Resonance Raman Study on Intact Pea Phytochrome and Its Model Compounds: Evidence for Proton Migration during the Phototransformation

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ABSTRACT: Resonance Raman (RR) scattering from intact pea phytochrome was observed in resonance with the blue band at ambient temperature. The relative populations of the red-light-absorbing form (P_r) and far-red-light-absorbing form (P_{fr}) under laser illumination were estimated from the absorption spectra. The most prominent RR band of P_r obtained by 364-nm excitation under 740-nm pumping exhibited a frequency shift between H_2O and D_2O solutions, but that of P_{fr} obtained by 407-nm excitation under 633-nm pumping did not, indicating a distinct difference in a protonation state of their chromophores. Since the protonation level of a whole molecule of intact phytochrome remains unchanged between P_r and P_{fr}, this observation indicates migration of a proton from the chromophore of P_r to the protein moiety of P_{fr}. As model compounds, octaethylbiliverdin (OEBV-h_3), its deuterated and ^{15}N derivatives, and their protonated forms were also studied with both RR and ^1H and ^{15}N NMR spectroscopies. The RR spectrum of the protonated form, for which the protonation site was determined to be C-ring pyrrole nitrogen by NMR, displayed a deuteration shift corresponding to that of P_r, suggesting a similar protonated structure for the pyrrolic rings of P_r. The RR spectral difference between OEBV-h_3 and OEBV-d_3 and that between H_2O and D_2O solutions of P_{fr} suggested that the N–H protons of the A-, B-, and D-rings of intact phytochrome are replaced with deuterons in D_2O. A role of the 7-kDa segment of phytochrome is discussed on the basis of RR spectral differences between the intact and large phytochromes.

Phytochrome is a chromoprotein which acts as a photoreceptor for a variety of light-triggered morphogenetic responses in green plants (Lagarias, 1985; Furuya, 1987). The chromoprotein has two distinct spectral forms called a red-light-absorbing form (P_r)[1] and a far-red-light-absorbing form (P_{fr}), which are interconvertible by illumination with red and far-red light, respectively. The chromoprotein has been recently shown to have two isoforms, named types I and II depending on the light sensitivity of their amounts in tissues (Furuya, 1989). The type I phytochrome used in this study, consists of two identical subunits with N-terminal chromophoric and C-terminal nonchromophoric domains (Tokutomi et al., 1989), the former of which contains a 2,3-dihydrobiliverdin chromophore (Lagarias & Rapoport, 1980; Rudiger et al., 1985). The tetrapyrrole chromophore is thought to be deprotonated at C-ring as shown in Figure 1a, although it has not been established yet.

The type I phytochrome is biosynthesized in the biologically inactive form (P_r) in the dark and is transformed to the active form (P_{fr}) upon red-light illumination, which triggers physiological responses in green plants. The molecular mechanism by which phytochrome regulates a great diversity of morphogenetic responses is still obscure. To understand it, elucidation of the primary process ($P_r \rightarrow P_{fr}$) of the light signal transduction is quite important. So far, the *Z,Z,Z* to *Z,Z,E* configurational changes in the tetrapyrrole chromophore (Rudiger et al., 1983; Thummler et al., 1983; Farrens et al., 1989; Fodor et al., 1990) and/or proton migration (Moon et al., 1985; Tokutomi et al., 1988a) have been proposed as the chemical event in the P_r to P_{fr} phototransformation. Several lines of evidence for a light-induced conformational change in the protein moiety have also been presented (Wong et al., 1986; Chai et al., 1987; McMichael et al., 1990). In order to know further details of the photoreaction mechanism, it is requisite to explore the structural differences between P_r and P_{fr} and to relate these changes to the physiological function.

Resonance Raman (RR) spectroscopy is a powerful technique for studying the structure of chromophore in chromoproteins (Spiro, 1987). Since Raman excitation in resonance with the first absorption band of P_r brings about intense fluorescence background, RR technique had not been successfully applied to phytochrome until Fodor et al. (1988) measured the RR spectra of oat intact phytochrome at 77 K by using far-red-light excitation to avoid much of the inherent fluorescence. Song and his co-workers have employed surface-enhanced resonance Raman spectroscopy (SERRS) using silver colloids as the adsorbing substrate (Rospendowski et al., 1989; Farrens et al., 1989). In both kinds of studies, the sample solutions were frozen, which might cause possible changes in tertiary and/or quaternary structures, and more-

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[1] Abbreviations: RR, resonance Raman; SERRS, surface-enhanced resonance Raman spectroscopy; OEP, octaethylporphyrin dianion; OEBV-h_3, octaethylbiliverdin; OEBV-d_3, N-deuterated octaethylbiliverdin; OEBV-h_4^+, protonated octaethylbiliverdin; OEBV-$^{15}N_4$-h_3, ^{15}N-substituted OEBV-h_3; BVDE-h_3, biliverdin dimethyl ester; P_r and P_{fr}, red-light- and far-red-light-absorbing forms of phytochrome; I_{700} and I_{bl}, intermediates observed in the phototransformation of phytochrome; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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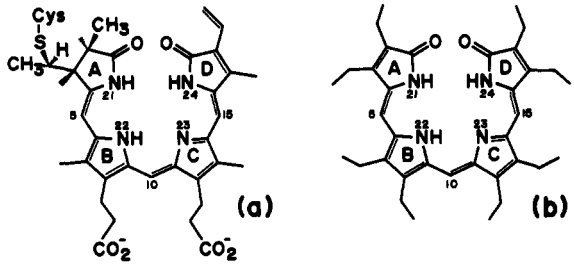

FIGURE 1: Structure of phytochrome chromophore (a) and OEBV-h_3 (b). Numbering of pyrrole rings and atoms is denoted in the figure. The deprotonated pyrrole ring is defined as C-ring for OEBV-h_3, although the deprotonated pyrrole ring of phytochrome has not been established yet.

over, SERRS may suffer from a possible structural change of the protein which is induced by the adsorption onto the substrate.

