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Surface enhanced resonance Raman scattering (SERRS) as a probe of the structural differences between the Pr and Pfr forms of phytochrome

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Surface enhanced resonance Raman scattering (SERRS) spectra have been obtained from the active, far-red light absorbing (Pfr) and biologically inactive (Pr) forms of phytochrome adsorbed on silver colloids. Substantial differences between the SERRS spectra of the two forms in the low and high wavenumber regions are observed using 406.7 nm wavelength excitation. These differences reinforce those seen with 413.1 nm wavelength excitation in the high wavenumber region. Simultaneously, extensive differences are observed in the SERRS obtained from the same form in the low wavenumber region using 406.7 nm, as compared with 413.1 nm wavelength excitation. The relative intensity differences observed for the two forms, and those obtained using two slightly different excitation wavelengths to illuminate the same form, suggest that some type of subtle, protein-controlled structural variation is responsible for the spectroscopic differences. A Z → E isomerization during the Pr → Pfr phototransformation is consistent with the SERRS data, although the overall chromophore conformations are most likely conserved for the native Pr- and Pfr-phytochrome species. Slight out-of-plane ring twisting, accompanying the Pr → Pfr photoisomerization, may be responsible for the large difference in the spectroscopic properties of the native Pr and Pfr chromophores.

Surface enhanced resonance Raman scattering (SERRS) spectroscopy; Resonance Raman spectroscopy; Low temperature spectroscopy; (*Avena sativa*)

1. INTRODUCTION

Phytochrome is a light receptor protein found in a wide variety of plants [1]. Biologically inactive phytochrome (Pr) is synthesized in the dark and upon irradiation with red light is converted into an active, far-red light absorbing form (Pfr). It has been postulated that the reversible Pr ⟶ Pfr phototransformation of phytochrome involves a photoisomerization around the C-15 methine bridge of the tetrapyrrolic chromophore, changing from a Z,Z,Z configuration in Pr to a Z,Z,E configuration in Pfr [2] (fig.1). In this model, the Pr chromophore becomes more exposed in the Pfr form [3]. Oxidation of the chromophore with tetranitromethane takes place preferentially at the C-15 methine bridge, indicating that the protein moiety around the pyrrole D-ring is sufficiently flexible to allow for some movement of this ring [4]. The room temperature UV-Vis spectra of the two forms are shown in fig.2.

Recently, resonance Raman spectra have been obtained for the Pr and Pfr forms of phytochrome using laser excitation at 752 nm [5]. However, the observation of preresonance Raman scattering from Pr and *discrete* resonance Raman scattering (RRS) from Pfr, using 752 nm wavelength excitation in both cases, renders interpretation of differences in band intensities and frequencies problematic.

Excitation of the electronic transition responsible for the Soret band of either form (Pr λ_{max} = 379 nm; Pfr λ_{max} = 406 nm) also results in an intense fluorescence background. In order to quench this fluorescence, we have employed surface enhanced resonance Raman scattering (SERRS) spectroscopy for the study of both Pr and Pfr forms of the protein [6]. Our results indicate that use of citrate-reduced silver colloid suspensions (sols) preserves the native structure of the two forms at 77 K [6] and allows use of low sample concentrations (10^{-8} M).

Differences between the SERRS spectrum of Pr and Pfr using 406.7 nm excitation are reported here for the first time. SERRS spectra obtained using the 406.7 nm laser line are also different from those obtained using the 413.1 nm line in a number of respects, and these differences will be discussed.

2. MATERIALS AND METHODS

Native 124-kDa oat phytochrome [7] was isolated from etiolated Garry oat seedlings (*Avena sativa*), as in [8]. The phytochrome solutions (A_{666} = 1.4, SAR = 0.92 [9]) were converted to either the Pr or Pfr forms by irradiation with 660 nm or 730 nm actinic light, respectively. All protein samples were dialyzed and dissolved in 20 mM potassium phosphate buffer (KPB), pH 7.8 with 1 mM EDTA present.

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A SPEX Triplemate monochromator/spectrograph using backscattering optics was used to record the SERRS spectra [6]. Spectra were recorded at 77 K (in liquid nitrogen) using a laser excitation wavelength of 406.7 nm. Twenty-five spectra were averaged. At 77 K, no significant photodegradation was observed over the time period of the measurement (approximately 2 min). Furthermore, the liquid nitrogen prevents photochemical conversion and photocycling [10].

