Resonance Raman Spectra of the Intermediates in Phototransformation of Large Phytochrome: Deprotonation of the Chromophore in the Bleached Intermediate†

Yasuhisa Mizutani,‡ Satoru Tokutomi,§ and Teizo Kitagawa*,‡

Institute for Molecular Science and Graduate University for Advanced Studies, Okazaki National Research Institutes, Myodaiji, Okazaki 444, Japan, and National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan

Received June 14, 1993; Revised Manuscript Received October 14, 1993⊛

ABSTRACT: Resonance Raman (RR) scattering from type A large phytochrome of pea was measured at cryogenic as well as ambient temperatures to determine an intermediate in which deprotonation of the chromophore takes place. The RR bands of the red-absorbing (P_r) and far-red-absorbing forms (P_{fr}) of large pea phytochromes at ambient temperatures are almost the same in their frequencies as those of the intact form reported previously (Mizutani et al., 1991). The RR spectrum of large phytochrome excited at 364 nm at −120 °C, where P_r and a photointermediate, I_{700} (=lumi-R), are trapped, showed a strong band at 1625 cm^{-1} with a shoulder at 1648 cm^{-1} in the C=C stretching region. The shoulder disappeared, and a new band appeared at 1597 cm^{-1} upon raising the temperature to −80 °C, where transformation from I_{700} to meta-R_a proceeds. The RR spectra remained unchanged until −10 °C, indicating that the RR spectra of meta-R_b and meta-R_c are close to that of meta-R_a, and we call them comprehensively the bleached intermediate, I_{bl}. At ambient temperatures where photo-steady-states among a few species are attained, strong RR bands were observed at 1625 and 1599 cm^{-1} upon excitation at 364 nm under simultaneous far-red illumination, and the 1599-cm^{-1} band was appreciably intensified under simultaneous red- instead of far-red illumination. By comparison of these spectra with those at low temperatures, the 1625- and 1599-cm^{-1} bands were reasonably assigned to P_r and I_{bl}, respectively. A chemically prepared model of the bleached form, P_{bl}, also gave a prominent band at 1599 cm^{-1}. The RR spectrum obtained by 407-nm excitation under red illumination showed two prominent bands at 1631 and 1591 cm^{-1}, which were previously assigned to P_{fr}. The 1625-cm^{-1} band of P_r exhibited a downward shift in D_2O, whereas the prominent C=C stretching bands of I_{bl}, P_{fr}, and P_{bl} did not. This indicates distinct differences in protonated structures of the chromophore between P_r and the other three species. Frequencies and relative intensities of RR bands in the 1650–1000-cm^{-1} region of I_{bl} resemble those of P_{fr} but not those of P_r, meaning that chromophore configurations of I_{bl} and P_{fr} are alike but dissimilar to that of P_r. These findings suggest that the configurational changes of the chromophore such as E–Z photoisomerization are involved in the process prior to the formation of I_{700} and that deprotonation of the chromophore follows it in a subsequent process yielding I_{bl}.

Phytochrome is a photoreceptor in green plants, converting absorbed light energy into physiological signals (Furuya, 1987; Rüdiger & Thümmler, 1991; Sage, 1992). The pigment can exist in two stable and photoconvertible forms: a physiologically inactive P_r[1] form (λ_{max} 667 nm) and an active P_{fr} (λ_{max} 730 nm) form. Formation of P_{fr} from P_r on absorption of red light triggers many morphogenetic responses. This molecule consists of two identical subunits with two major domains (Tokutomi et al., 1989; Nakasako et al., 1990) and a 2,3-dihydrobiliverdin chromophore in each one (Grombein et al., 1975; Lagarias & Rappoport, 1980). For chemical events in the phototransformation processes from P_r to P_{fr}, E–Z isomerization of the chromophore (Rüdiger et al., 1983; Thümmler & Rüdiger, 1983; Farrens et al., 1989; Fodor et al., 1990) and proton migration (Sarkar & Song, 1981; Tokutomi et al., 1982; Moon et al., 1985; Tokutomi et al., 1988b) have been proposed. However, molecular mechanisms of light-signal transduction have not been well understood. To elucidate this, more detailed structural information on all the intermediates present in the phototransformation process from P_r to P_{fr} is required.

Flash photolysis (Cordonnier et al., 1981; Inoue et al., 1990; Schaffner et al., 1990) and low-temperature absorption studies (Eilfeld & Rüdiger, 1984, 1985) have demonstrated that phototransformation of P_r to P_{fr} proceeds via at least two spectrally distinct intermediates. The first intermediate, I_{700}, appears within nanoseconds following actinic flash and is characterized by the hyperchromically shifted absorbance near 700 nm compared with P_r. The second intermediate, I_{bl}, is characterized by a reduced absorbance around 700 nm with no corresponding increase of abosrbance at nearby wavelengths. The intermediates I_{700} and I_{bl} are equivalent to lumi-R and meta-R of Kendrick and Spruit (1977), respectively. At low temperatures, the phototransformation stops at an intermediate step specific to a given temperature. The phototransformation processes reported so far (Eilfeld &

† 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. was supported by the fellowship of Japan Society for the Promotion of Science for Japanese Junior Scientists. This paper is dedicated to Professor W. Rüdiger on the occasion of his 60th birthday.

