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Spectroscopic Determination of Organic Material Part 3 Some Resonance Raman Spectroscopies for Molecular Change of Pea Phytochrome: Several Molecular Differences in Chromophore Protonation between Red Absorption and Far-red Absorption Types

by

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Introduction

This experiment is one of the series of "Spectroscopic Determination of Organic Material."[1,2,3] In this case, resonance Raman scattering is applied to analyse the specific property of pea phytochrome which molecular structure is changed with the surrounding characteristics of irradiated light. Phytochrome has various interesting properties, for example: phototransformation, isomerization, photoreconversion, protonation, that are determined and analysed using some suitable instruments. When phytochrome absorbs the specific light, the absorption spectrum changes reversiblely. So, the phytochrome is considered as a receptor of reversible red·far-red light reaction.

Especially, the specific structural changes of phytochrome irradiated with red light, phytochrome irradiated with far-red light, and one kind of intermediate compound which is bleached form, are studied by several methods from various points. In this paper, the application of resonance Raman scattering obtained some significant analytical results for these compounds.

The phytochrome is one precious photoreceptor chromoprotein in green plants and shows some reversible morphogenetic and developmental responses to red and far-red light irradiation.[4,5] And the phytochrome is constitutes from two typical specific different subunits[6] that each subunit has a 2,3-dihydrobiliverdin chromophore individually.[7,8]

Also, a photoreversible transformation between a red absorbed form (P_R) and a far-red absorbed form (P_{FR}) undergoes through the intermediate form (I_{BL}) which is like a bleached form. The isomerization of the chromophore[9,10,11] and the proton migration[12,13,14] were proposed for phototransformation and the chromophore with an undecapeptide from P_R using nuclear magnetic resonance spectroscopy[8], however, the chromophore structures in P_R and P_{FR} proteins were not established yet.

On the other hand, resonance Raman spectroscopy[8] was used to study the structure of chromophores in chromoprotein[15]) as one good method. Generally, it is very strong technique against water that infrared apectroscopy is very weak in, but it is very weak in fluorescence. So, the application to phytochrome was unsuccessful for a long time, because phytochorome preparation has the very strong fluorescence.

Recently, resonance Raman spectra of oat photochrome in P_R were obtained at 77K with far-red excitation[16,17], also the Surface Enhanced Resonance Raman (SERR) of oat phytochrome was studied with blue exitation[18,19,20]. But, the observation of the resonance Raman spectra of P_R and P_{FR} in a natural state at surrounding temperature is indispensable to obtain the information about structural differences between those two pytochrome forms. Therefore, the resonance Raman spectrum of phytochrome in a natural state was determined by the author using the two color excitation techniques, and the blue excited resonance Raman spectra of the P_R and P_{FR} of pea phytochrome at 288K are reported by the author in this paper showing the different protonated structures between P_R and P_{FR}.

This experiment was carried out at the institutes in

Laboratory of Biology

Okazaki, Japan; Institute for Molecular Science and National Institute for Basic Biology.

Experimental and Results

Pea phytochrome was isolated with the reported method which was precipitated by ammonuium sulfate [$(ND_4)_2SO_4$ in D_2O for the deuterated precipitation], 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} ($\epsilon M = 1.3 \times 10^{5\ cm^{-1}}$).

Resonance Raman spectra were determined for 50 $\mu\ell$ of the phytochrome preparation in the specific micro-spinning cell which was drived at 1600 rpm and kept at 288 ± 3 K by flushing with (flowing in) cold nitrogen gas. And they were detected by a photodiode array (PAR 1420) attached to a Spex 1401 double monochrometer. The Raman scattering was excited at 406.7 nm (Kr^+ laser) or 363.8 nm (Ar^+ laser) under continuous irradiation to another spot of the cell at 740.0 nm (Ti-doped sapphire laser) or at 632.8nm (He/Ne laser) to bias the equilibrium in photosteady state toward either P_R and P_{FR}, respectively. But, in the photosteady state under double laser irradiation, the third component[21, 22], which was previously demonstrated to have the absorption spectrum close to that of the bleached form and was considered to be the same as I_{BL}[23], was appreciably present besides P_R and P_{FR}.

The relative population of P_R, P_{FR}, and I_{BL}, under double laser irradiation was estimated separately from visible absorption spectra that were determined by irradiating the continuously stirred sample solution in the sample compartment of the spectrophotometer (Shimazu UV-240) with the two laser beams.