Silver sols were prepared as described by Kelly et al. [11]. 75 μl of 1% ascorbic acid was added to 2.9 ml of sol prior to protein addition, in order to 'activate' the sol. The pH of the sol was then between pH 5.5 and 6.0. Before addition of the phytochrome samples, the slightly acidified sols were chilled to 0°C on ice.

3-μl aliquots of the phytochrome solution were added to the chilled sol solutions. The final concentration of the phytochrome in the sol was approximately 1×10^{-6} M. Immediate neutralization (pH 7.0 - 7.5) was accomplished by adding 200 μl of 200 mM KPB to the sol/phytochrome solution. Approximately 1 ml of glycerol was added to the sol/phytochrome solution in order to stabilize the sol during freezing. This enabled the sol to form a uniform glass at 77 K. All manipulations were performed in the dark or under dim green light.

3. RESULTS AND DISCUSSION

Phytochrome possesses a tetrapyrrole chromophore as the photosensitive prosthetic group. SERRS has been applied to the study of phytochrome and its model compounds [6]. It was found that a citrate-reduced silver sol is the most efficacious enhancing medium with which to study these photolabile proteins.

The SERRS spectra, excited with the 406.7 nm laser line, clearly differentiate between the Pr and Pfr forms of phytochrome in the high (fig.3) and medium-to-low (fig.4) wavenumber regions. The most salient differences between the two forms in the high wavenumber region are observed for the 1624, 1596, 1458, 1414, 1252 and 1163 cm^{-1} bands. Significantly, one of the greatest differences in the SERRS spectra between the Pfr and Pr forms is the relative intensities of the 1620 vs 1596 cm^{-1} bands. Unfortunately, a complete normal coordinate analysis has not been performed on the tetrapyrrole chromophore of phytochrome. However, Schneider et al. [12] have kindly provided us with a preprint of a normal coordinate analysis performed on a simpler three membered ring model compound. Also tentative assignments have been made for RRS from biliverdin dimethyl ester [13] and A-dihydrobilindione [14]. The latter is related to the extended chromophore of the protein, C-phycocyanin which has been invoked as a model for phytochrome studies because of its structural and spectral similarities to phytochrome [15]. Both the normal coordinate analyses by Schneider et al. and those in [13,14] appear to indicate that the higher C_α-C_m [16] stretching wavenumber belongs to the C_{15}-C_{16} bond at approximately 1620 cm^{-1}. The wavenumber of the C_α-C_m bond between C_{10} and C_{11} is calculated to appear at approximately 1590 cm^{-1}.

Based upon the above tentative assignments, the difference between the Pr and Pfr spectra can be rationalized in terms of the model proposed for the Pr to Pfr transformation, which involves a Z,Z,Z to Z,Z,E

