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Resonance Raman spectra of large pea phytochrome at ambient temperature

Difference in chromophore protonation between red- and far red-absorbing forms

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Resonance Raman (RR) scattering from large pea phytochrome was observed at ambient temperature for the first time by using a micro-spinning cell and the two color excitation technique. The relative population of the red-absorbing form (P_r), the far red-absorbing form (P_{fr}), and the bleached intermediate (I_{bl}) under laser illumination was estimated from the absorption spectra. The RR spectrum of P_r obtained by the 363.8 nm excitation under the 740.0 nm pumping exhibited a frequency shift between the H_2O and D_2O solutions, but those of P_{fr} and I_{bl} obtained by the 406.7 nm excitation under the 632.8 nm pumping did not, indicating a distinct difference in their protonation levels, presumably in the protonation of ring C.

Phytochrome; Resonance Raman; Vibrational spectra; Protonation state of photochrome

1. INTRODUCTION

Phytochrome (P) is a noble photoreceptor chromoprotein in green plants and performs a variety of reversible morphogenetic and developmental responses upon red/far red light illumination [1,2]. This molecule consists of two identical subunits [3], with a 2,3-dihydrobiliverdin chromophore in each one [4,5]. It undergoes a photoreversible transformation between a red-absorbing form (P_r) and a far red-absorbing form (P_{fr}) via intermediates like a bleached form (I_{bl}). Although isomerization of the chromophore [6-8] and proton migration [9-11] have been proposed for the phototransformation and a structure of the chromophore with an undeca-peptide from P_r was determined by NMR spectroscopy [5], the chromophore structures in the P_r and P_{fr} proteins have not been clarified yet.

Resonance Raman (RR) spectroscopy is a powerful technique for studying the structure of chromophores in chromoproteins [12]. However, application of this technique to P had long been unsuccessful due to the intense fluorescence of phytochrome preparations. Recently RR spectra of oat P in P_r form were obtained at 77K with far red excitation [13,14] and also a surface enhanced resonance Raman (SERR) study of oat P was reported with blue excitation [15-17]. However, in order to obtain information on structural differences between the two phytochrome forms, it is indispensable to observe the RR spectra of P_r and P_{fr} forms in a natural state at ambient temperature. Accordingly, we have studied the RR spectra of P in a natural state by using a two color excitation technique, and report here the blue excited RR spectra of the P_r and P_{fr} forms of large pea P at 288K for the first time. We propose that the P_r and P_{fr} forms have different protonated structures.

2. EXPERIMENTAL

Large pea phytochrome was isolated according to reported method* [18], precipitated by ammonium sulfate [$(ND_4)_2SO_4$ in D_2O for the deuterated preparation] and resuspended in either 50 mM HEPES and 1 mM Na_2EDTA, pH 7.8 or 50 mM CHES and 1 mM Na_2EDTA, pH 9.0 to a final concentration of 4.5–8.0 cm^{-1} in terms of A_{667} ($\epsilon_M = 1.3 \times 10^5\ cm^{-1}$). RR spectra were measured for 50 μl of the P preparation in a micro spinning-cell at 1600 rpm, kept at 288 ± 3 K by flushing with cold N_2 gas, and detected by a photodiode array (PAR 1420) attached to a Spex 1401 double monochromator. Raman scattering was excited at 406.7 nm (Kr^+ laser) or 363.8 nm (Ar^+ laser) under continuous illumination to another spot of the cell at 740.0 nm (Ti-doped sapphire laser) or at 632.8 nm (He/Ne laser) to

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*Large pea phytochrome was prepared from 7 day-old etiolated seedlings of pea (*Pisum sativum* cv. Alaska) as described [18]. The specific absorbance ratio (A_{667}/A_{280}) of the present sample was 0.98. Its purity was estimated to be more than 95% as judged from the SDS polyacrylamide gel electrophoresis.

