT: Ultraviolet resonance Raman (UV RR) spectra excited at 244 nm were observed for pea intact, large, and small phytochromes at pH 7.8. Raman bands assignable to Trp residues dominated the UV RR spectra. The intensity ratios of Trp W7 doublet bands, $I(1358)/I(1342)$, of all three phytochromes in the red light-absorbing form (P_r) were almost the same as that of an aqueous Trp solution, indicating that most of the six and four Trp residues in the 59-kDa chromophoric and the C-terminal 59-kDa nonchromophoric domains, respectively, reside in hydrophilic microenvironments in P_r. This ratio increased under red light illumination, where photoequilibria are attained between P_r and the far-red-absorbing form (P_{fr}) for intact and small phytochromes and among P_r, a bleached intermediate (I_{bl}), and P_{fr} for large phytochromes. The increase of the intensity ratio was most prominent for small phytochromes. These observations suggest that the microenvironments around some Trp residues become more hydrophobic due to conformational changes induced by phototransformation from P_r to I_{bl} and that the hydrophobicity increase occurs mainly in the chromophoric domain. Among the six Trp residues in the chromophoric domain, Trp^{365} and Trp^{567} are likely candidates for those involved in this hydrophobicity increase. The intensity distribution of the amide I band shows little β-sheet in both P_r and P_{fr} of the intact, large, and small phytochromes and indicates that α-helices and nonregular structure are less populated in the chromophoric domain than in the N-terminal 6-kDa segment and the C-terminal nonchromophoric domain.

Phytochrome is a chromoprotein found in green plants and serves as a photoreceptor for a variety of photoregulatory processes (Furuya, 1987; Rudiger & Thummuler, 1990; Sage, 1992). The chromoprotein is categorized into three members, types A, B, and C (Scharrock & Quail, 1989). The phytochrome that is rich in etiolated tissues and disappears under light is called type A, and that found in both etiolated and green tissues with a much less amount is called type B. While the two kinds of phytochrome have proven to play different roles in morphogenetic responses (Kendrick & Nagatani, 1991), both have two similar photoconvertible forms, that is, a physiologically inactive red-absorbing form (P_r)[1] and an active far-red-absorbing form (P_{fr}). Type A phytochrome used in this study consists of two identical subunits with a 2,3-dihydrobiliverdin chromophore in each (Lagarias & Rapoport, 1980).

The primary structures of phytochromes from several plants have been determined from nucleotide sequences (Hershey et al., 1985; Sato, 1988; Kay et al., 1989; Sharrock & Quail, 1989). Their secondary structures have been studied by CD spectra (Vierstra et al., 1987; Chai et al., 1987; Sommer & Song, 1990) and also by various predictive methods (Parker & Song, 1990). The tertiary and quaternary structures were investigated by electron microscopy (Jones & Erickson, 1989; Tokutomi et al., 1989; Nakasako et al., 1990), by quasi-elastic light scattering (Sarker et al., 1984), and by small-angle X-ray scattering (Tokutomi et al., 1989; Nakasako et al., 1990). These methods, however, are not always effective for studying structural changes induced by the phototransformation due to their low resolution.

Information regarding the physiological aspects of phytochrome-dependent regulations has been sufficiently accumulated, and currently a molecular mechanism of signal transduction, especially from phytochrome to its second partner, is of interest. An approach to this problem entails characterization of light-induced structural changes. Recent vibrational spectroscopic studies made some progress in exploring the primary photoprocess of the chromophore (Fodor et al., 1988, 1990; Farrens et al., 1989; Rospendowski et al., 1989; Siebert et al., 1990; Tokutomi et al., 1990; Mizutani et al., 1991; Hildebrandt et al., 1992), but the accompanied conformational changes in the protein moiety, which lead to signal transduction, remain to be clarified.

So far, biochemical studies, such as chemical modification (Smith & Cyr, 1988; Eilfeld et al., 1988), monoclonal antibody binding (Schneider-Poetsch et al., 1989), or enzymatic modification (Wong et al., 1986; Grimm et al., 1988;

† This work was supported in part by Grants-in-Aid for Scientific Research 0766 to Y.M. and 02454560 to S.T. and by Grant 04NP0301 to T.K. for the new program "Intelligent Molecular Systems with Controlled Functionality" from the Ministry of Education, Science, and Culture of Japan. Y.M. and S. K. were supported by the fellowship of Japan Society for the Promotion of Science for Japanese Junior Scientists. This paper is dedicated to the late Professor Issei Harada.

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[1] Abbreviations: UV RR, ultraviolet resonance Raman; P_r and P_{fr}, red- and far-red-light-absorbing forms of phytochrome, respectively; I_{bl}, bleached intermediate of phytochrome; SAR, specific absorption ratio; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; Trp, tryptophan; Tyr, tyrosine; Phe, phenylalanine.