In order to study the structural changes of the chromophore accompanied by phototransformation, it is highly desirable to observe the RR spectra of phytochrome in a natural state at an ambient temperature. Accordingly, we adopted Soret excitation in Raman scattering to avoid the interference by fluorescence and the two-color excitation technique to modulate the population of either P_r or P_{fr} under photo steady state, and we successfully measured the RR spectra of the "large" pea phytochrome, which lacks amino acid residues corresponding to 7 kDa from the "intact" form (Lumsden et al., 1985), in a solution at 16 °C for the first time (Tokutomi et al., 1990).

Recently Siebert et al. (1990) applied FTIR spectroscopy to phytochrome and its model compounds, pointing out that both P_r and P_{fr} are protonated contrary to our results on large phytochrome (Tokutomi et al., 1990). Furthermore, Fodor et al. (1990) observed far-red-excited RR spectra of P_{fr} at room temperature and concluded that P_{fr} is protonated. Therefore, we examined the protonation problem with intact phytochrome and report here the Soret-excited RR spectra of the P_r and P_{fr} forms of intact pea phytochrome at 16 °C, which demonstrate that P_r and P_{fr} have different protonated structures, similar to the case of the large pea phytochrome. Based on the present observations and earlier results about the light-induced proton release and uptake by pea phytochromes (Tokutomi et al., 1988), possible proton migration during the phototransformation is deduced. In addition, we specify the protonation site in the chromophore from the structural studies on the model compound octaethylbiliverdin. Its structure and numbering of pyrrole rings and atoms are illustrated in Figure 1b, where the deprotonated pyrrole ring is tentatively defined as C-ring.

Materials and Methods

Phytochrome Preparation. Intact pea phytochrome consisting of two subunits with M_r of 121 000 (determined by SDS–PAGE) was isolated from 7-day-old etiolated seedlings of pea (*Pisum sativum* cv. Alaska) as described previously (Tokutomi et al., 1988b). The protein was precipitated by ammonium sulfate [$(ND_4)_2SO_4$ in D_2O for the deuterated preparation] and resuspended in 50 mM HEPES and 1 mM Na_2EDTA, pH 7.8 (D_2O was used for the deuterated preparation). All the preparation procedures were carried out under dark or dim green light. The specific absorbance ratio (A_{667}/A_{280}) of the present sample was 0.90 and its purity was estimated to be higher than 95% from SDS–PAGE.

Synthesis of Model Compounds. Octaethylbiliverdin (OEBV-h_3) and its ^{15}N derivative (OEBV-$^{15}N_4$-h_3) were synthesized from octaethylporphyrinatoiron(III) chloride [(OEP)$Fe^{III}Cl$] and its ^{15}N derivative, respectively, according to the reported method (Bonnett & Dimsdale, 1972; Cavaleiro & Smith, 1972) with some modifications about reaction temperature, time, and pH, which raised the yield from 5% to 35%. The N-deuterated form of octaethylbiliverdin (OEBV-d_3) was obtained by mixing a small amount of D_2O with the $CHCl_3$ solution of OEBV-h_3. Protonation of OEBV-h_3 (or -d_3) was accomplished by adding 1 N HCl (or DCl) to the $CHCl_3$ solution of OEBV-h_3 (or -d_3) according to the procedures described by Margulies and Stockburger (1984).

Measurements of RR Spectra. Fifty microliters of the phytochrome solution with the concentration of 8.0 cm^{-1} in terms of A_{667} in the P_r form, which corresponds to 61 μM on the basis of $\epsilon_M = 1.32 \times 10^5$ cm^{-1} for the intact oat phytochrome (Lagarias et al., 1987), was put into a micro spinning cell (diameter = 5 mm, 1600 rpm) and kept at 16 ± 3 °C by flushing with cold N_2 gas. RR scattering was excited by the 407-nm line of a Kr^+ ion laser (Spectra Physics, Model 2016) or the 364-nm line of an Ar^+ ion laser (Spectra Physics, Model 2045), dispersed with a double monochromator (SPEX 1404, and detected with a diode array detector with an image intensifier (PAR 1421HQ). The data were processed with an OMA III system (PAR 1460). The Raman spectra were calibrated with indene for each excitation line, and errors in band positions for well-defined peaks are ±2 cm^{-1}.

The sample solution in the spinning cell was illuminated with either far-red (740 nm) or red (633 nm) light at another spot during the blue-light excitation in order to bias the equilibrium of photo steady state toward P_r or P_{fr}, respectively. The illuminating spot of the red (or far-red) light was located at the downstream side of the blue-light illuminating spot of the spinning cell as close as possible for making the interval after the red (or far-red) light illumination longest in one turn of the cell. The far-red light was generated by a Ti-doped sapphire laser (Spectra Physics, Model 3900) pumped by an Ar^+ ion laser (Spectra Physics, Model 164). The red light was generated by a He/Ne laser (NEC GLS5800).

To estimate the relative population of P_r and P_{fr} under the photo steady state attained by double-laser illumination, the absorption spectra of the phytochrome solutions with concentrations from 15 to 23 μM were measured under the same illumination conditions as used for measurements of RR spectra; the two laser beams were introduced to a magnetically stirred sample solution in the cuvette placed in a spectrophotometer (Shimadzu, UV240). Although a slight increase in sample turbidity was noticed over time, it was confirmed that the sample preserved its original photoreversibility and gave no degradation or aggregation products in the SDS–PAGE patterns after the double-laser illumination experiments. The absorption spectrum of P_r was measured after saturating irradiation with far-red light. The spectrum of P_{fr} was determined by subtracting the spectrum of P_r from that observed after saturating irradiation with red light, in which it was assumed that the red-light-irradiated pea intact phytochrome is composed of 84% P_{fr} and 16% P_r on the basis of the method reported by Vierstra and Quail (1983).