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bias the equilibrium in photosteady state toward either P_r or P_{fr}, respectively. However, in the photosteady state under double laser illumination, the third component [19,20], which was previously demonstrated to have an absorption spectrum close to that of the bleached form and is considered to be the same as I_{bl} [21], was appreciably present besides P_r and P_{fr}. The relative population of P_r, P_{fr}, and I_{bl}, under double laser illumination was estimated separately from visible absorption spectra** that were measured by irradiating the continuously stirred sample solution in the sample compartment of a spectrophotometer (Shi-madzu UV-240) with the two laser beams. In order to modulate the population of I_{bl}, RR spectra were measured at different pH's [21]. Although slight increase in sample turbidity was observed as lapse of time, after the double laser illumination experiments the sample was confirmed to preserve its original photoreversibility and showed no degradation or aggregation products in SDS polyacrylamide gel electrophoresis patterns.

3. RESULTS AND DISCUSSION

RR spectra of the red-light illuminated P observed upon excitation at 406.7 nm are depicted in Fig. 1, and the relative populations of P_r, P_{fr} and I_{bl} forms are described in the figure caption. Under red light illumination, where the main component is I_{bl} (44% at pH 7.8, increasing to 80% at pH 9.0) [21], two prominent RR bands are observed at 1630 and 1590 cm^{-1} and overall spectral patterns at pH 7.8 (spectrum A) and at pH 9.0 (spectrum C) are alike. It is noted that the spectral pattern for the D_2O solution at pD 7.8 (spectrum B) also resembles that of the H_2O solution. These RR spectra cannot be ascribed to P_r because of its negligible population for spectrum (C). It is unreasonable to ascribe these RR spectra to I_{bl} due to the following fact.

Fig. 2 shows the RR spectra of far red-light illuminated P probed at 406.7 nm. The relative populations of P_r, P_{fr} and I_{bl} under this illumination condition are given in the figure caption. Under far red illumination at pH 9.0 (spectrum C) the population of I_{bl} reaches as high as 79%, but it does not give the 1590 cm^{-1} band shown in Fig. 1(A). Therefore, we assign the 1590 cm^{-1} band to P_{fr} (The bands at 1546 and 1521 cm^{-1} in Fig. 1A, which are rather obscured in Fig. 2A,C, would also arise from P_{fr}). Selective enhancement of RR bands of P_{fr} is likely to happen, since the absorbance of P_{fr} is larger than twice of those of P_r and P_{bl} [22] at 406.7 nm.

Under the far red illumination (spectra A and C in Fig. 2), on the other hand, contribution from P_{fr} is negligible (0–4%) while the populations of P_r and I_{bl} at pH 7.8 (44 and 56%, respectively) are appreciably

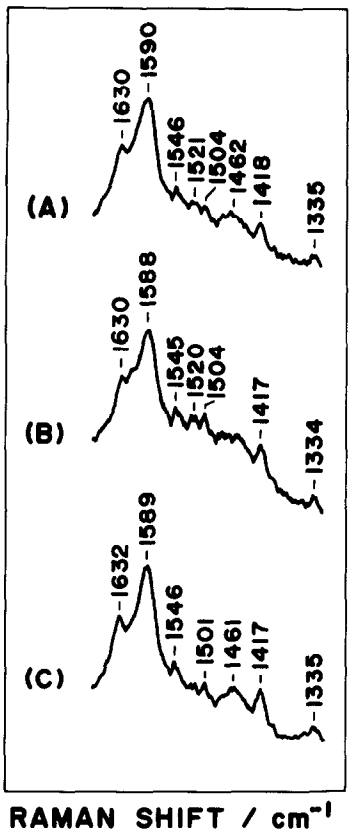

Fig. 1. RR spectra of large pea phytochrome under red light illumination (632.8 nm) at pH 7.8 in H_2O (A), at pD 7.8 in D_2O (B) and pH 9.0 in H_2O (C). Excitation, 406.7 nm. Relative populations of P_r, P_{fr} and I_{bl} under individual illumination conditions are as follows; P_r 20%, P_{fr} 36%, I_{bl} 44% for (A); P_r 18%, P_{fr} 32%, I_{bl} 50% for (B); P_r 0%, P_{fr} 20%, I_{bl} 80% for (C).