0006-2960/93/0432-6916$04.00/0

McMichael et al., 1990), have provided useful information concerning the light-induced conformational changes in the protein moiety. These probe-mediated methods, however, may possibly bring in some side effects and lead to observation of secondary and indirect effects in an extreme case.

Ultraviolet resonance Raman (UV RR) spectroscopy has recently been shown to be a powerful technique to investigate protein structure (Rava & Spiro, 1984; Johnson et al., 1984; Bajdor et al., 1987; Mayne & Hudson, 1987; Harada et al., 1986; Miura et al., 1988; Ames et al., 1992). Some side chain modes of aromatic amino acid residues, which are selectively enhanced by tuning the excitation wavelength, are sensitive to the microenvironment in proteins [see Kitagawa and Kaminaka (1992) for a review]. Very recently, UV RR spectra were observed for the intact phytochrome by Harada and co-workers (Toyama et al., 1993), although detailed analysis has not been carried out yet. Here we present UV RR spectra of pea intact, large, and small phytochromes obtained by 244-nm excitation and will discuss differences in protein topography between P_r and P_{fr}.

MATERIALS AND METHODS

Phytochrome and Amino Acid Preparations. The type A intact (subunit molecular mass 121 kDa) and large phytochromes (subunit molecular mass 114 kDa) were isolated from 7-day-old etiolated seedlings of pea (*Pisum sativum* cv. Alaska) as described by Tokutomi et al. (1988). Pea small phytochrome was prepared as a monomeric polypeptide by tryptic digestion of the large phytochrome according to Yamamoto and Furuya (1983). Concentrated phytochrome samples were prepared by ammonium sulfate precipitation followed by resuspension in a 50 mM HEPES and 1 mM Na_2EDTA solution, pH 7.8. The concentrated solutions were dialyzed against the same buffer solution to remove residual ammonium sulfate completely. This procedure was indispensable to prevent the sample with high concentrations from aggregation during UV RR measurements. All the preparation procedures were carried out under a dark or dim green safe light.

The specific absorbance ratios (SAR = A_{667}/A_{280}) of the intact, large, and small phytochromes were 0.90–0.95, and 0.95–0.98, and 0.70, respectively, and their purities were estimated from SDS–PAGE to be higher than 90, 95, and 95% for the intact, large, and small phytochromes, respectively. Each preparation was used for only a single UV RR measurement. Absorption spectra measured after Raman experiments indicated a 5–10% decrease in the SAR, presumably due to partial bleaching of the chromophore, and full photoreversibility for the intact phytochrome and no change in SAR and full photoreversibility for large and small phytochromes. The SDS–PAGE pattern after Raman experiments indicated no degradation into smaller polypeptides for all three phytochromes. Concentrations of the intact, large, and small phytochrome solutions were determined optically by using values of $\epsilon_{667} = 1.32 \times 10^5$ (Lagarias et al., 1987), 1.30×10^5, and 1.16×10^5 cm^{-1} (Yamamoto, personal communication) for their P_r forms, respectively. They were adjusted to 8.0 (61 μM), 8.0 (62 μM), and 16.0 cm^{-1} (138 μM) for Raman measurements of intact, large, and small phytochromes, respectively. For UV RR measurements of aromatic amino acids, the guaranteed grade reagents of tryptophan, tyrosine, and phenylalanine (Wako Pure Chemical Industries, Ltd.) were used without further purification and dissolved in the same buffer as used for phytochrome solutions.

Measurements of UV RR Spectra. The excitation light of UV RR scattering was obtained from an excimer-pumped dye laser system (Lambda Physik EMG104MSC/FL3002) with coumarin 480 dye. The 488-nm emission (3–4 mJ/pulse) of the dye laser was loosely focused on a β-BaB_2O_4 crystal to generate the second harmonic. The angle of the crystal axis against the direction of 488-nm light was adjusted suitably for phase-matching. The generated 244-nm light was led to the sample point after separation from the fundamental by a Pellin–Broca prism and focused in a rectangular shape by two cylindrical lenses on the sample (Kaminaka et al., 1990). The pulse width and the repetition rate were 10 ns and 100 Hz, respectively. The average power of the 244-nm line was 10–20 μJ/pulse at the sample point. Under this condition, no spectral difference was detected between the two UV RR spectra which were measured successively with the same amino acid solutions, indicating no degradation of these aromatic amino acids by UV irradiation.