* To whom correspondence should be addressed.

‡ Institute for Molecular Science.

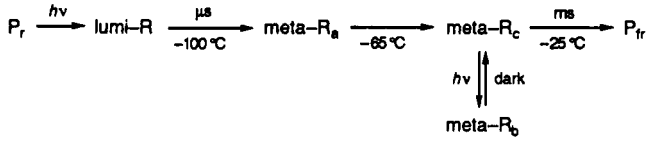
§ National Institute for Basic Biology.

⊛ Abstract published in *Advance ACS Abstracts,* December 15, 1993.

[1] Abbreviations: RR, resonance Raman; P_r and P_{fr}, red- and far-red-absorbing forms of phytochrome, respectively; P_{bl}, species with so-called bleached absorbing spectra of phytochrome; I_{bl}, comprehensive name of photointermediates of phytochrome with bleached absorption spectra accumulated in the photo-steady-state at ambient temperatures; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/94/0433-153$04.50/0

Rüdiger, 1985) are summarized as



where the order of the decay times of each intermediate at room temperature and the characteristic temperatures below which the subsequent processes are blocked are indicated. Recently, a new photoproduct with a decay time of 24 ps has been reported (Kandori et al., 1992), and this species gives an absorption maximum near 680 nm distinct from that of I_{700} (Kandori et al. submitted for publication).

In order to obtain structural information about the photochemical events during these phototransformation processes, RR spectroscopy is a powerful technique, since it can selectively provide information related to the *in situ* structures of chromophores in chromoproteins (Spiro, 1987). However, application of this technique to phytochrome has long been unsuccessful due to its intense fluorescence. Recently, RR spectra of phytochrome have been measured by a few groups: RR spectra of oat phytochrome with far-red excitation (Fodor et al., 1988, 1990; Hildebrandt et al., 1992), of oat phytochrome with surface-enhanced Raman effects upon blue excitation (Farrens et al., 1989; Rospendowski et al., 1989), and of pea phytochrome by using the two-color excitation technique (Tokutomi et al., 1990; Mizutani et al., 1991). In the last one, we used deep blue excitation for probing Raman scattering to avoid the interference by inherent fluorescence, and simultaneously illuminated the sample by red or far-red light to increse the population of either P_{fr} or P_r in the photo-steady-state. Our previous study of intact pea phytochrome (Mizutani et al., 1991) indicated different protonated structures of the chromophore between P_r and P_{fr} similar to those in the large phytochrome (Tokutomi et al., 1990), but in the present study, we succeeded in obtaining RR spectra of I_{bl} at cryogenic temperatures. We also extended the measurements to a chemically prepared model of bleached species, P_{bl}, which gives rise to a so-called bleached spectrum similar to I_{bl}, at an ambient temperature. On the basis of these observations together with the spectra of P_r and P_{fr} obtained with signal-to-noise ratios higher than those in the previously reported ones, we discuss structural changes of the chromophore and proton migration in the phototransformation process.

MATERIALS AND METHODS

Phytochrome Preparation. Type A large phytochrome of pea, consisting of two equivalent subunits of molecular mass 114 kDa (determined by SDS–PAGE), was isolated from 7-day-old etiolated seedlings of pea (*Pisum sativum* cv. Alaska) as described previously (Tokutomi et al., 1988a). The purified protein was concentrated through ammonium sulfate precipitation [$(ND_4)_2SO_4$ in D_2O for the deuterated preparation] followed by resuspension into a small volume of 50 mM HEPES and 1 mM Na_2EDTA, pH 7.8 [D_2O was used for the deuterated preparation, but the pD value indicated by the pH meter was used instead of pD = pH + 0.4, since the correction was not confirmed with this protein and the small change of pH around 7.8 alters only slightly relative populations of P_r, P_{fr}, and I_{bl} in a photo-steady-state; see Tokutomi et al. (1990)]. In this preparation, the phytochrome solution was dialyzed against the HEPES–EDTA solution to remove remaining ammonium sulfate completely. On account of the last procedure no turbidity increase was observed during the measurements, and this greatly improved the quality of the present RR spectra compared with those of previous studies (Tokutomi et al., 1990). All preparation procedures were carried out under dark or dim green light. The specific absorbance ratio (A_{667}/A_{280}) of the present sample was between 0.93 and 0.98.

A model of P_{bl} was prepared by alkaline treatment, since the addition of bleaching reagents such as zinc ions (Sommer & Song, 1990) or soyasaponin (Konomi et al., 1982) to the highly concentrated phytochrome solutions induced some precipitation. In brief, large phytochrome precipitated with ammonium sulfate was resuspended in 50 mM CHES and 1 mM Na_2EDTA at pH 9.0, and then its pH was raised to 11.0 by adding 5 M NaOH. The solution was irradiated with red light until the absorption spectrum showed complete bleaching. Immediately after the end of the irradiation, 5 M HCl was added to lower the pH to 9.0. The alkaline treatment at pH 11.0 yielded a stable bleached spectrum at pH 9.0 similar to those of soyasaponin-treated (Konomi et al., 1982), tryptic digestion-treated (Yamamoto & Furuya, 1983), and zinc ion-treated (Sommer & Song, 1990) phytochromes, although alkaline treatment lower than pH 11.0 showed reversion to P_r at pH 9.0.