Measurements of NMR Spectra. The 400-MHz ^1H NMR and 40.5-MHz proton-decoupled ^{15}N NMR spectra were observed with a JEOL JNM GX400 pulse Fourier transform NMR spectrometer. The chemical shifts were measured downfield in parts per million relative to $Si(CH_3)_4$ for ^1H nuclei and to 2.9 M $^{15}NH_4Cl$ dissolved in 1 N HCl for ^{15}N as a standard (Srinivasan & Lichter, 1977). The pulse width and pulse delay were 13 μs and 2 s, respectively, for ^{15}N nuclei.

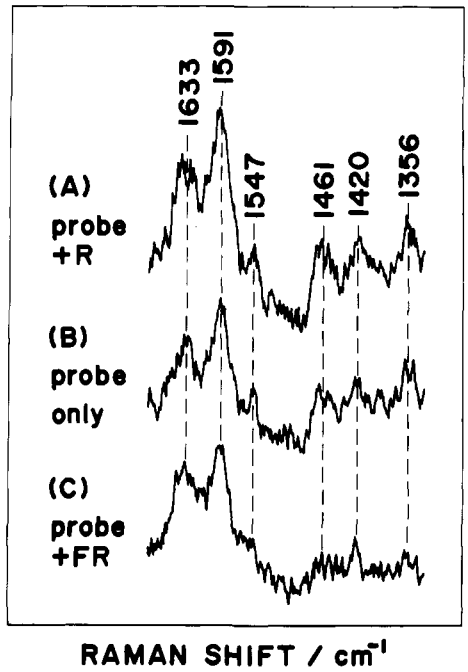

FIGURE 2: Effects of red or far-red illumination on RR spectra excited at 407 nm (5 mW). The experimental conditions are common to the three spectra except for illumination with red (A, 633 nm, 15 mW) or far-red light (C, 740 nm, 7 mW). Spectrum B was obtained without illumination of the second laser. The three spectra are represented in the same scale.

RESULTS

Since P_r and P_{fr} of the intact pea phytochrome have the second absorption band around 380 and 410 nm, respectively, the excitation of Raman scattering at 364 and 407 nm is expected to selectively enhance the intensity of Raman bands of P_r and P_{fr}, respectively. However, as the wavelength of the probe light of Raman scattering approaches the absorption maximum, the probe light would increasingly induce the photoreaction, although the light intensity is made as low as possible, and we might observe the photoreacted species after several turns of the spinning cell. In order to circumvent this problem, the sample was illuminated with the second laser (pump beam). The effect of the second laser illumination was examined with the spectra of intact phytochrome excited at 407 nm, as illustrated in Figure 2, where three spectra were observed under the same conditions except for illumination of the second laser and are represented in a common scale. Spectrum B was obtained without the pump beam. Spectrum B was obtained without the pump beam. Upon red-light illumination (spectrum A), the intensity of Raman scattering was enhanced by a factor of 1.3 without a change of the spectral pattern. This is consistent with the population increase of P_{fr} upon red-light illumination in the photo steady state determined by the absorption spectroscopy. Upon far-red-light illumination, on the other hand, those bands became weaker and the relative intensities were slightly altered, as shown by spectrum C. Therefore, it is reasonable to assign the bands intensified by red-light illumination to P_{fr}.

Figures 3 and 4 show the RR spectra of intact pea phytochrome excited at 407 nm under red-light (633-nm) illumination and at 364 nm under far-red-light (740-nm) illumination, respectively (A, H_2O solution; B, D_2O solution). The RR spectra of large pea phytochrome in H_2O observed previously under the same conditions as this measurement (Tokutomi et al., 1990) are also displayed as spectra C for comparison. The RR spectral pattern of the intact phytochrome (A) resembles that of the large phytochrome (C), except for

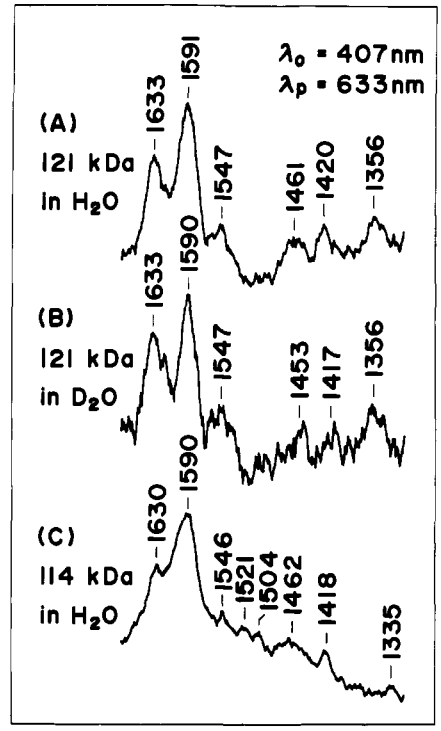

FIGURE 3: RR spectra of intact (A and B) and large (C) pea phytochrome excited at 407 nm (5 mW) under red-light illumination (633 nm) at pH 7.8. Spectra A and C are for H_2O solutions, and spectrum B is for D_2O solution. Accumulation time was 320 s. Relative populations of P_r, P_{fr}, and I_{bl} under individual illumination conditions are as follows: P_r 45% and P_{fr} 55% for (A); P_r 40% and P_{fr} 60% for (B); P_r 20%, P_{fr} 36%, and I_{bl} 44% for (C).