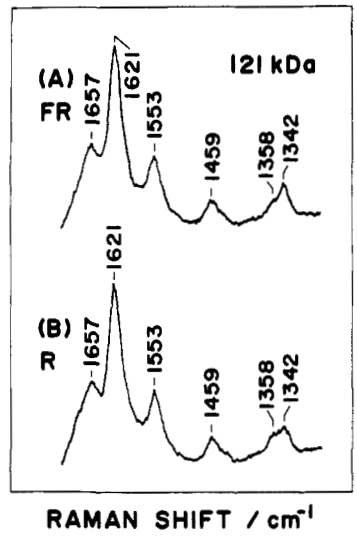

FIGURE 1: UV RR spectra of a 61 μM intact pea phytochrome solution (50 mM HEPES/1 mM Na_2EDTA, pH 7.8) at 16 °C. Spectra A and B are obtained under far-red and red light illumination, respectively.

About 50 μL of the phytochrome solution was put into an ESR tube (diameter = 5 mm), spun at 1600 rpm, and kept at 16 ± 3 °C by flushing with cold nitrogen gas. The sample solution in the spinning cell was always illuminated with either far-red light (740 nm) or red light (660 nm) during the Raman measurements in order to bias the equilibrium of the photo-steady-state toward P_r or P_{fr}, respectively. The far-red or red light was obtained by passing the radiation from a 300-W tungsten lamp through a 660-nm (FWHM, 3.0 nm) or a 740-nm interference filter (FWHM, 10.5 nm) and ca. 10-mm path of water. Raman scattered light was dispersed by a double monochromator (Spex 1403) and detected with an intensified photodiode array (PAR 1421HQ). The monochromator is specially modified for UV RR measurements so as to eliminate the intense visible fluorescence of Trp and to allow for UV Raman light to pass through with high efficiency (Kaminaka & Kitagawa, 1992). Data were processed with an OMA system PAR 1460. Raman shifts were calibrated with cyclohexane.

RESULTS

Figure 1 shows UV RR spectra of pea intact phytochrome at pH 7.8. Spectra A and B were obtained under far-red and red light illumination, respectively. Most of the sample molecules for spectrum A are in P_r, although UV probe light induces slight phototransformation (Sarker & Song, 1982). For spectrum B, a photo-steady-state, in which P_{fr} is domi-

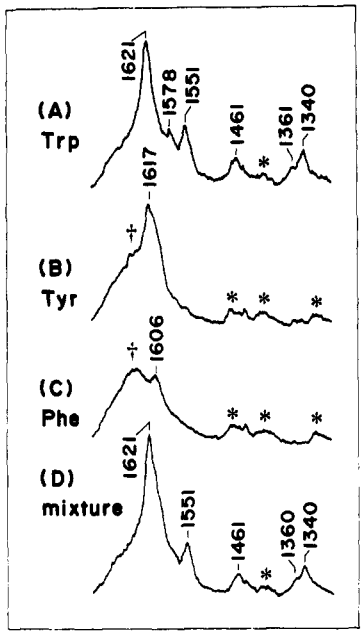

FIGURE 2: UV RR spectra of Trp (A), Tyr (B), Phe (C), and their mixture (D) in the same buffer (50 mM HEPES/1 mM Na_2EDTA, pH 7.8) as used for the intact phytochrome at 16 °C. Concentrations are (A) 0.61 mM, (B) 1.32 mM, (C) 2.20 mM, and (D) 0.61, 1.32, and 2.20 mM for Trp, Tyr, and Phe, respectively. These concentrations are stoichiometrically equivalent to that in a 61 μM pea intact phytochrome solution. Spectra A–C are depicted on a common scale for comparison. Asterisks and daggers indicate UV RR bands of HEPES and water, respectively.

nantly populated, is attained (Tokutomi et al., 1986). Absorption spectra measured just after the UV RR measurements confirmed the undetectable effects of UV irradiation on the photoequilibrium. Therefore, the differences between spectra A and B should be attributed to different molecular structures between P_r and P_{fr}. A small but clear increase was observed in the intensity ratio of doublet bands at 1358 and 1342 cm^{-1}, $I(1358)/I(1342)$, and, in addition, a shoulder around 1670 cm^{-1} is relatively intensified in spectrum B compared to spectrum A; except for this, no difference was detected between spectra A and B. These spectral features are essentially the same as those in the 240-nm-excited RR spectra reported by Toyama et al. (1993).

The UV RR spectra of an aqueous solution of Trp, Tyr, Phe, and their mixture at pH 7.8 excited at 244 nm are shown in Figure 2. The concentrations of each amino acid in the three single-component solutions (spectra A, B, and C) are adjusted so that they are equal to the actual concentrations of the corresponding residues in the intact phytochrome solution (61 μM) used for Figure 1. Accordingly, the spectrum of their mixture solution (spectrum D) quantitatively corresponds to spectra shown in Figure 1. From comparison of spectrum D with spectra A, B, and C in Figure 2, it is evident that the spectrum of the amino acid mixture solution is dominated by Trp bands. Major bands at 1621, 1551, and 1461 cm^{-1} and the doublet band at 1360 and 1340 cm^{-1} are assigned to the W1, W3, W5, and W7 bands of Trp, respectively (Takeuchi & Harada, 1986).