Measurements of RR Spectra. About 50 μL of phytochrome solution with a concentration of 8.0 cm^{-1} in terms of A_{667} (P_r form), which corresponds to 62 μM on the basis of $\epsilon_M = 1.30 \times 10^5$ cm^{-1} M^{-1} for the large pea phytochrome (Yamamoto, unpublished result), 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). The sample solution in the spinning cell was illuminated with either far-red (740 nm) or red (660 nm) light from the side of the cell during the Raman measurements in order to bias the equilibrium of the photo-steady-state toward either P_r or P_{fr}, respectively. In the room temperature experiments, the red or the far-red light was obtained by passing the radiation from a 300-W tungsten lamp through a 660-nm (FWHM, 3 nm) or 740-nm (FWHM, 10.5 nm) interference filter and ca. 10-mm path of water.

Measurements of RR spectra at low temperature were performed with a sample containing 67% (v/v) glycerol (glycerol-d_3 for deuterated preparation) by using a cryostat cooled by cold N_2 gas. The final concentrations of phytochrome, HEPES, and Na_2EDTA after the addition of glycerol were the same as those used in the room temperature experiments. The sample temperature was controlled within ±2 °C by adjusting the flow rate of the gas. The sample irradiated saturatingly with far-red light was put into the micro spinning-cell and then cooled gradually to −120 °C in the cryostat in the dark. After the temperature at which the RR spectrum should be measured was reached, the sample was illuminated with red light for 3 min, and then RR spectra were measured. As a source of red light in the low-temperature experiments, 633-nm light obtained from a He/Ne laser (NEC GLS 5800) was used.

Raman scattered light was 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 differences in sensitivity of individual pixels of the diode array detector were corrected by the white light:

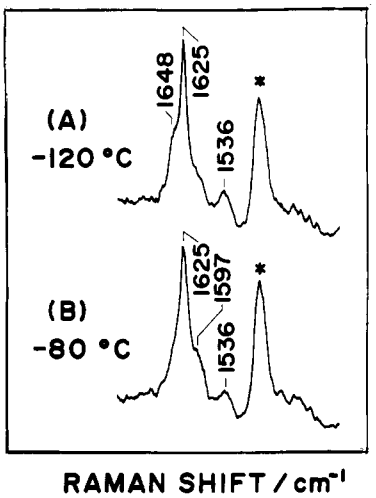


FIGURE 1: RR spectra of pea large phytochrome observed at −120 (A) and −80 °C (B) by 364-nm excitation under red illumination (633 nm). The bands marked by an asterisk arise from glycerol.

the observed spectra were divided by the spectrum of white light. The background due to fluorescence was removed by subtraction of the curve generated by a suitable polynomial function.

RESULTS

Figure 1A shows the RR spectra in the C=C stretching region of large phytochrome at −120 °C measured with 364-nm excitation under red illumination. Under this condition where P_r and I_{700} (=lumi-R) should be trapped (Eilfeld & Rüdiger, 1985), the observed RR spectrum is composed of a strong band at 1625 cm^{-1} with a shoulder at 1648 cm^{-1} and a minor band at 1536 cm^{-1}. Upon raising the temperature to −80 °C (Figure 1B), where thermal conversion from I_{700} to meta-R_a proceeds (Eilfeld & Rüdiger, 1985), the shoulder at 1648 cm^{-1} disappeared, and a new band appeared at 1597 cm^{-1}, while the relative intensity of the 1536-cm^{-1} band decreased slightly. When the temperature was raised to −10 °C, at which a mixture of meta-R_b and meta-R_c is generated (Eilfeld & Rüdiger, 1985), the RR spectrum was unaltered. Therefore, it is likely that the three meta-R species give rise to similar RR spectra. Since these species and other intermediates in the back-reaction present in the photo-steady-state cannot be distinguished from each other by visible absorption and RR spectra at ambient temperatures, these species are comprehensively called I_{bl} in this study.

On the basis of the temperature dependence, the bands at 1625, 1648, and 1597 cm^{-1} are assigned to P_r, I_{700}, and I_{bl}, respectively. The 1536-cm^{-1} band might be common to P_r, I_{700}, and I_{bl}. The low-temperature RR spectra were also measured for the D_2O solution. A deuteration shift of the 1625-cm^{-1} band to a lower wavenumber was recognizable, but its spectral quality was not high enough to determine the precise peak positions of the minor 1648- and 1597-cm^{-1} bands mainly due to strong fluorescence background in the D_2O solution. Although the origin is unknown, similar enhancement of fluorescence in a deuterated solution has been reported with oat phytochrome (Sarkar & Song, 1981).