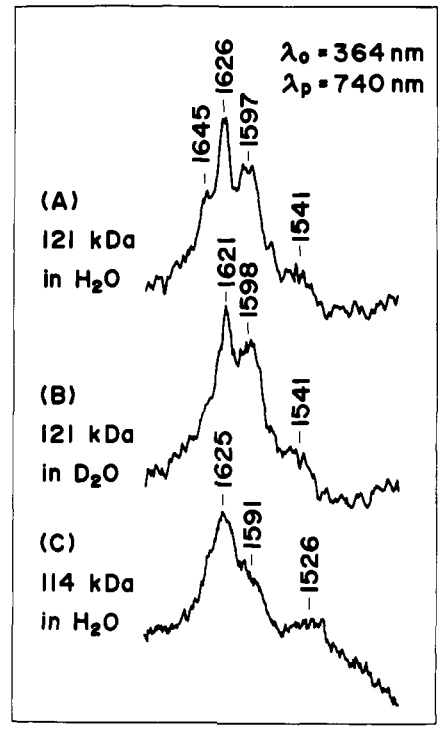

FIGURE 4: RR spectra of intact (A and B) and large (C) pea phytochrome excited at 364 nm (5 mW) under far-red-light illumination (740 nm) at pH 7.8. Spectra A and C are for H_2O solutions, and spectrum B is for D_2O solution. Accumulation time was 320 s. Relative populations of P_r, P_{fr}, and I_{bl} under individual illumination conditions are as follows: P_r 55% and P_{fr} 45% for (A); P_r 55% and P_{fr} 45% for (B); P_r 66%, P_{fr} 5%, and I_{bl} 29% for (C).

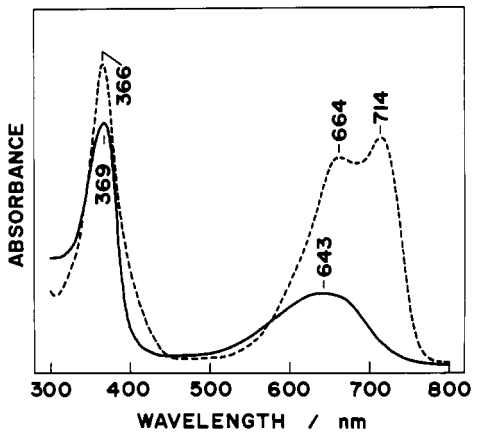

FIGURE 5: Absorption spectra of OEBV-h_3 (solid line) and its protonated form (OEBV-h_4^+, broken line) in chloroform.

the presence of a band at 1356 cm^{-1} and absence of bands near 1521 and 1504 cm^{-1} in the spectrum of P_{fr} (Figure 3A).

The spectra in Figures 3 and 4 arise from a photo-steady-state mixture of phytochrome generated by two laser illuminations. The visible absorption specra of the large pea phytochrome under the two-color excitation were well simulated by the weighted sum of the spectra of P_r, P_{fr}, and I_{bl}, in which I_{bl}, the bleached intermediate, was accumulated under red-light illumination (Tokutomi et al., 1986, 1990). The absorption spectra of the intact pea phytochrome under the two-color excitation, however, can be well reproduced by the weighted sum of the spectra of only P_r and P_{fr}. This coincides with the observation that I_{bl} is hardly accumulated with the pea intact phytochrome under red-light illumination (Tokutomi et al., 1986). The relative populations of P_r and P_{fr} under the double-laser illumination in this experiment are described in the figure captions. When the sample was illuminated with either red or far-red light in addition to the blue light, the population of P_{fr} or P_r, respectively, increased 1.3–2 times, similarly to that of the large phytochrome.

Major RR bands of the large phytochrome in Figure 3C were previously assigned to P_{fr} judging from the relative populations of the three components present under the two-laser illumination (Tokutomi et al., 1990). This supports attribution of spectra A and B in Figures 3 to P_{fr}. Although the assignment of the RR spectrum of the large phytochrome shown in Figure 4C had some ambiguity, that is, to P_r and/or the I_{bl}, those in Figures 4A,B can be assigned to P_r, since the relative population of I_{bl} in this experiment is negligible. The spectral pattern of Figure 4A did not change when the pump wavelength was changed to red.

In Figure 4A, the most prominent Raman band of P_r is observed at 1626 cm^{-1} and exhibits a downshift to 1621 cm^{-1} upon deuteration (B). A similar deuteration shift was observed for the large phytochrome at the same excitation conditions as this experiments (Tokutomi et al., 1990) and for oat phytochrome with far-red excitation at 77 K (Fodor et al., 1988). In contrast, the RR spectra of P_{fr} shown in Figure 3 give different features; although none of the double-bond stretching RR bands around 1550–1650 cm^{-1} shows deuteration shifts similar to the case of the large phytochrome (Tokutomi et al., 1990), the RR bands at 1461 and 1420 cm^{-1} in H_2O shift to 1453 and 1417 cm^{-1} in D_2O, respectively. All these observations suggest that the protonation structures of the chromophores of P_r and P_{fr} are different.

Figure 5 shows the absorption spectra of OEBV-h_3 (solid line) and its protonated form (OEBV-h_4^+) in $CHCl_3$ (broken line). The absorption spectrum of OEBV-h_3 gives a broad and

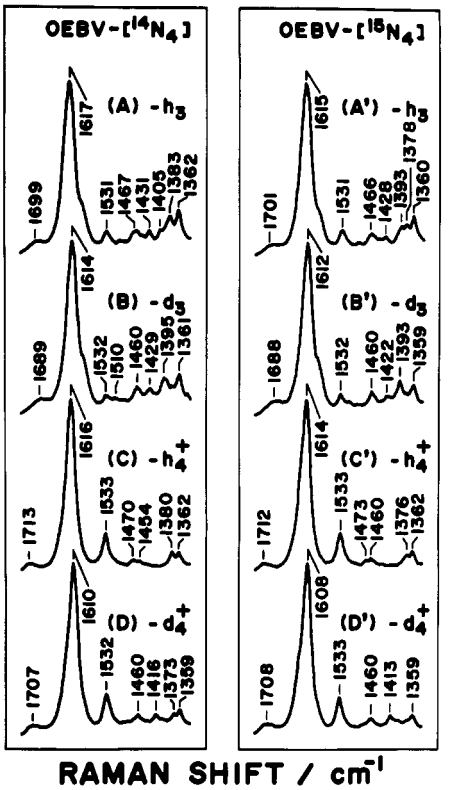

FIGURE 6: Left panel: RR spectra of OEBV-h_3 (A), OEBV-d_3 (B), OEBV-h_4^+ (C), and OEBV-d_4^+ (D) in chloroform. Excitation was at 364 nm (5 mW); accumulation time was 320 s. Right panel: RR spectra of OEBV-$^{15}N_4$-h_3 (A), OEBV-$^{15}N_4$-d_3 (B), OEBV-$^{15}N_4$-h_4^+ (C), and OEBV-$^{15}N_4$-d_4^+ (D) in chloroform. Excitation was at 364 nm (5 mW); accumulation time was 320 s.