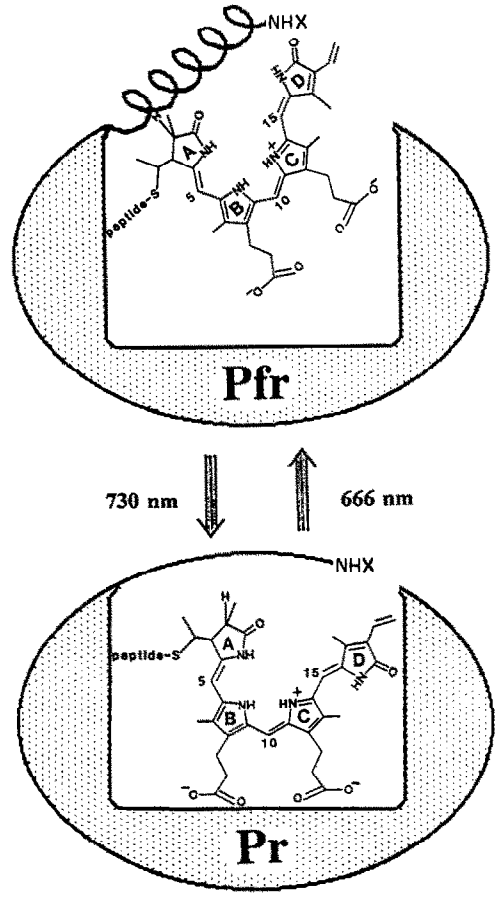

Fig.1. Representation of the phototransformation of the Pr chromophore to the Pfr chromophore. The overall semi-extended conformation of the tetrapyrrolic chromophore is retained during the transformation, with conservation of the exocyclic dihedral angle at ring D by a chromophore-apoprotein interaction. The protonation of ring C is based upon recent work by Fodor et al. [5a]. The model also incorporates the postulated reorientation and more exposed nature of the Pfr chromophore, as well as the increase in alpha-helical folding of the N-terminus [19]. X = $COCH_3$, as determined by Grimm et al. [20]. The postulated slight out-of-plane twisting of ring D for the Pfr chromophore (see text) is not shown.

photoisomerisation about the C_{15}-C_{16} bond. Calculated results [17] and crystal structure analysis of model compounds [18] have shown that a discernible difference exists in the exocyclic dihedral angles of helical Z-isomers as compared to E-isomers. The Z,Z,Z-forms have been predicted (and shown) to adopt a fully cyclic conformation in free chromophores, exhibiting little difference in their exocyclic dihedral angles. Thus, their overall conformation (and π-conjugation network) is essentially helical. On the other hand, the exocyclic dihedral angles of E-forms are twisted approximately 20 degrees greater than in their Z-forms. This twist may perturb the π-conjugation of the system and decouple the pyrrole and lactam ring π-systems, which should result in a decrease in the RRS from the shared pyrrole/lactam C_α-C_m stretching mode in Pfr. Localization of the electronic transition on the pyrrole rings could explain the large

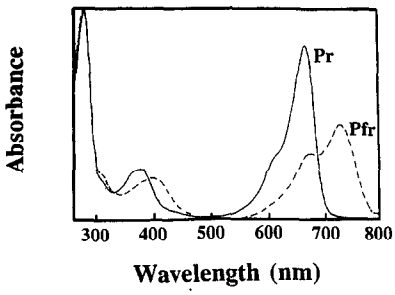

Fig.2. UV-Vis absorption spectra of the Pr and Pfr forms of phytochrome in 20 mM KPB, pH 7.8, 298 K. The specific absorbance ratio of this sample (absorbance at 666 nm to (protein) absorbance at 280 nm) is 0.88.

relative intensity increase of the band due to the pyrrole/pyrrole C_α-C_m stretching mode (1596 cm^{-1}).

The large relative intensity changes observed for the 1252 cm^{-1} band (assigned to the C_m-H in-plane bending mode, where C_m is the methine bridge between rings C and D [13]) of Pfr and Pr can also be explained by this mechanism. As the relative intensity of the 1624 cm^{-1} band decreases in Pfr, that of the 1252 cm^{-1} decreases as well. This is consistent with coupling of the pyrrole/lactam C_α-C_m stretching mode and the C_m-H in-plane bending mode. The low wavenumber region of the phytochrome spectrum is even more difficult to assign than the high wavenumber region. However, differences between the Pr and Pfr forms can be clearly seen (fig.4). In particular, the intensities of the 824 and 605 cm^{-1} bands relative to the other bands of Pr are markedly increased compared to those of Pfr. Bands in the medium-to-low wavenumber region are usually attributed to C-C bending and C-H in-plane and out-of-plane bending modes. The fact that relative intensity differences, rather than wavenumber shifts, are observed in this region, suggests that some subtle protein-controlled structural difference exists between the chromophores of the two forms. The influence of the protein may not affect the normal mode character of the chromophore vibrations, but could affect the relative intensity of Raman scattering from a given mode. This is consistent with the general observation that RRS intensities reflect excited state properties, whereas the peak positions reflect ground state structure. Thus, the absence of *new and intense* bands in the spectrum of one form as compared to the other supports the hypothesis that protein-induced structural differences are primarily responsible for the spectroscopic differences.

Spectral differences between the two forms of phytochrome in the high and low wavenumber region are consistent with those observed with 413.1 nm excitation [6]. However, in contrast, the Pr and Pfr spectra are quite different in the low wavenumber region when 406.7 nm excitation is used. Again, this suggests that protein-induced differences, rather than large scale structural variations, exist between the two forms of the phytochrome chromophores. The wavenumbers and relative band intensities of modes in the middle-to-low wavenumber region are expected to be more susceptible to the influence of the protein, than in the high wavenumber region.