RR spectra of the pea large phytochrome at 16 °C excited at 364 nm are displayed in Figure 2. The left and right spectra were obtained for H_2O and D_2O solutions, respectively. The top (A and A′) and the middle spectra (B and B′) were obtained under far-red and red light illumination, respectively, and the bottom traces (C and C′) are the weighted differences between

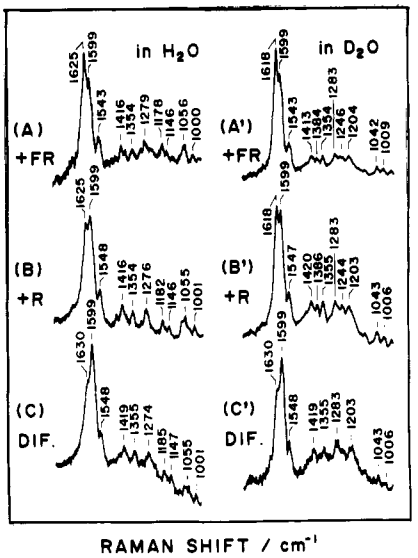


FIGURE 2: RR spectra of pea large phytochrome at 16 °C observed by 364-nm excitation at pH 7.8 in H_2O (left) and in D_2O (right). Spectra A and A′ were obtained under far-red illumination (740 nm) and spectra B and B′ under red illumination (660 nm). Spectra C and C′ are weighted differences; $C = B - kA$ and $C' = B' - k'A'$. Details about the difference calculations are described in the text.

the upper two ($C = B - kA$, $C' = B' - k'A'$). The weight factors, k and k', were adjusted so that the 1625/1618-cm^{-1} bands disappear and no negative peaks were generated.

Two prominent bands were observed in the C=C stretching region at 1625 and 1599 cm^{-1} under far-red illumination. In a D_2O solution, the former band showed −7-cm^{-1} shift, while the latter exhibited no shift. Large pea phytochrome has been shown to attain photo-steady-states among P_r, P_{fr}, and I_{bl} under red (Tokutomi et al., 1986) and blue (Tokutomi et al., 1990) illumination at ambient temperatures. Here, I_{bl} can be defined by a visible absorption spectrum, but may not always mean a single species as mentioned above. I_{700} is not populated due to its short lifetime (approximately microseconds). The 1625/1618-cm^{-1} bands cannot be assigned to P_{fr} due to the following reasons. First, P_{fr} has its second absorption band around 408 nm, while P_r and I_{bl} have them around 380 nm (Eilfeld & Rüdiger, 1985), much closer to the excitation wavelength (364 nm), and are expected to have a stronger resonance enhancement effect. Second, the population of P_{fr} is negligible under far-red illumination. However, it could not be determined in the previous study (Tokutomi et al., 1990) whether the bands arose from P_r or P_{bl}. In this study, the assignment of the 1625/1618-cm^{-1} bands to P_r became evident from the low-temperature experiments which demonstrated the presence of the 1625-cm^{-1} band at −120 °C where I_{bl} was not formed (Figure 1). This band showed a downward shift on deuteration (data not shown).

The 364-nm-excited RR spectrum of the species whose population increased under red illumination is shown by traces C and C′ in Figure 2. They give a strong band at 1599 cm^{-1}, which is remarkably close to the 1597-cm^{-1} band of the spectrum at −80 °C (Figure 1B), where I_{bl} appears. The visible absorption spectra at ambient temperature also indicate the increased population of I_{bl} under red illumination than far-red illumination (Tokutomi et al., 1990). Therefore, it is most likely that the 1599-cm^{-1} band in Figure 2C,C′ arises from I_{bl}.

The difference spectra (Figure 2, bottom) have a strong band at 1599 cm^{-1} with a shoulder at 1630 cm^{-1} and a minor band at 1548 cm^{-1} in the C=C stretching region. All these

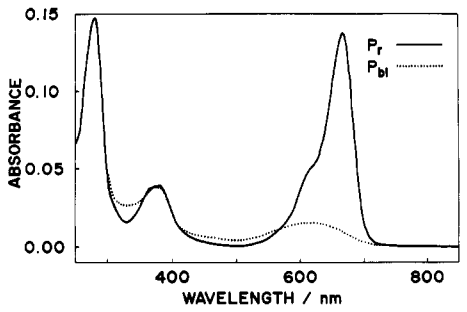


FIGURE 3: Absorption spectra of pea large phytochrome in P_r (solid line) and P_{bl} forms (dotted line). P_{bl} was obtained by red light irradiation after alkaline treatment as described under Materials and Methods.

bands and the bands at 1419- and 1335-cm^{-1} did not show any deuteration shift. However, in D_2O the band of the H_2O solution at 1274 cm^{-1} is upward-shifted by 9 cm^{-1}, the doublet at 1185/1147 cm^{-1} of the H_2O solution disappears, and new bands appear at 1244/1203 cm^{-1}. The D_2O-sensitive bands are considered to be RR spectral characteristics of I_{bl} and ascribed to modes associated with the pyrrole rings A, B, and D. The spectra for P_r were also calculated by subtracting spectrum C (or C') from spectrum B (or B'). This gave only two bands, a strong one at 1625/1618 cm^{-1} and a weak and broad one at 1530 cm^{-1}, but not other features (data not shown). This means that the RR spectrum of P_r is not resonance-enhanced strongly at this excitation wavelength except for the strong two double-bond stretching modes.