Comparison of Figure 1A with Figure 2D demonstrates that the major spectral features of intact phytochrome in P_r are almost the same as those of the corresponding amino acid mixture, except for the presence of a prominent amide I band at around 1660 cm^{-1}. Accordingly, major peaks of the UV RR spectra of intact phytochrome at 1621, 1553, and 1459

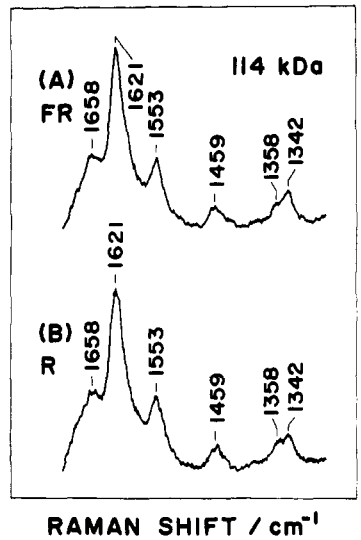

FIGURE 3: UV RR spectra of a 62 μM pea large phytochrome solution (50 mM HEPES/1 mM Na_2EDTA, pH 7.8) at 16 °C. Spectra A and B are obtained under far-red and red light illumination, respectively.

cm^{-1}, and the doublet peaks at 1358 and 1342 cm^{-1}, are assigned to Trp W1, W3, W5, and W7 bands, respectively. It is noted that the Raman bands of HEPES (marked by an asterisk in Figure 2), which can serve as an internal intensity standard for both the protein and amino acid solutions, are much weaker for the protein solution, indicating that the RR intensities of Trp residues are more enhanced in the protein than in the aqueous medium. Since the power density of the excitation laser light is sufficiently low, this intensity difference would not be caused by saturation effects (Ludwig & Asher, 1988; Teraoka et al., 1990). It is probably due to the red shift of the B_b band (~220 nm) in the protein as deduced by Toyama et al. (1993), since the intensity ratio of the W1 and W3 bands ($R = I_{W1}/I_{W3}$), which has been pointed out to be sensitive to the position of the B_b band (Harada et al., 1990), is also larger for the amino acid solution ($R = 3.0 \pm 0.2$) than for the protein solution (2.6 ± 0.2 for spectrum A and 2.5 ± 0.2 for spectrum B).

Figure 3 shows UV RR spectra of pea large phytochrome, which lacks 6-kDa N-terminal and probably 1-kDa C-terminal polypeptides from the intact molecule (Yamamoto & Tokutomi, 1989). Spectrum A was obtained under far-red light illumination, in which most of the molecules are in P_r. The spectral feature of Trp bands is almost the same as that of the intact phytochrome in P_r (Figure 1A), indicating that the removal of those polypeptides scarcely affects the microenvironments around Trp residues. The intensity of the amide I band relative to that of Trp W1 is slightly smaller than that of intact phytochrome (compare Figure 3A with Figure 1A).

Spectrum B was obtained under red light illumination. Large phytochrome has been shown to accumulate a bleached intermediate (I_{bl}) under red light illumination and to attain a photo-steady-state among P_r, P_{fr}, and I_{bl} (Tokutomi et al., 1986). Spectrum B, however, is almost the same as that of the intact phytochrome (Figure 1B) despite the accumulation of I_{bl}. The red light-induced increase in $I(1358\ cm^{-1})/I(1342\ cm^{-1})$, besides a slight change of the band shape around 1670 cm^{-1}, is also observed with the large phytochrome, and their extents are almost the same as that of the intact phytochrome. The change in the intensity ratio of the doublet bands was reproducible in repeated measurements of P_r and P_{fr} for an identical sample, confirming no degradation of Trp residues by UV irradiation under the present conditions. Except for

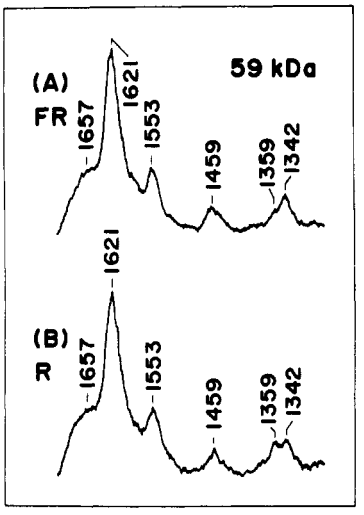

FIGURE 4: UV RR spectra of a 138 μM pea small phytochrome solution (50 mM HEPES/1 mM Na_2EDTA, pH 7.8) at 16 °C. Spectra A and B are obtained under far-red and red light illumination, respectively.

this, no significant difference was observed between under red and far-red light illumination. This strongly suggests that I_{bl} does not adopt a specific protein structure regarding Trp residues.