weak band centered at 643 nm and a strong band at 369 nm. Upon addition of 1 N HCl, the absorption maximum of the red band shifted to 664 nm with a new band centered at 714 nm and the overall absorption intensity increased. The 369-nm band also slightly shifts to 366 nm. These spectral changes were accompanied by an isosbestic point at 575 nm, suggesting that the spectral change is caused by binding of a single proton to OEBV-h_3. The protonation site to OEBV-h_3 was determined to be pyrrole nitrogen of C-ring by NMR (vide infra). These features closely resemble those of BVDE-h_3 reported by Margulies and Stockburger (1979).

The left panel of Figure 6 shows RR spectra of OEBV-h_3 (A), OEBV-d_3 (B), OEBV-h_4^+ (C), and OEBV-d_4^+ (D). A prominent band is observed at 1617 cm^{-1} in addition to many weak bands in spectrum A. This pattern is characteristic of the RR spectra of linear C=C-conjugated systems like carotenoids. These RR spectra did not change when spinning of the Raman cell was abolished, the laser power was raised, or the sample was cooled to −50 °C. Upon N-deuteration of A-, B-, and D-rings, the 1617- and 1467-cm^{-1} bands exhibited downward shifts by −3 and −7 cm^{-1}, respectively, while the bands at 1431 and 1362 cm^{-1} shifted little. It is inferred that the bands of OEBV-h_3 at 1467, 1431, and 1362 cm^{-1} correspond to the RR bands of P_{fr} at 1461, 1420, and 1356 cm^{-1}, respectively, from the similar behavior of the corresponding bands upon deuterium substitution and proximity of their frequencies in the model compound and the intact phytochrome.

The RR spectrum of OEBV-h_3 changed definitely upon protonation of C-ring as shown by spectrum C. The changes include disappearance of the Raman bands at 1431 and 1405 cm^{-1} and the frequency shifts and intensity changes of the

bands around 1700 and 1600 cm^{-1}. The 1699-cm^{-1} band in spectrum A is shifted to higher frequency by 14 cm^{-1} upon protonation and to a slightly lower frequency upon deuteration. Judging from its frequency, this band would arise from the C=O stretching mode of A- and D-rings. The increase of the C=O stretching frequency upon protonation is in agreement with the FTIR data (Siebert et al., 1990). Presumably protonation at C-ring appreciably changes the conjugation system and thus all the double-bond stretching force constants. These RR spectral changes resemble those of BVDE-h_3 (Margulias & Stockburger, 1979).

The most prominent Raman band of the protonated form at 1616 cm^{-1} exhibits a deuteration shift of −6 cm^{-1}. This corresponds well to the deuteration shift of the most prominent band of P_r of the intact phytochrome at 1626 cm^{-1} by −5 cm^{-1} (Figure 4A,B) and that of the large phytochrome at 1625 cm^{-1} by −7 cm^{-1}, and accordingly, this observation strongly suggests that the structures of the P_r chromophore in the two kinds of phytochromes are similar to that of OEBV-h_4^+.

The corresponding RR spectra of the ^{15}N-substituted octaethylbiliverdin (OEBV-$^{15}N_4$) are shown in the right panel of Figure 6. The most prominent band of OEBV-$^{15}N_4$-h_3 appears at 1615 cm^{-1} (1614 cm^{-1} for the protonated form), which is little shifted from that of OEBV-$^{14}N_4$ [although the $^{14}N/^{15}N$ isotopic frequency shifts are comparable to experimental uncertainty (2 cm^{-1} per one pixel), the shifts were confirmed by three independent measurements and also by difference calculations]. If this band involved 30% of the C=N stretching character, it would exhibit a frequency shift as large as 8 cm^{-1} on the basis of a simplified diatomic calculation. In fact, the C=N stretching RR band of (OEP)Ni^{II} showed the ^{15}N shift of −6 cm^{-1} for the totally symmetric mode at 1383 cm^{-1} (Kitagawa et al., 1976), which was deduced to contain ca. 50% of the C=N stretching character and to shift by −9 cm^{-1} through the normal coordinate calculations (Abe et al., 1978). Therefore, the 1617-cm^{-1} band of OEBV-h_3 in Figure 6A is not primarily associated with the C=N stretching vibration. However, the 2-cm^{-1} shift due to the ^{15}N substitution implies a small coupling with the C=N stretching mode irrespective of the protonation at C-ring. It is stressed for the 1617-cm^{-1} band that the deuteration shift is fairly large for the protonated form but the ^{15}N shift is small. It is most likely that the 1617-cm^{-1} band has the largest contribution from the methine-bridge C=C stretching vibrations adjacent to C-ring and some contributions from other C=C and the C=N stretching modes.

The highest frequency band (1699 cm^{-1}) in the left panel of Figure 6A shows little shift upon ^{15}N substitution but an appreciable shift to lower frequencies upon N-deuteration (10–13 cm^{-1}). The shift size is close to the deuteration shift of the amide I band, and it is reasonable to assign it to the C=O stretching mode of the lactam. While most of the RR bands show ^{15}N frequency shifts smaller than 4 cm^{-1}, the 1405-cm^{-1} band of OEBV-h_3 and the 1429-cm^{-1} band of OEBV-d_3 exhibit downshifts by 7–12 cm^{-1} upon ^{15}N substitution and seem to disappear upon protonation to C-ring. These may be associated mainly with the C=N stretching mode of C ring.