The photo-steady-state of large phytochrome under red illumination is affected by pH (Tokutomi et al., 1986). Accumulation of I_{bl} becomes remarkable in alkaline solutions, and, therefore, the stable bleached form is obtained by alkaline treatment as described under Materials and Methods. The absorption spectrum of this species is shown in Figure 3 together with that of P_r. This spectrum bears close resemblance to those obtained by red light irradiation to a tryptic-digested fragment (Yamamoto & Furuya, 1983), to large phytochrome in the presence of soyasaponin I (Konomi et al., 1982) or 2-anilinonaphthalenesulfonic acid (Hahn & Song, 1981), and to intact phytochrome in the presence of zinc ions (Sommer & Song, 1990). Accordingly, this species can be regarded as a model of the bleached species, P_{bl}. The absorption spectrum represented by the dotted line in Figure 3 closely resembles that of I_{bl} (Eilfeld & Rüdiger, 1984, 1985). The CD spectrum of I_{bl} at low temperature (Burke et al., 1972) is also similar to that of P_{bl} obtained with the 39-kDa fragment of pea phytochrome (Yamamoto & Furuya, 1983).

RR spectra of P_{bl} in H_2O and D_2O solutions are shown in Figure 4A and Figure 4B, respectively. The RR spectrum of P_{bl} in H_2O (Figure 4A) is close to that of I_{bl} (Figure 2C). In the C=C stretching region, a strong band was observed at 1599 cm^{-1} with weak shoulders at 1636 and 1554 cm^{-1}, which correspond to the prominent band at 1599 cm^{-1} with shoulders at 1630 and 1548 cm^{-1} of I_{bl}. The 1415-, 1355-, 1274-, 1056-, and 1001-cm^{-1} bands of P_{bl} seem to correspond to the 1419-, 1355-, 1274-, 1055-, and 1001-cm^{-1} bands of I_{bl}, although the 1247-cm^{-1} band of P_{bl} is missing in the spectrum of I_{bl} and, conversely, the 1147-cm^{-1} band of I_{bl} is missing in that of P_{bl}.

Deuteration of P_{bl} did not affect the 1599-cm^{-1} band as in the case of I_{bl}, but it induced splitting of the 1415-cm^{-1} band into 1408 and 1384 cm^{-1} and shifts of the 1274-, 1176-, 1056-, and 1001-cm^{-1} bands to 1282, 1197, 1045, and 1008 cm^{-1}, respectively. Similar deuteration effects were observed with I_{bl}, e.g., shifts of 1274-, 1185-, 1055-, and 1001-cm^{-1} bands

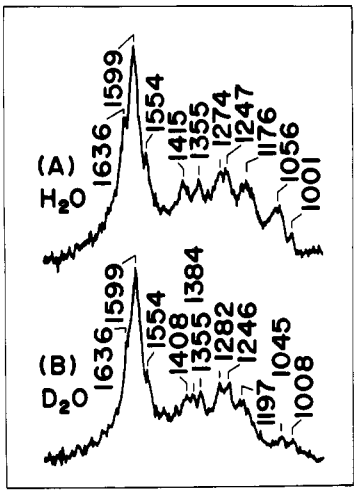


FIGURE 4: RR spectra of P_{bl} of pea large phytochrome in H_2O (A) and D_2O (B) solutions. Excitation, 364 nm. Spectra were obtained under simultaneous light illumination.

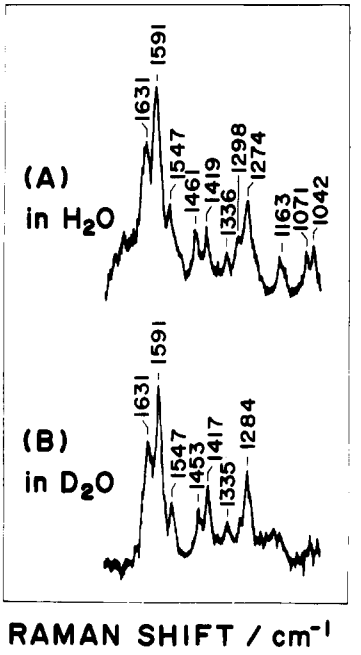


FIGURE 5: RR spectra of large pea phytochrome at 16 °C obtained by 407-nm excitation under red illumination (660 nm) in H_2O (A) and D_2O (B).

to 1283, 1203, 1043, and 1006 cm^{-1}. The strong 1599-cm^{-1} band is presumably characteristic of the chromophore structure with a bleached absorption spectrum as seen for I_{bl} and P_{bl}. Although P_{bl} obtained by alkaline treatment does not show the photoreversibility to P_r which P_{bl}'s prepared by other methods preserve, the present P_{bl} can serve as a model for the chromophore structure of I_{bl} on the basis of their RR spectra.