The UV RR spectra of the small phytochrome, which lacks the N-terminal 6-kDa and C-terminal 59-kDa polypeptides and thus corresponds to the 59-kDa chromophoric domain of phytochrome, are depicted in Figure 4. Spectrum A obtained under far-red light illumination arises dominantly from P_r. The UV RR bands of Trp residues are almost the same as those of the intact and large phytochromes in P_r. However, the intensity of the amide I band relative to those of Trp bands showed a marked decrease compared with those of the intact and large phytochromes (compare Figure 4A with Figures 1A and 3A). This is not due to intensity enhancement of Trp bands, since I_{W1}/I_{W3} (=2.8 ± 0.2) is larger than the value for the intact phytochrome and, therefore, resonance effects from the B_b band should be rather decreased for the small phytochrome. Spectrum B was obtained under red light illumination. The red light-induced increase in the relative intensity of the W7 doublet is more pronounced than those of the large and intact phytochromes (compare Figure 4B with Figures 1B and 3B). In addition, an increase in the Raman intensity around 1670 cm^{-1} is also observed with the small phytochrome.

DISCUSSION

Three Phytochrome Preparations. In the present study, three phytochromes with different molecular sizes, as illustrated in Figure 5A, were used to locate Trp residues responsible for the red light-induced spectral changes. Proteolytic studies of both oat and pea phytochromes have revealed that the subunit consists of a flexible N-terminal 6-kDa segment, the successive 59-kDa chromophoric domain, and the C-terminal 59-kDa nonchromophoric domain (Figure 5A). Models for the organization of these domains in a phytochrome molecule have been proposed on the basis of small-angle X-ray scattering (Tokutomi et al., 1989; Nakasako et al., 1990) and electron microscopic studies (Jones & Erickson, 1989; Tokutomi et al., 1989; Nakasako et al., 1990).

The intact and large phytochromes have a dimeric structure of subunits. The latter lacks the N-terminal 6-kDa segment (Met^1–Ser^{52} in the present preparation) and probably a ~1-kDa C-terminal segment (Yamamoto & Tokutomi, 1989). The spectral difference between the two phytochromes, therefore, will inform us of the roles of the lacking segments in molecular structure and function. Small phytochrome adopts a monomeric structure with an amino acid sequence from Ser^{63} to Lys^{597}, which corresponds to the 59-kDa chromophoric domain (Figure 5A). It seems likely that tryptic cleavage from the whole molecule does not modify the chromophoric domain significantly, since removal of the N-terminal segment and the C-terminal nonchromophoric domain has little effect on the character of photoreversible transformation (Yamamoto, 1987). Accordingly, the difference in the UV RR spectra between the large and small phytochromes will allow us to separate the spectral information of one major domain from that of the other.

It is noted that the C-terminal 59-kDa nonchromophoric domain contains two contact sites (Val^{623}–Ser^{673} and Asn^{1049}–Gln^{1129}) of the two subunits of a molecule [Edgerton & Jones, 1992; Figure 5C-(b)] and also a transmitter module of bacterial sensory proteins [Schneider-Poetsch et al., 1991; Figure 5C-(c)]. From the reported amino acid sequence (Sato, 1988) and the tryptic sites (Yamamoto & Tokutomi, 1989), the number of aromatic residues (Trp, Tyr, and Phe) in the intact, large, and small phytochrome monomers are counted as (10, 21, and 36), (10, 20, and 34), and (6, 14, and 18), respectively, and are used in the analysis below. It should be noted that the N-terminal 6-kDa segment has one Tyr and two Phe but no Trp.

Assignment of Major UV RR Bands to the Trp Resonance. Major UV RR bands of all three phytochromes are assignable to Trp residues except for the amide I band at 1657 cm^{-1}. Locations of six and four Trp residues in the chromophoric and the nonchromophoric domains, respectively, are indicated in Figure 5B. It has been shown that the W7 doublet around 1350 cm^{-1} arises from the Fermi resonance between a skeletal stretching fundamental and a combination of the indole ring, and accordingly that the intensity ratio $I(1360)/I(1340)$ is sensitive to the microenvironment around the ring (Harada & Takeuchi, 1986; Miura et al., 1988); the ratio is less than 1 for Trp residues exposed to aqueous medium, about 1 for those in contact with aromatic amino acid residues, and greater than 1 for those in contact with aliphatic side chains, unless there are special stacking or hydrogen-bonding interactions, and it was confirmed that this empirical rule is applicable also to UV excited spectra (Harada et al., 1986).