In order to control the population of I_{BL}, resonance Raman spectra were determined at the different pHs[23]. Although the slight increase in the sample turbility was observed as lapse, after the double laser irradiation experiments the sample was confirmed to preserve its original photoreversibility and showed no degradation or aggregation products in SDS polyacrylamide gel electrophoresis patterns. In this paper, the least important main related charts are showed in the following description.

In Fig. 1, the determined resonance Raman spectra of pea phytochrome under red light (632.8 nm) at pH 7.8 in H_2O (A) at pD 7.8 in D_2O (B), and at pH 9.0 in H_2O (C) are showed. The excitation at that time was 406.7 nm. And the relative populations of P_R, P_{FR}, and I_{BL} under the condition of individual irradiation 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). Under the red irradiation, where the main component is I_{BL} (44% at pH 7.8, increasing to 80% at pH 9.0[23], the two prominent resonance Raman bands are observed at 1630 and 1590 cm^{-1}. And the overall

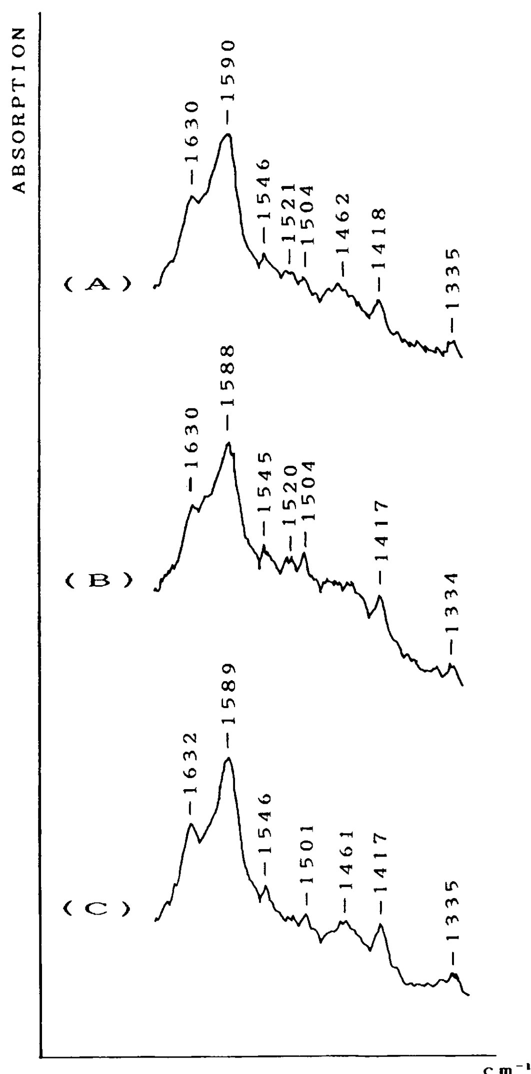

Fig. 1 Raman Shift

spectral patterns at pH 7.8 (spectrum A) and at pH 9.0 (spectrum C) are like.

In Fig. 2, the determined resonance Raman spectra of pea phytochrome under far-red light irradiation (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.

The excitation at that time is 406.7 nm. And the relative populations of P_R, P_{FR}, and I_{BL} under the condition of individual irradiation 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).

In Fig. 3, the determined resonance Raman spectra of pea phytochrome under far-red light irradiation (740.0 nm) at pH 7.8 (A and B) and at pH 9.0 (C and D). These Spectra (A) and (B) are for H_2O solutions and the Spectra (B) and (D) are for D_2O solutions. The excitation at that time is 363.8 nm. And the relative populations of P_R, P_{FR}, and I_{BL} under the condition of individual irradiation 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).