RR spectra of red-illuminated phytochrome observed with 407-nm excitation are depicted in Figure 5. In the C=C stretching region, there are two prominent RR bands at 1631 and 1591 cm^{-1} and a weaker band at 1547 cm^{-1}, which are assignable to P_{fr}. Although the presence of these bands was pointed out previously, its spectrum was of much lower signal-to-noise ratio (Tokutomi et al., 1990). In this study, the RR spectrum became more reliable, and it became evident that these three bands showed no deuteration shifts. However, the 1461-cm^{-1} band is shifted to 1453 cm^{-1}, and the 1298/1274-cm^{-1} doublet appears as a singlet at 1284 cm^{-1} in D_2O.

In addition, the RR bands at 1163, 1071, and 1042 cm^{-1} in H_2O solution are considerably weakened in D_2O solution. These bands are presumably associated with the A, B, and D rings, and deuterium substitution of the NH groups of these rings is evident.

DISCUSSION

Different Protonation Structures of the Chromophore between P_r and I_{bl}. It has been proposed previously that the chromophores of P_r and P_{fr} are different in their protonation structures for the following reasons (Tokutomi et al., 1990; Mizutani et al., 1991). The major RR band in the C=C stretching region of P_r (1625 and 1626 cm^{-1} for large and intact pea phytochromes, respectively) showed a deuteration shift, while the corresponding bands of P_{fr} (1631 and 1591 cm^{-1} for large and 1633 and 1591 cm^{-1} for intact pea phytochromes) did not. It was inferred that this difference arose from protonation of the C-ring nitrogen of the tetrapyrrole chromophore in P_r (Fodor et al., 1988; Mizutani et al., 1991) and its deprotonation in P_{fr} (Mizutani et al., 1991). The present results confirmed this proposal and furthermore suggest different protonated structures of the chromophore between P_r and I_{bl}, since the 1599-cm^{-1} band of I_{bl} did not show a deuteration shift while the 1625-cm^{-1} band of P_r showed it. This would also be explained by deprotonation of the C-ring nitrogen of the chromophore in I_{bl}.

These bands are probably associated with the C=C and C=N stretching vibrations of pyrrole ring C. Note that pyrrole rings A, B, and D are always protonated and their C–NH–C bonds are not involved in the π conjugation chain. However, the C=N bond of the C ring would be involved in the π conjugation and would be vibrationally coupled with the C=C stretching modes. Therefore, double-bond stretching modes near the C ring would reflect protonation of the C ring more sensitively than other modes.

Some RR bands of P_{fr} in the 1500–1000-cm^{-1} region showed deuteration effects (Figure 5). Similar deuteration effects are observed in the RR spectra of P_r plus I_{bl} (Figure 2B) and of I_{bl} (Figure 2C). These presumably reflect the exchange of imino protons of the A, B, and D rings of tetrapyrrole with a deuteron in a D_2O solution, and presumably similar deuteration shifts would be observed for P_r.

Involvement of Proton Migration in the Phototransformation of Phytochrome. It is known that there is apparently no net uptake or release of protons between P_r and P_{fr} for intact phytochrome (Tokutomi, 1986) but that the protonated structures of their chromophores are distinct (Mizutani et al., 1991). On the other hand, pea large phytochrome has been shown to release or take up protons on phototransformation depending on the medium pH (Tokutomi et al., 1982). It was deduced from the observed correlation of proton release with the formation of I_{bl} or P_{bl} (Tokutomi et al., 1988b) and also from the effects of pH on phototransformation (Tokutomi et al., 1986) that a proton is released during the phototransformation process from P_r to I_{bl}.

We have explored that the RR spectra of pea intact phytochrome are similar to those of large phytochrome in both the P_r and P_{fr} forms, although this technique reveals only the vibrational spectrum of the chromophore. The present observation has provided clear evidence that a proton is detached from the chromophore in the process from P_r to I_{bl}. Several studies on the photoreaction kinetics of intact oat phytochrome have revealed that the phototransformation process from P_r to I_{700} is unaffected by deuteration (Ruzsicska et al., 1985; Aramendia et al., 1987; Brock et al., 1987).

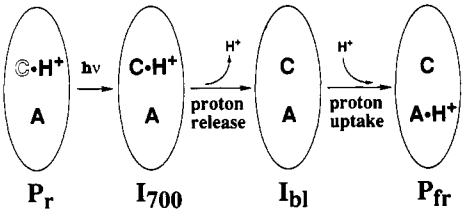


FIGURE 6: Proposed model for the phototransformation of phytochrome. The symbols "C" and "A" represent the chromophore and a particular amino acid residue of the protein, respectively. Different styles of the symbol "C" represent different chromophore configurations.