The relative intensity $I(1358)/I(1342)$ of intact phytochrome in P_r (Figure 1A) is small and almost the same as that of the corresponding amino acid mixture solution (Figure 2D), suggesting average hydrophilic microenvironments for 10 Trp residues. This is in marked contrast to the case of Trp residues of a membrane photoreceptor protein, bacteriorhodopsin (Harada et al., 1990). The intensity ratios for the large (Figure 3A) and small phytochromes (Figure 4A) in P_r are almost the same as that of the intact phytochrome (Figure 1A). This means that six and four Trp residues in the chromophoric and nonchromophoric domains, respectively, have similar hydrophilic microenvironments and that the presence of the N-terminal 6-kDa segment has little influence on the microenvironments around Trp residues in P_r.

Red Light-Induced Hydrophobicity Increase around Trp Residues. The intensity ratio of the W7 doublet band of the intact phytochrome increased slightly upon conversion to P_{fr} (Figure 1B), indicating that the hydrophobicity around Trp residue(s) increased in P_{fr} due to some conformational changes

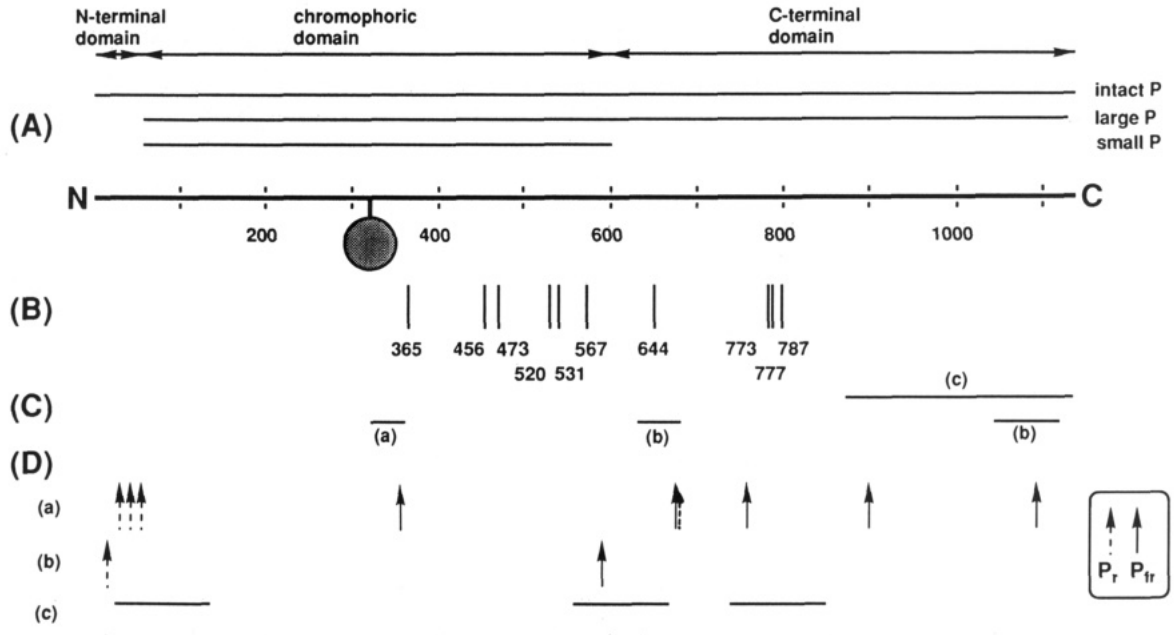

FIGURE 5: Peptide map. (A) Domain structures; (B) locations of Trp residues; (C) regions proposed to be related to the functions; (D) sites which show different behavior between P_r and P_{fr} on molecular modification. (C-a) PEST sequence (Rogers et al., 1986); (C-b) contact sites for dimerization (Edgerton & Jones, 1992); (C-c) region analogous to the transmitter module of bacterial sensor proteins (Schneider-Poetsch et al., 1991). (D-a) Proteolysis sites (Grimm et al., 1988; Lagarias & Mercurio, 1985); (D-b) phosphorylated sites by cAMP-dependent protein kinase (Wong et al., 1986; McMichael & Lagarias, 1990); (D-c) ubiquitin binding sites (Shanklin et al., 1989). In (D-a) and (D-b), solid and dashed arrows denote the sites which are preferably modified in P_r and P_{fr}, respectively. The shaded circle denotes the tetrapyrrole chromophore.

induced by phototransformation. The red light-induced hydrophobicity increase was observed with the large phytochrome (Figure 3B). Although the population of I_{bl} plus P_{fr} in the large phytochrome is comparable to that of P_{fr} in the intact phytochrome under red light illumination at pH 7.8 (Tokutomi et al., 1990), the degree of the increase of hydrophobicity is almost the same between the two phytochromes. This means that the intensity ratio of the W7 doublet of I_{bl} is almost the same as that of P_{fr}, being larger than that of P_r, and suggests that the conformational changes responsible for it are induced during the phototransformation process from P_r to I_{bl}.