The 400-MHz ^1H NMR spectra of OEBV-h_3 in deuterated chloroform ($CDCl_3$) are shown in Figure 7A. The triplet signals at 1.0–1.3 ppm and the quartet signals at 2.2–2.7 ppm are assigned to methyl and methylene protons of ethyl groups, respectively. The broad band at 1.7 ppm is due to water contamination in the sample. The singlet peaks at 5.9 and 6.6 ppm are assigned to the methine protons at positions 5 and

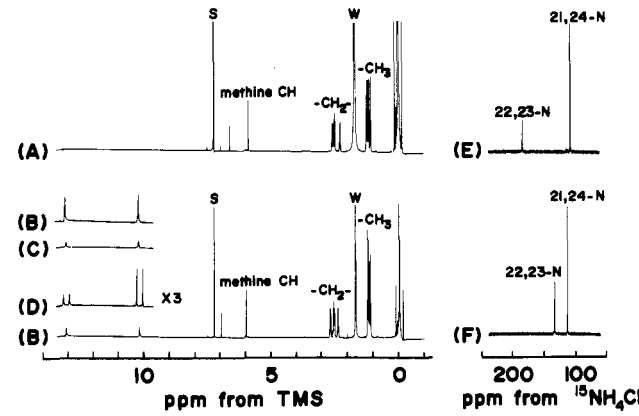

FIGURE 7: ^1H NMR spectra (400 MHz) (A, B, C, and D) and proton-decoupled ^{15}N NMR spectra (40.5 MHz) (E and F) of octaethylbiliverdin and its protonated form in $CDCl_3$. (A) OEBV-$^{14}N_4$-h_3; (B) OEBV-$^{14}N_4$-h_4^+; (C) OEBV-$^{14}N_4$-d_4^+; (D) OEBV-$^{15}N_4$-h_4^+; (E) OEBV-$^{15}N_4$-h_3; (F) OEBV-$^{15}N_4$-h_4^+. The resonances marked by S and W are due to solvent ($CHCl_3$) and water, respectively.

15 and to that at position 10, respectively, since the former is twice as strong as the latter. The protons of pyrrole NH are not observed as a result of line broadening.

Upon protonation, while the chemical shifts of ethyl groups are scarcely affected, the methine protons show downfield shifts, as shown by spectrum B. New signals appear at 10.1 and 13.0 ppm, each corresponding to two protons in intensity. As shown by spectrum C, these signals are weakened when DCl is used instead of HCl. Therefore these protons are exchangeable with the protons in the solvent. With the ^{15}N-enriched compound, the proton signals are split into doublets with an N–H coupling constant of 94 Hz for the 10.1 ppm signal and 93 Hz for the 13.0 ppm signal, which are typical values of one-bond NH couplings and close to that of the ^{15}N enriched $OEPH_4^{2+}$ [$^1J(^{15}N–H)$ = 93 Hz] (Kawano et al., 1978). These coupling constants clearly indicate that the corresponding protons bind to the nitrogen atoms. Probably, the 13.0 and 10.1 ppm signals are assignable to the B- (and C-) and the A- and (D-) rings, respectively. Since both OEBV and protonated OEBV have C_2 symmetry in the NMR time scale, distinction between B- and C-rings is impossible. A positive charge of OEBV-h_4^+ is delocalized and the downshift of the C_{10}–H proton signal indicates this.

The 40.5-MHz proton-decoupled ^{15}N NMR spectra of OEBV-$^{15}N_4$-h_3 and its protonated form in $CDCl_3$ at −50 °C are shown in Figure 7, spectra E and F, respectively. The signal at 107.1 ppm is assignable to the nitrogens of A- and D-rings with the lactam skeleton, because the chemical shift is comparable to that of 2-pyrrolidinone (98.6 ppm), Δ^3-pyrrolin-2-one (98.1 ppm), and Δ^4-pyrrolin-2-one (124.0 ppm) (Fronza et al., 1977). Since the chemical shift of 184.5 ppm is close to that of imidazole (184.5 ppm) (Schuster & Roberts, 1979) and ^{15}N-$OEPH_2$ (160.6 ppm) (Kawano et al., 1978), the signal is assignable to the nitrogens of B- and C-rings. In contrast with ^{15}N-$OEPH_2$, the signal at 184.5 ppm does not collapse into two peaks, even at low temperature (−50 °C). This implies that the proton exchange between B- and C-rings is faster than the relaxation of the ^{15}N nuclei.

Upon protonation, while the nitrogen resonance of A- and D-rings is little affected, the nitrogen resonance of B- and C-rings shows a large upfield shift by 51 ppm. It is known that the nitrogen resonance of pyridine shifts to upfield by ca. 100 ppm upon protonation (Witanowski et al., 1977). The upfield shift of 51 ppm can be reasonably interpreted as an average of the shifts for the hydrogen-attached B-ring and the

protonated C-ring nitrogens. A similar upfield shift was also reported for protonation of $OEPH_2$ to $OEPH_4^{2+}$ (Kawano et al., 1978). This result also evidently shows that protonation occurs on nitrogen of C-ring.

Discussion

Protonation of the Chromophore. The RR spectra of oat phytochrome in P_{fr} excited at 752 nm were recently reported by Fodor et al (1990). Four RR bands were observed above 1300 cm^{-1} for P_{fr}: 1622, 1599, 1552, and 1312 cm^{-1}. Contrary to the present results, all bands exhibited deuteration shifts (to 1618, 1591, 1494, and 1060 cm^{-1}, respectively), and therefore, it was deduced that P_{fr} was protonated similarly to P_r. The RR bands of P_{fr} at 1461, 1420, and 1356 cm^{-1} in Figure 3 cannot be seen in the reported RR spectra excited at 752 nm, and other peak frequencies differ by more than the experimental errors in the two studies. Such discrepancies are also seen for the spectra of P_r. It is unlikely that the spectral differences are attributable to the different sources of the phytochrome (pea and oat).