Table 1: Comparison of RR Bands (cm^{-1}) and Their Deuteration Shifts for I_{bl}, P_{bl}, and P_{fr}

I_{bl}			P_{bl}			P_{fr}		
H_2O	D_2O	$\Delta\nu$[a]	H_2O	D_2O	$\Delta\nu$	H_2O	D_2O	$\Delta\nu$
1630	1630	0	1636	1636	0	1631	1631	0
1599	1599	0	1599	1599	0	1591	1591	0
1548	1548	0	1554	1554	0	1547	1547	0
						1461	1453	−8
1419	1419	0	1415	1408	−7	1419	1417	−2
				1384				
1355	1355	0	1355	1355	0	1336	1335	−1
						1298		
1274	1283	+9	1274	1282	+8	1274	1284	+10
			1247	1246	−1			
1185	(1203)	(+18)	1176	1197	+21	1163		
1147								
1055	(1043)	−12	1056	1045	−11	1071		
1001	(1006)	(+5)	1001	1008	+7	1042		

[a] Frequency difference between in H_2O and in D_2O buffers.

Therefore, proton detachment from the chromophore should take place in the process from I_{700} to I_{bl}.

Proton uptake, on the other hand, is proposed to occur during the phototransformation process later than the formation of I_{bl} (Tokutomi et al., 1986, 1988b). The presence of the N-terminal 6-kDa segment in the intact phytochrome, which makes the absorption spectrum intact (Vierstra & Quail, 1982) and protects the chromophore of P_{fr} from access of the oxidative reagent (Hahn et al., 1984; Thümmler et al., 1985) or of protons (Tokutomi et al., 1986), apparently cancels the proton transfer between a whole molecule and the external medium so that no net gain or loss of protons takes place in the phototransformation from P_r to P_{fr} of the intact phytochrome (Tokutomi et al., 1988b). These results suggest proton migration from the chromophore to an amino acid residue protected by the 6-kDa segment. Figure 6 schematically illustrates the chemical events involved in the phototransformation process.

Involvement of Isomerization in the Phototransformation of Phytochrome. The E–Z photoisomerization around the C=C bond between the C and D rings of the tetrapyrrole chromophore is proposed as the most likely event for the primary photoreaction of phytochrome (Rüdiger et al., 1983; Thümmler & Rüdiger, 1983; Farrens et al., 1989; Fodor et al., 1990). Such a change in the chromophore structure is expected to yield some RR spectral changes in the relative intensities and frequencies of RR bands in the C=C stretching region. The frequencies of the RR bands in this region are compared among I_{bl}, P_{fr}, and P_{bl} in Table 1, where deuteration shifts are also indicated for reference. The RR spectral patterns of these three species are rather close although precise band positions are somewhat altered among them.

RR spectral features of P_r in this region differ markedly from those of P_{fr} and I_{bl}. Upon Raman excitation in deep

blue, P_r gives a single strong band at 1625 cm^{-1} in the double-bond stretching region above 1550 cm^{-1} and extremely weak features in the fingerprint region, whereas I_{bl} and P_{fr} give two prominent bands around 1630 and 1590–1600 cm^{-1} and many weak bands in the fingerprint region, suggesting some configurational differences between the chromophores of P_r and the other two species. This supports photoisomerization as the primary event in the photoreaction of phytochrome.

CONCLUDING REMARKS

RR spectra were measured for large phytochrome at low temperatures, and the RR spectral characteristics of I_{bl} have been determined for the first time. The major C=C stretching RR bands in the photo-steady-state at ambient temperatures were reasonably assigned to either P_r, I_{bl}, or P_{fr}. The different deuteration effects between P_r and the other two species suggest that the protonated structure of the chromophore differs between them. In consideration of the previous results on proton transfer between the intact phytochrome and medium during the phototransformation (Tokutomi et al., 1988b), we propose that deprotonation of the chromophore occurs in the process from I_{700} to I_{bl} and that protonation to the protein moiety occurs in the process from I_{bl} to P_{fr} for intact phytochrome. RR spectra of a model-bleached species with the absorption spectra of P_{bl} were also determined in H_2O and D_2O for the first time, and it was found that the species giving rise to the so-called bleached absorption spectrum has a chromophore structure similar to that of I_{bl}. General features of the RR bands in the C=C stretching region differ markedly between P_r and the other two species, supporting the proposal of photoisomerization as the primary event of phototransformation.

REFERENCES

Aramendia, P. F., Ruzsicska, B. P., Braslavsky, S. E., & Schaffner, K. (1987) *Biochemistry 26*, 1418–1422.

Brock, H., Ruzsicska, B. P., Arai, T., Schlamann, W., Holtzwarth, A. R., Braslavsky, S. E., & Schaffner, K. (1987) *Biochemistry 26*, 1412–1417.

Burke, M. J., Pratt, D. C., & Moscowitz, A. (1972) *Biochemistry 11*, 4025–4031.

Cordonnier, M. M., Mathis, P., & Pratt, L. H. (1981) *Photochem. Photobiol. 34*, 733–740.

Eilfeld, P., & Rüdiger, W. (1984) *Physiol. Plant 60*, 383–388.

Eilfeld, P., & Rüdiger, W. (1985) *Z. Naturforsch. 40C*, 109–114.