The red light-induced hydrophobicity increase was more prominent for small phytochrome, where accumulation of the I_{bl}–like intermediate is much less compared with that of large phytochrome (Yamamoto & Furuya, 1983), and therefore this is attributed to P_{fr} formation. The hydrophobicity around Trp residues in the small phytochrome is comparable to that of light-adapted bacteriorhodopsin (Harada et al., 1990). Since the relative intensity of the doublet reflects an average of the values for the 10 Trp residues present, the larger degree of hydrophobicity increase for the small phytochrome compared with those for the intact and large phytochromes may be explained by either a larger relative population of the Trp residues which are involved in a photoinduced structural change in the chromophoric domain and/or stronger hydrophobic interactions between the indole ring and the vicinal aliphatic groups for Trp residues of the small phytochrome. Since tryptic cleavage had little influence on the chromophoric domain (Yamamoto, 1987), we are tempted to adopt the former; that is, Trp residues involved in the hydrophobicity increase are located in the 59-kDa chromophoric domain, and their relative contributions are larger with the small phytochrome than with the large and intact phytochromes.

It is worth noting that the extra 6-kDa segment in the intact phytochrome has little influence on the hydrophobicity increase on P_{fr} formation despite the fact that this segment is known to play a major role in preserving the spectral properties of P_{fr} intact (Vierstra & Quail, 1983) and protecting the chromophore of P_{fr} from the access of oxidants (Hahn et al., 1984; Thummuler et al., 1985) or of protons (Tokutomi et al., 1988) and thus to have preferential interactions with the chromophoric domain in P_{fr}.

Localization of Trp Residue(s) Involved in the Hydrophobicity Increase. Amino acid sequences of type A phytochromes reported so far have revealed that they have higher homology among those of the same cotyledonous plants (80–90%) than those of two different cotyledonous plants (60–70%) (Hershey et al., 1985; Kay et al., 1989; Sharrock et al., 1990; Sato, 1988). Interestingly, the positions of the 10 Trp residues of type A phytochromes are conserved irrespective of the cotyledonous group, suggesting that they play some essential roles in the functioning of phytochrome.

As mentioned above, the Trp residues responsible for the hydrophobicity increase are considered to be located in the chromophoric domain, where six Trp residues (Trp^{365}, Trp^{456}, Trp^{473}, Trp^{520}, Trp^{531}, and Trp^{567}) are located. It has been shown that Glu^{354}, which is the 32nd residue from the chromophore-bound Cys^{321} and in the so-called PEST sequence (Rogers et al., 1986) (Figure 5C), becomes exposed on phototransformation to P_{fr} in oat intact phytochrome (Grimm et al., 1988) (Figure 5D-a). It means that a conformational change should occur near Glu^{354} upon the phototransformation and this may cause different alterations in microenvironments around the individual amino acid residues involved. Accordingly, Trp^{365} close to Glu^{354} is one of the probable candidates for the residue in the hydrophobicity change. This Trp might also be related to the signal emission for rapid turnover of type A phytochromes (Roger et al., 1986).

Another probable candidate for the microenvironmental change is Trp^{567}. It was demonstrated recently that Ser^{598} (Figure 5D-b) in the hinge region between the chromophoric and the C-terminal nonchromophoric domains is phosphorylated preferentially in P_{fr} (MacMichael & Lagarias, 1990).

This Ser residue is located in one of the reported ubiquitin binding sites (Figure 5D-c) that are exposed in P_{fr} (Shanklin et al., 1989). Binding assay using monoclonal antibodies also proposes exposure of this region in P_{fr} (Schneider-Poetsch et al., 1989).

Trp^{570} in oat phytochrome, which corresponds to Trp^{567} in pea phytochrome, was supposed to be responsible for the quenching of Trp fluorescence by cationic ions in P_{fr} (Singh & Song, 1990). All these results indicate that conformational changes occur in the hinge region on phototransformation, which suggests the involvement of Trp^{567} in the hydrophobicity increase based on the same reason described above.