Linear dichroism measurements of the absorption spectrum (Chae & Song, 1975) determined the angle between the transition dipoles for the near-UV and red bands of P_r to be 68°. This suggests that different parts of the chromophore contribute to the two transitions. Accordingly, different kinds of vibrational modes would be resonance-enhanced upon the near-UV and red excitations. If the red excitation preferentially probes vibrations attributed to the A-, B-, and D-rings, it should exhibit deuteration effects irrespective of protonation to the chromophore, since the three rings have exchangeable protons. Fodor et al. (1990) observed a single N–H bending band for three N–H groups of A-, B-, and D-rings and three C=C or C=N stretching bands for P_{fr}. The number of observed bands is much less than the number of modes expected even for the limited part of the molecule. Consequently, the similar deuteration effects observed for P_r and P_{fr} do not necessarily demonstrate that the chromophore is protonated. In contrast, the present observation revealed a clear difference between the deuteration effects of the C=C stretching modes of P_r and P_{fr}; this cannot be explained by the idea that both P_r and P_{fr} are protonated, unless only the C-ring proton of P_{fr} is nonexchangeable. On the other hand, if the near-UV excitation preferentially probes the C-ring moiety, the difference in deuteration shifts between the P_r and P_{fr} could be reasonably ascribed to the protonation effects. The 1617-cm^{-1} band of OEBV-h_3 in the near-UV-excited RR spectrum (Figure 6) gave a shift of −3 cm^{-1} for deuteration of three pyrrole rings, while the protonated form twice as large as a deuteration shift (−6 cm^{-1}).

The FTIR study by Siebert et al. (1990) demonstrated that the difference spectrum between P_r and P_{fr} is altered between H_2O and D_2O in both the positive and negative components. They therefore proposed that both P_r and P_{fr} are protonated. As mentioned above, because three pyrrole rings are deuterated even for the nonprotonated form, deuteration effects are not diagnostic unless the absorption bands associated with C-ring are specified. This is practically impossible at the present stage, since there are too many bands to be assigned. On the other hand, the FTIR difference spectrum between P_r and P_{fr} provided only a few difference peaks around 1690–1710 cm^{-1}. Since protonation to C-ring causes an upshift of the lactam C=O stretching band (Figure 6), the FTIR data are compatible with a model that the protonation level differs between P_r and P_{fr}.

Song and his co-workers reported SERR spectra of oat phytochrome adsorbed on colloidal Ag sols at 77 K (Rospendowski et al., 1989). The SERR spectrum excited at 407 nm has features in common with the present spectra: they reported peaks at 1624, 1596, 1458, 1414, and 1354 cm^{-1} for P_{fr} in the frequency region above 1350 cm^{-1}, which correspond to the bands at 1633, 1591, 1461, 1420, and 1356 cm^{-1} in Figure 3A of the present work. Although the 1547-cm^{-1} band in Figure 3A is not resolved in the SERR spectrum, a shoulder is recognizable at the lower frequency side of the strong 1596-cm^{-1} band of their reported spectrum [Figure 3 of Rospendowski et al. (1989)]. The 1354-cm^{-1} band in the SERR spectrum appears considerably weaker than the 1356-cm^{-1} band in Figure 3A. This might be due to the difference in temperature, because Fodor et al. (1990) obtained slightly different spectra for P_r at 77 and 300 K upon excitation at 752 nm. Alternatively, the differences in the relative peak intensities could be explained by the mode dependence of SERR enhancement, which depends on the orientation of the polarizability tensor of a given vibration relative to the surface normal of the Ag particles. When the stated instrumental error in the SERR work (±3 cm^{-1}) and the better spectral resolution of the present spectrum are taken into consideration, the present solution spectrum and the SERR spectrum seem to be in agreement. However, there may possibly be intrinsic slight differences in frequencies and relative peak intensities between the RR spectra of pea and oat phytochromes, since the two species have different amino acid sequences.

Photoreaction. Phototransformation of phytochrome is considered to involve the *Z,Z,Z* to *Z,Z,E* isomerization of the chromophore (Rudiger et al., 1983; Thummler et al., 1983; Farrens et al., 1989; Fodor et al., 1990) and/or proton migration (Moon et al., 1985; Tokutomi et al., 1988a) and is known to proceed via a few intermediates (Rudiger, 1987): $P_r \rightarrow I_{700}$ (=lumi-R) $\rightarrow I_{bl}$ (=meta-R) $\rightarrow P_{fr}$. It was reported previously that there is no net gain or loss of protons between P_r and P_{fr} as a whole molecule of intact pea phytochrome (Tokutomi et al., 1988). However, the present experiments demonstrated that the protonation state of the chromophore is definitely different between P_r and P_{fr}. Accordingly, a proton should be transferred from the chromophore to an amino acid residue in the process of phototransformation from P_r to P_{fr}. Several studies on the photoreaction kinetics have revealed that proton translocation is not involved in the photoreaction from P_r to I_{700} (Aramendia et al., 1987; Brock et al., 1987). Therefore, the proton migration from the chromophore to an amino acid residue should occur in the processes $I_{700} \rightarrow I_{bl}$ and/or $I_{bl} \rightarrow P_{fr}$. The observation that the large phytochrome releases a proton during the phototransformation process from P_r to I_{bl} (Tokutomi et al., 1988a) suggests that the former may be the case.