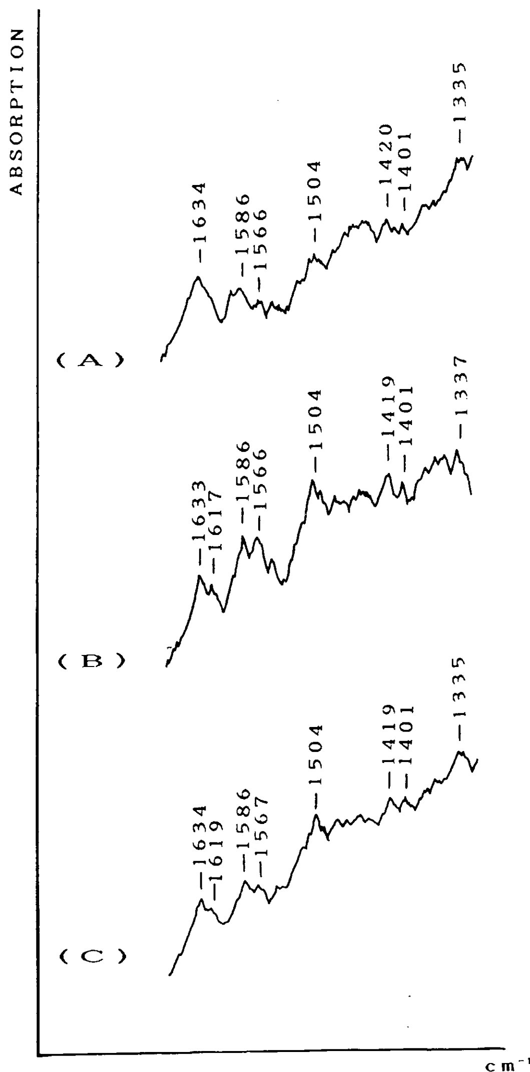

Fig. 2 Raman Shift

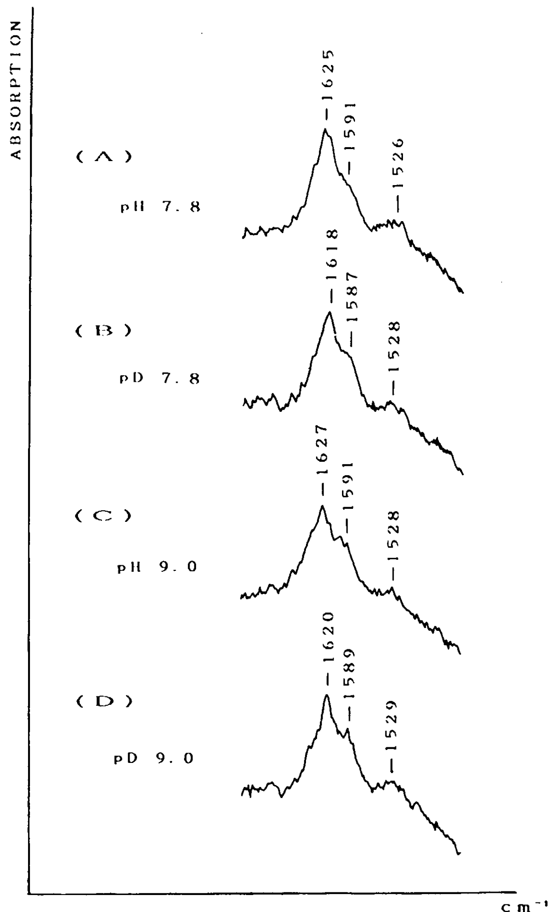

Fig. 3 Raman Shift

Discussion

All plant contains phytochrome except fungi. The phytochrome is very important compound for autotroph.

The phytochrome changes the absorption spectrum respectively, when the specific light is absorbed. And it is chromoprotein which controls various biofunctions at the same time. Butler et al. discovered it as a photoreceptor of red and far-red reversible photoreaction.

When P_R and P_{FR} are irradiated by light, they show a photo-interconversion. And the unstable intermediate which was short life time(span) was discovered with flush decomposition or determination of super low temperature absorption spectroscopy. P_R is stable in solution or cell and is unchangeable for a long time, but P_{FR} converts to P_R after a short time when it allows to stand under dark condition. Its phenomenon is called as P_{FR} dark conversion.

P_{FR} inactivates under the dark condition to the state which losses the light reversibility. Its phenomenon is called as P_{FR} decay (dark inactivation reaction).

At the room temperature, the red light absorbed form or the far-red light absorbed form has the absorption maxima at 280, 382, 664 nm or 280, 400, 724 nm respectively. Also, the intermediate form is observed between P_R and P_{FR}. Phytochrome is detected and analysed quantitatively by the application of the change of specific absorption spectrum or immunochemically. Chromophore contains ring opened tetrapyrrol per subunit that molecular weight is about 1.2×10^5. Phytochrome molecule dissolves in cytoplasm or adsorbs on a membrane of intercellular granule. Phytochrome is produced in biosynthesis. Phytochrome is produced in biosynthesis under dark condition as P_R. As a cell is irradiated with the light and P_{FR} is produced, phytocrome transfers to a membrane system.