changed at pH 9.0 (17 and 79%). Except for an additional band at 1619 cm^{-1}, the general characteristics of spectra (A) and (C) are alike. In the D_2O solution at pD 7.8 (spectrum B) the band at 1619 cm^{-1} was also observed at 1617 cm^{-1} and the overall spectrum was close to spectrum (A). Since the Schiff base C=NH stretching vibration of retinoid proteins around 1640 cm^{-1} is shifted to 1620 cm^{-1} similarly upon N-deuteration and unprotonation [23], it is likely that the 1634 cm^{-1} band in spectrum (A) is overlapped with a band which contains appreciable contribution from the C=NH stretching coupled with the N-H bending vibration, and only the coupled band exhibits a frequency shift to 1619 cm^{-1} at pH 9.0 or upon N-deuteration at pD 7.8.

Since P_r has the absorption maximum around 380 nm, deep blue excitation of RR spectra was examined. The 363.8 nm excited RR spectra obtained under far red illumination are displayed in Fig. 3, where spectra (A) and (B) are for pH 7.8 and spectra (C) and (D) for pH 9.0. The main component under illumination at 740.0 and 363.8 nm is P_r at pH 7.8 (53–66%) and I_{bl} at pH 9.0

**The absorption spectra of the large phytochrome measured under double laser illumination can be simulated by digital addition of the spectra of P_r, P_{fr} and I_{bl}. As the spectrum of P_{fr}, the spectrum of oat intact phytochrome calculated by Rudiger and Eilfeld (in [2], p. 18) was used by shifting the absorption maximum from 730 to 724 nm. The spectrum of I_{bl} was regarded the same as that of P_{bl} obtained for an alkaline solution of the pea phytochrome after red light illumination. The relative populations of each component were calculated by solving three simultaneous linear equations set up for the absorptions at three different wavelengths.

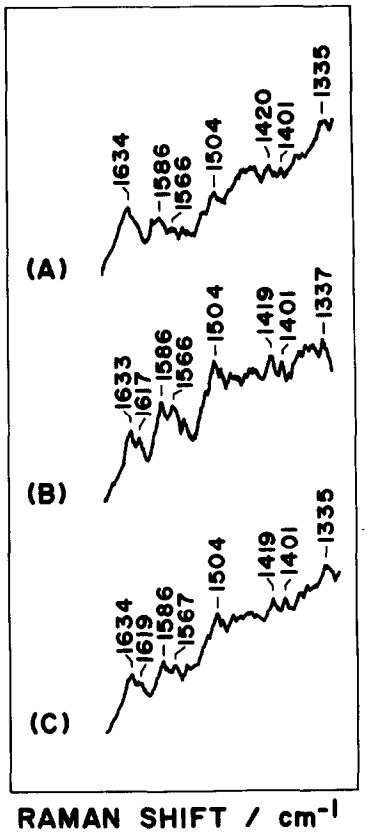

Fig. 2. RR spectra of large pea phytochrome under far red light illumination (740.0 nm) at pH 7.8 in H_2O (A), pD 7.8 in D_2O (B) and at pH 9.0 in H_2O (C). Excitation, 406.7 nm. Relative populations of P_r, P_{fr} and I_{bl} under individual illumination conditions are as follows; P_r 44%, P_{fr} 0%, I_{bl} 56% for (A); P_r 42%, P_{fr} 0%, I_{bl} 58% for (B); P_r 17%, P_{fr} 4%, I_{bl} 79% for (C).