Farrens, D. L., Holt, R. E, Rospendowski, B. N., Song, P.-S., & Cotton, T. M. (1989) *J. Am. Chem. Soc. 111*, 9162–9169.

Fodor, S. P. A., Lagarias, J. C., & Mathies, R. A. (1988) *Photochem. Photobiol. 48*, 129–136.

Fodor, S. P. A., Lagarias, J. C., & Mathies, R. A. (1990) *Biochemistry 29*, 11141–11146.

Furuya, M., Ed. (1987) *Phytochrome and Photoregulation in Plants*, Academic Press, Tokyo.

Grombein, S., Rüdiger, W., & Zimmermann, H. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1709–1714.

Hahn, T.-R., & Song, P.-S. (1981) *Biochemistry 20*, 2602–2609.

Hahn, T.-R., Song, P.-S., Quail, P. H., & Vierstra, R. (1984) *Plant Physiol. 74*, 755–758.

Hildebrandt, P., Hoffman, A., Lindemann, P., Heibel, G., Braslavsky, S. E., Schaffner, K., & Schrader, B. (1992) *Biochemistry 31*, 7957–7962.

Inoue, Y., Rüdiger, W., Grimm, R., & Furuya, M. (1990) *Photochem. Photobiol. 52*, 1077–1083.

Kandori, H., Yoshihara, K., & Tokutomi, S. (1992) *J. Am. Chem. Soc. 114*, 10958–10959.

Kendrick, R. E., & Spruit, C. J. P. (1977) *Photochem. Photobiol. 26*, 201–214.

Konomi, K., Furuya, M., Yamamoto, K. T., Yokota, T., & Takahashi, N. (1982) *Plant Physiol. 70*, 307–310.

Lagarias, J. C., & Rapoport, H. (1980) *J. Am. Chem. Soc. 102*, 4821–4828.

Mizutani, Y., Tokutomi, S., Aoyagi, K., Horitsu, K., & Kitagawa, T. (1991) *Biochemistry 30*, 10693–10700.

Moon, D.-K., Jeen, G.-S., & Song, P.-S. (1985) *Photochem. Photobiol. 42*, 633–641.

Nakasako, M., Wada, M., Tokutomi, S., Yamamoto, K. T., Sakai, J., Kataoka, M., Tokunaga, F., & Furuya, M. (1990) *Photochem. Photobiol. 52*, 3–12.

Rospendowski, B. N., Farrens, D. L., Cotton, T. M., & Song, P.-S. (1989) *FEBS Lett. 258*, 1–4.

Rüdiger, W., & Thümmler, F. (1991) *Angew. Chem., Int. Ed. Engl. 30*, 1216–1228.

Rüdiger, W., Thümmler, F., Cmiel, E., & Schneider, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6244–6248.

Ruzscicska, B. P., Braslavsky, S. E., & Schaffner, K. (1985) *Photochem. Photobiol. 41*, 681–688.

Sage, H. (1992) *Pigment of the Imagination; A History of Phytochrome Research*, Academic Press, San Diego.

Sarkar, H. K., & Song, P.-S. (1981) *Biochemistry 20*, 4315–4320.

Schaffner, K., Blaslavsky, S. E., & Holzwarth, A. (1990) *Pure Appl. Chem. 62*, 1421–1426.

Sommer, D., & Song, P.-S. (1990) *Biochemistry 29*, 1943–1948.

Spiro, T. G., Ed. (1987) *Biological Applications of Raman Spectroscopy*, Wiley, New York.

Thümmler, F., & Rüdiger, W. (1983) *Tetrahedron 39*, 1943–1951.

Thümmler, F., Eilfeld, P., Rüdiger, W., Moon, D.-K., & Song, P.-S. (1985) *Z. Naturforsch. 40C*, 215–218.

Tokutomi, S., Yamamoto, K. T., Miyoshi, Y., & Furuya, M. (1982) *Photochem. Photobiol. 35*, 431–433.

Tokutomi, S., Inoue, Y., Sato, N., Yamamoto, K. T., & Furuya, M. (1986) *Plant Cell Physiol. 27*, 765–773.

Tokutomi, S., Kataoka, M., Sakai, J., Nakasako, M., Tokunaga, F., Tasumi, M., & Furuya, M. (1988a) *Biochim. Biophys. Acta 953*, 297–305.

Tokutomi, S., Yamamoto, K. T., & Furuya, M. (1988b) *Photochem. Photobiol. 47*, 439–445.

Tokutomi, S., Nakasako, M., Sakai, J., Kataoka, M., Yamamoto, K. T., Wada, M., Tokunaga, F., & Furuya, M. (1989) *FEBS Lett. 247*, 139–142.

Tokutomi, S., Mizutani, Y., Anni, H., & Kitagawa, T. (1990) *FEBS Lett. 269*, 341–344.

Vierstra, R. D., & Quail, P. H. (1982) *Planta 156*, 158–165.

Yamamoto, K. T., & Furuya, M. (1983) *Plant Cell Physiol. 24*, 713–718.