After phytochorme is irradiated with light, the red far-red light reversible reaction is caused in organism. Also, there is one case that phytochrome acts as phytoreceptor of irreversible reaction.

The first order molecular structure of protein of phytochrome shows two types from the results of amino acid sequence and base sequence of gene at least. And each one of them is called as I type or II type respectively. The each monomer is consituted from a subunit that molecular weight is about 1.2×10^5. And it in solution exists as dimer. Phytochrome is hydrolysed easily with hydrolytic enzyme to a fragment that molecular weight is 6×10^4, but the spectroscopic property does not change so much. Moreover, if the molecular weight is decreased to 4.5×10^5, the absorption maxima of P_R shifts from 666 nm to 659 nm. P_{FR} changes a bleached form that the absorption is very small, a light reversible absorption spectrum between P_{659} and P_{BL} is observerd. A chromophore contains one ring opened tetrapyrrol per each unit. Phytochrome is biosynthesized as P_R. The expression of gene of I type is caused by the control of transcription through phytochrome itself. And II type exists in almost constant amount in spite of m RNA level, protein level, and light or dark state. Recently, there is one suggestion as follows; I type is related to the gene expression of chlorophyll a/b bond protein and the pigment formations of anthocyan and other from the analytical results of light morphogenesis and mutant (plant). In the case that phytochrome exists in membrane system, if a polarized light which has the vibration plane in the several directions is irradiated and the physiological reaction undergoes, the most effective polarized plane changes about 90° reversiblely between P_R and P_{FR}.

In Fig. 1, it is noted that the spectral pattern for D_2O solution at pD 7.8 (spectrum B) also resembles that of the H_2O solution. These resonance Raman spectra cannot be ascribed to P_R because of its negligible population for spectrum (C). It is unreasonable to ascribe these resonance Raman spectra to I_{BL} due to the following fact.

In Fig. 2, under fa-red irradiation at pH 9.0 (spectrum C) the population of I_{BL} reached as high 79%, but it did not give the 1590 cm^{-1} band shown in Fig. 1(A). Therefore, the author assigns the 1590 cm^{-1} band to P_{FR}. These bands at 1546 cm^{-1} and 1521 cm^{-1} in Fig. 1(A), which are rather abscured in Fig. 2(A) and 2(C), would also arise from P_{FR}. The selective enhancement of resonance Raman bands of P_{FR} is likely to happen, because the absorbance of P_{FR} is larger than twice of those of P_R and I_{BL} at 406.7 nm.

Under the far-red irradiation (spectrum A and C in Fig. 2) the contribution from P_{FR} is negligible (0 ~ 4%) while the population of P_R and I_{BL} at pH 7.8 (44% and 56% respectively) are appreciable changed at pH 9.0 (17% and 79% respectively).

Except for an additional band at 1619 cm^{-1}, the general characteristics of spectra (A) and (C) are like. In the D_2O solution at pD 7.8 (spectrum B), the band at 1619 cm^{-1} was also determined at 1617 cm^{-1} and the overall spectrum was close to spectrum(A). As 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[24], it is likely that the 1634 cm^{-1} band in spectrum(A) is overall with a band which contains appreable 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} pH 9.0 or upon N-deuteration at pD 7.8. As P_R has the absorption maximum around 380 nm, the deep blue excitation of resonance Raman spectra was examined. The 363.8 nm excited resonance Raman spectra obtained under far-red irradiation are displayed in Fig. 3, where spectrum (A) and (B) are for pH 7.8 and spectra (C) and (D) for pH 9.0. The main component under irradiation at 740.0 nm and 363.8 nm is P_R at pH 7.8 (53 ~ 66%) I_{BL} at pH (79 ~ 83%) respectively.

Nevertheless, the resonance Raman spectra at pH 7.8 and 9.0 have no marked difference. This point implies that P_R and I_{BL} have similar resonance Raman spectra in the skeletal stretching region or that only one of the two species show resonance Raman bands upon this excitation wave length.

However, the most prominent band at 1625 cm^{-1} in H_2O (A) is shifted to 1618 cm^{-1} in D_2O (B) and the similar change is seen at pH 9.0. Depending upon the previous finding by Fodor et al.[16, 17], a resonance Raman 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 probably arises from the C = NH[25] or C = C stretching vibration[26, 27] of the pyrrole ring C. So, pyrrole rings A, B, and D, are always protonated and therefore deuterated in D_2O but the N-H bonds of those rings are not contained in conjugation chain. Therefore, the vibrations localized at the NH groups of rings A, B, and D are not resonance enhanced in the present excitation wavelength.