Large pea phytochrome lacks the N-terminal polypeptide of Ser-1 to Ser-51 (Yamamoto & Tokutomi, 1989) compared with the intact phytochrome (Lumsden et al., 1985). The molecular mass of the N-terminal polypeptide is calculated to be 5.6 kDa, which suggests that the large phytochrome also lacks a C-terminal segment (Yamamoto & Tokutomi, 1989) like oat phytochrome (Grimm et al., 1986). The 7-kDa segment(s) of the intact phytochrome is reported to be necessary for appearance of the characteristic spectral properties of P_{fr} (Vierstra & Quail, 1982; Lumsden et al., 1985). Without the 7-kDa segment(s), the population ratio of constituents present in the photo steady state is different between the H_2O and D_2O solutions, although with it the D_2O effect is absent (Sarkar & Song, 1981; Moon et al., 1985). Furthermore, the 7-kDa segments prevent the chromophore from reacting with exogenous reagents (Baron & Epel, 1983; Hahn et al., 1984;

Thummler et al., 1985). In the spectra shown in Figure 3, the 1356-cm^{-1} band of intact phytochrome was shifted to 1335 cm^{-1} with the large phytochrome. Both bands did not exhibit a frequency shift in D_2O. Therefore, they cannot be assigned to the N–H bending mode. It seems to be most plausible to assign them to the methine-bridge C–H in-plane bending mode. If this is the case, the frequency difference between the large and intact P_{fr} implies that the 7-kDa segments interact with the chromophore and induce some structural changes at the methine-bridge moiety. Since the 1461-cm^{-1} band of intact P_{fr} is shifted to 1453 cm^{-1} in D_2O, bulk water should reach the chromophore even in the presence of the 7-kDa peptides in either P_{fr} or other intermediates during the photocycle. However, with the intact phytochrome, the population ratio of constituents present in the photo steady state is hardly affected by bulk pH and I_{bl} is not accumulated in it (Tokutomi et al., 1986), suggesting that the 7-kDa peptides have an acceptor/donor residue and behave to have a shield effect for proton translocation between bulk water and the chromophore.

Phycocyanin, the light-harvesting component of the photosynthetic apparatus of cyanobacteria (blue-green algae) and of some red algae, contains a chromophore similar to that of phytochrome (the vinyl substituent of the D-ring is replaced by an ethyl group in phycocyanin). The protonated structure of its chromophore was recently studied by using a FT-Raman technique (Sawatzki et al., 1990), and it was found that the C=C stretching RR bands around 1635 cm^{-1} were shifted to lower frequency by 5–6 cm^{-1} in D_2O as in the case of P_r. Its molecular structure at 2.1-Å resolution was revealed by Schirmer et al. (1987); the chromophores arch around aspartate residues and the nitrogens of pyrroles B and C are within hydrogen-bonding distance of one of the carboxylate oxygens. Since the pK_a value of the cation of the P_r chromophore in an unfolded protein is 5.4 (Grombein et al., 1975), the tetrapyrrole would ordinarily be deprotonated at C-ring at pH 7.8 of the present solution. Therefore, the change in the protonation state of the chromophore in phytochrome must be induced by a change of the unique protein environment as in phycocyanin. It is plausible that the protonation/deprotonation of the chromophore during the phototransformation is switched by the distance between the nitrogen of C-ring and a proton-donating (or -accepting) amino acid residue(s) like in rhodopsin (Sakmar et al., 1989) and bacteriorhodopsin (Lin & Mathies, 1989).

The protonation effect on the most prominent Raman band of OEBV-h_3 is very small. This shows that the protonation itself little alters the RR spectral feature in the C=C stretching regions. In contrast, the RR spectral features of P_r and P_{fr} around C=C stretching region are much different with each other in both large and intact phytochromes. As a plausible explanation for it, one may note the presence of specific interactions between the chromophore and the apoprotein for P_{fr}, since the first absorption band of intact P_{fr} is bathochromically shifted in comparison with the absorption band of the P_{fr} chromopeptide (Rudiger, 1987). Such interactions may make the Raman spectra of the intact phytochrome different from those of the chromophore without apoprotein. It would be unlikely that the specific interactions ceased to exist in P_r if the structure of the chromophore remained unaltered between P_r and P_{fr}. The photoisomerization from *Z,Z,Z* to *Z,Z,E* may bring about the specific interaction between the chromophore and the protein moiety for P_{fr}.

In conclusion, the present RR experiments demonstrate that the protonation state of the chromophore is distinctly different between P_r and P_{fr} of intact pea phytochrome. The model compound study strongly suggests that the binding site of the additional proton in the P_r chromophore is the pyrrole nitrogen of the C-ring. Since there is no net gain or loss of protons between intact pea P_r and P_{fr}, the proton released from the chromophore of P_r would be migrated to a protein moiety of P_{fr}.

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Rate and Mechanism of the Assembly of Tropomyosin with Actin Filaments†

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ABSTRACT: The rate of assembly of tropomyosin with actin filaments was measured by stopped-flow experiments. Binding of tropomyosin to actin filaments was followed by the change of the fluorescence intensity of a (dimethylamino)naphthalene label covalently linked to tropomyosin and by synchrotron radiation X-ray solution scattering. Under the experimental conditions (2 mM $MgCl_2$, 100 mM KCl, pH 7.5, 25 °C) and at the protein concentrations used (2.5–24 μM actin, 0.2–3.4 μM tropomyosin) the half-life time of assembly of tropomyosin with actin filaments was found to be less than 1 s. The results were analyzed quantitatively by a model in which tropomyosin initially binds to isolated sites. Further tropomyosin molecules bind contiguously to bound tropomyosin along the actin filaments. Good agreement between the experimental and theoretical time course of assembly was obtained by assuming a fast preequilibrium between free and isolatedly bound tropomyosin.

Tropomyosin is a long rodlike molecule associated with actin filaments (Bailey, 1948; Martonosi, 1962; Laki et al., 1962). In non-muscle cells both free actin filaments and actin filaments covered with tropomyosin have been detected. In cultured human lung cells tropomyosin has been found to associate with newly formed actin filaments after a lag time of a few minutes (Lazarides, 1976). Tropomyosin regulates the assembly and stability of actin filaments and the actin-activated myosin ATPase (Ebashi et al., 1969; Spudich et al., 1972; Jahnke & Heilmeyer, 1983). Further, it stabilizes actin filaments against spontaneous fragmentation or fragmentation by severing proteins and retards binding of monomers to the

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