Among the four Trp residues in the C-terminal domain, Trp^{644} is within one of the two contact sites of subunits (Edgerton & Jones, 1992) (Figure 5C), and the dimeric structure is preserved in P_{fr}. Since this residue is presumably buried in α-helices of the contact site, its microenvironment may not be altered by the phototransformation. The other three Trp residues (Trp^{773}, Trp^{777}, and Trp^{787}) lie in close vicinity to one another in one of the ubiquitin binding sites (Figure 5D-c), which is supposed to be exposed on phototransformation from P_r to P_{fr} (Shanklin et al., 1989), and may behave in a similar way on phototransformation. If they undergo the hydrophobicity change upon phototransformation, the UV RR spectra of the intact and large phytochromes should have exhibited a large intensity change regarding the Trp W7 doublet. The larger UV RR spectral change in small phytochrome, which lacks the three Trp's, again supports the idea that the Trp residues involved in the hydrophobicity increase are located in the chromophoric domain. However, this does not exclude the possibility of subtle alteration in the surface topography of Trp^{773} and Trp^{777} during the phototransformation as reported from their fluorescence quenching studies (Singh & Song, 1990).

It is reported that the hydrophobicity of the large phytochrome as a whole increases upon phototransformation from P_r to P_{fr} (Yamamoto & Smith, 1981). After removal of the C-terminal nonchromophoric domain, the hydrophobicity of the molecule as a whole decreases, and the red light-induced hydrophobicity increase was not detected (Tokutomi et al., 1981). Unfortunately, such hydrophobicity was determined in terms of the affinity of the whole molecule to an aliphatic group and cannot be compared directly to the microenvironmental change around Trp residues.

Contents of α-Helices and Random Coils in the Chromophoric Domain. A prominent amide I band is expected around 1675–1665 cm^{-1} for β-sheets and β-turns, but it was not observed in all three phytochromes both under far-red and under red light illuminations. This is consistent with the small content of β-sheets and β-turns revealed by far-UV CD studies (45–55% α-helices, 25–30% nonregular structures, 20–25% β-turns, and little β-sheets) (Viertra et al., 1987; Chai et al., 1987; Sommer & Song, 1990). The intensity of the amide I band around 1660 cm^{-1}, which should arise from α-helices and nonregular structures (Miyazawa et al., 1958; Krimm & Bandekar, 1986), relative to that of the Trp W1 band is largest for the intact phytochrome (Figure 1), slightly smaller with the large phytochrome (Figure 3), and by far smaller with the small phytochrome (Figure 4), although the ratios of molecular weights per single Trp residue are 1.23:1.16:1.0. The small difference between the intact and large phytochromes is understandable if the 6-kDa segment contains an average amount of α-helix (Parker & Song, 1990). The marked difference between the small and intact phytochromes means that α-helices and nonregular structures are much less populated in the chromophoric domain than in the other two. It seems more reasonable to assume that such character is intrinsic rather than endowed by protein digestion, since the character of photoreversible transformation of the chromophoric domain is preserved by the small phytochrome.

Change in α-Helix Content on Phototransformation from P_r to P_{fr}. The α-helical content is reported to increase by 3–5% upon phototransformation from P_r to P_{fr} for the intact phytochrome, but not for the large phytochrome, from far-UV CD measurements (Vierstra et al., 1987; Chai, et al., 1987). However, recent FTIR studies pointed out that the photoreversible change in the α-helix content is less thant 2 $\times 10^{-3}$ (Siebert et al., 1990). The present UV RR spectra suggest that the content ratio of β-sheet and β-turn to α-helix and nonregular structures estimated from the 1670-cm^{-1} intensity rather increases upon phototransformation from P_r to P_{fr}. This contradicts with the CD study of oat intact phytochrome. Unfortunately, the low resolution of the present UV RR spectra does not allow us to discuss the change of the peptide backbone conformation quantitatively, but the UV RR technique has the potential to determine it in the future.

Torsional Angle around the C_β–C_3 Bond of Tryptophan. The frequency of the Trp W3 band near 1550 cm^{-1} is correlated with the torsional angle $|\chi^{2,1}|$ around the C_β–C_3 bond of Trp. This frequency increases from 1542 to 1557 cm^{-1} when the $\chi^{2,1}$ angle increases from 61° to 117° (Miura et al., 1989). The center frequency and bandwidth of this band remained unchanged among the intact, large, and small phytochromes and also between their P_r and P_{fr} forms. This means that all Trp residues have similar $\chi^{2,1}$ values, which are estimated to be 102° from the reported relation (Miura et al., 1989), and that the $\chi^{2,1}$ angle is not altered by the phototransformation.

CONCLUDING REMARKS

The 244-nm-excited RR spectra of phytochromes mainly probe Trp residues. Most Trp residues are under hydrophilic microenvironments in P_r, but some Trp residues, presumably in the chromophoric domain, become hydrophobic on phototransformation to I_{b1}. Plausible candidates for these are Trp^{365} and Trp^{567}. The content of α-helices and random coils is smaller in the chromophoric domain than in the N-terminal 6-kDa segment and the C-terminal 59-kDa domain.

ACKNOWLEDGMENT

We are grateful to Prof. K. Manabe of Yokohama City University for stimulated discussion.

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