On the other hand, the deuteration shift of Raman band was not recognized for P_{FR} under 406.7 nm excitation and 632.8 nm irradiation. This point suggests that the protonation state of ring is different between P_{FR} and P_R (and/ or I_{BL}). And the lack of any shift in the deuterated sample of the far-red irradiated preparation [Fig. 2(B)] with both P_R and I_{BL} populations would be associated mainly with I_{BL}. Then, it suggests that the protonation level of I_{BL} is the same to that of P_{FR}. Then, the difference in protonation level is possibly related to proton release of the pea phytochrome during the phototransformation process from P_R to the intermediate bleached[14]. This point and the reported deuteration effect on the phototransformation kinetics[28] may suggest that the absorption spectral changes between lumi-R and I_{BL} (= meta R) detected at low temperatures[29] arises from deprotonation of ring C. Also, in Surface Enhanced Resonance Raman (SERR) spectra on Ag sol[18, 19, 20], the most intense resonance Raman band upon 413.1 nm excitation appears at the lower frequency for P_{FR} (1591 cm^{-1}) that for P_R (1615 cm^{-1}), in qualitative agreement with the present results. However, as 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, the discussion on this different in absolute frequencies between SERR and the present spectra would not be fruitful at this time. The detailed band assignments based on the model compounds are under progress in order to clarify the Z-E isomerization in the phytochrome chromophore.

Conclusion

The resonance Raman scattering induced from pea phytochrome was determined at the surrounding temperature using both the special micro-spinning cell that cold nitrogen gas was blowing from liquid nitrogen for the first step and the color (light) excitation technique.

The relative population of the red absorbed form (red absorption type, P_R), the far-red absorbed form (far-red absorption type, P_{FR}), and the intermediate bleached form (intermediate type, I_{BL}) under the condition of laser irradiation was estimated from the analytical results of absorption spectra.

Then, the resonance Raman spectrum of P_R determined with the 363.8 nm excitation under the condition of 740.4 nm pumping. Moreover, the spectrum showed the frequency shift between the H_2O solution

and the D_2O solution.

However, in the case of P_{FR} and I_{BL}, those spectra which were obtained with 406.8 nm pumping did not show the frequency shift. The phenomenon may mean an indication of distinct difference in these photonation levels and presumably it may be able to deduce the distinct difference in the protonation of ring C. Of course, P_R form and P_{FR} form have the different protonational product.

This resonance Raman scattering was one nice method for the structural analysis in the two forms using the special micro-spinning cell and two color excitation technique and the control surrounding temperature.

Summary

Resonance Raman scattering of pea phytochrome was observed at the surrounding temperature using both the special micro-spinning cell and the two color excitation technique.

The two techniques induced serveral successful results. The molecular structural properties of the red absorption type (red absorbed form, P_R), the far-red absorption type far-red absorbed form, P_{FR}), and the intermediate type (intermediate bleached form, I_{BL}) were determined under laser irradiation as the absorption spectra.

The resonance Raman spectrum of P_R obtained by the 363.8 nm excitation under 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 exhibit it indicating the distinct difference in their protonation levels.

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Foot note- As a pagination for one report was limited by budget, the description should be shortened. The rigorus restriction was one person one contribution one year.

有機物の分光学的測定

第3報 豆フイトクロームの分子変化に対するいくつかの共鳴ラマン分光法：赤吸収，と遠赤外吸収型間の発色団プロトン化におけるいくつかの分子相異について

堀 津 圭 佑

（平成6年9月28日受理）

豆フイトクロームの共鳴ラマン分散を特殊小型回転セルと2色励起方法を用いて，周囲温度で観測した．その2種の方法はいくつかの成功した結果を導いた．

赤吸収型(赤吸収形，P_R)，遠赤外吸収型(遠赤外吸収形，P_{FR})と中間型(中間漂白形，I_{BL})の分子構造上の性質が吸収スペクトルとして，レーザー照射のもとで測定された．

740.0nmポンピングのもとで，363.8nmにより得た P_R の共鳴ラマンスペクトルは H_2O と D_2O 溶液間において，振動数シフトを示した．しかし，632.8nmポンピングのもとで406.7nm励起により得た P_{FR} および I_{BL} のそれらは，それらのプロトン化レベルにおいて明白な相異を示す振動数シフトを示さなかった．