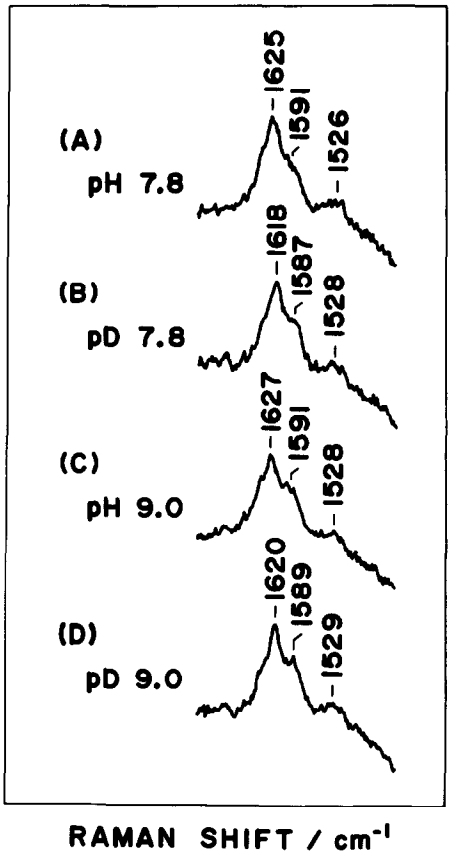

Fig. 3. RR spectra of large pea phytochrome under far red illumination (740.0 nm) at pH 7.8 (A and B) and at pH 9.0 (C and D). Spectra (A) and (C) are for H_2O solutions and spectra (B) and (D) are for D_2O solutions. Excitation, 363.8 nm. Relative populations of P_r, P_{fr} and I_{bl} under individual illumination conditions are as follows; P_r 66%, P_{fr} 5%, I_{bl} 29% for (A); P_r 53%, P_{fr} 4%, I_{bl} 43% for (B); P_r 13%, P_{fr} 4%; I_{bl} 83% for (C); P_r 16%, P_{fr} 5%, I_{bl} 79% for (D).

(79–83%). Nevertheless, the RR spectra at pH 7.8 and 9.0 have no marked difference. This implies that P_r and I_{bl} have similar RR spectra in the skeletal stretching region or that only one of the two species yields RR bands upon this excitation wavelength. However, the most prominent band at 1625 cm^{-1} in H_2O (A) is shifted to 1618 cm^{-1} in D_2O (B) and similar change is seen at pH 9.0. Based on the previous finding by Fodor et al. [13,14] that a RR band of oat P_r at 1626 cm^{-1} exhibited a downshift by 5 cm^{-1} in D_2O, it is more likely to assign the 1625–1627 cm^{-1} bands in spectra (A) and (C) to P_r. The 1625 cm^{-1} band presumably arises from the C = NH [24] or C = C stretching vibration [25,26] of the pyrrole ring C. Note that pyrrole rings A, B and D are always protonated and therefore deuterated in D_2O but the C-N bonds of those rings are not contained in the conjugation chain and therefore, the vibrations localized at the NH groups of rings A, B and D are not resonance enhanced in the present excitation wavelength.

The deuteration shift of Raman band was not recognized for P_{fr} under 406.7 nm excitation and 632.8 nm illumination (see Figs. 1 and 2). This strongly suggests that the protonation state of ring C is different between P_{fr} and P_r (and/or I_{bl}). The lack of any shift in deuterated sample of the far red illuminated preparation (Fig. 2B) with both P_r and I_{bl} populations would be associated mainly with I_{bl}. Then, this suggests that the protonation level of I_{bl} is the same as that of P_{fr}. The difference in protonation level is possibly related to a proton release of the large pea P during the phototransformation process from P_r to the bleached intermediate [11]. This and the reported deuteration effect on phototransformation kinetics [27] may also suggest that absorption spectral change between lumi-R and I_{bl} (= meta-R) detected at low temperature [28] arises from deprotonation of ring C.

In SERR spectra on Ag sol [15–17], the most intense RR band upon 413.1 nm excitation appears at lower frequency for P_{fr} (1591 cm^{-1}) than for P_r (1615 cm^{-1}), in qualitative agreement with the present results. However, since the reported SERR spectra of P_{fr} on the Ag electrode and Ag sol are significantly different with

each other and also from the present spectra, discussion on differences in absolute frequencies between the SERR and present spectra would not be fruitful at this moment. Detailed band assignments based on model compounds are under progress in order to clarify the Z-E isomerization in the P chromophore.

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