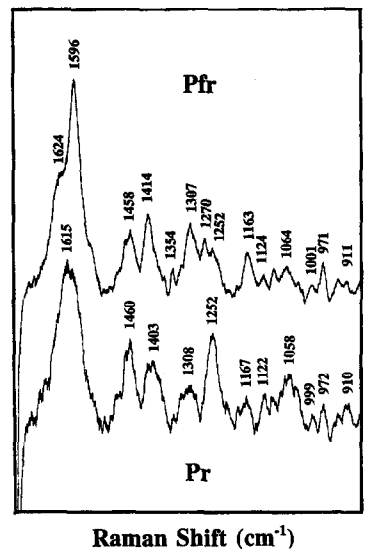

Fig.3. SERRS spectra in the high wavenumber region of the Pr and Pfr forms of phytochrome adsorbed on a silver sol. pH 7.5, 77 K. λ_{ex} = 406.7 nm. Laser power = 100 mW.

The emergence of new bands and substantial relative intensity differences in the SERRS spectra of a single form of phytochrome with a change of only 7 nm in excitation wavelength, suggests the existence of at least two, near-degenerate electronic states. The extreme sensitivity of the SERRS spectra to a small (7 nm) change in excitation wavelength, indicates that RRS selectivity predominates rather than surface propensity effects.

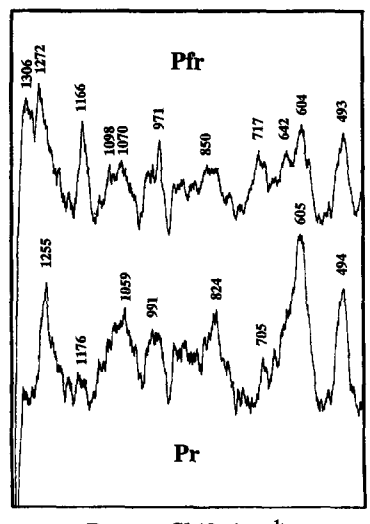

Fig.4. SERRS spectra in the medium-to-low frequency region of the Pr and Pfr forms of phytochrome adsorbed on a silver sol. pH 7.5, 77 K. λ_{ex} = 406.7 nm. Laser power = 100 mW.

4. CONCLUSION

SERRS spectra obtained using silver colloids are substantially different for the Pfr and Pr forms of phytochrome [6]. The spectra obtained for the same phytochrome species in the low frequency region are substantially different for 406.7 nm excitation as compared with 413.1 nm excitation. The observation of such large differences with such a small (7 nm) change in excitation wavelength reflects the complex nature of the electronic structures of Pfr and Pr. However, the observation of relative intensity, rather than frequency, differences between SERRS of the two forms indicates that a subtle, protein-controlled structural variation is responsible for the spectroscopic differences. It is postulated that the overall chromophore conformation is conserved following the primary photoisomerisation step in the Pr $\longrightarrow$ Pfr transformation. A slight out-of-plane ring twisting may occur in the case of Pfr. This could be responsible for the finely tuned light-sensing mechanism of phytochrome [6]. Future work will be carried out to obtain the SERRS excitation profile for both the Soret and Q-bands of the two protein forms. This information should provide new insights into the nature of the Pr $\longrightarrow$ Pfr transformation (from excited state properties) and the origin of the differences between the native chromophores of the Pr and Pfr forms.

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REFERENCES

[1] For reviews see: (a) Lagarias, J.C. (1985) Photochem. Photobiol. 42, 811–820; (b) Rüdiger, W. (1987) Photobiochem. Photobiophys. Suppl., 217–227. (c) Song, P.-S. (1988) J. Photochem. Photobiol. B: Biology 2, 43–57.

[2] (a) Thümmler, F. and Rüdiger, W. (1983) Tetrahedron 39, 1943–1951; (b) Rüdiger, W., Eilfeld, P. and Thümmler, F. (1985) in: Optical Properties and Structure of Tetrapyrroles (Blauer, G. and Sund, H. eds) pp. 349–369, Walter de Gruyter, Berlin.

[3] (a) Hahn, T.R., Song, P.-S., Quail, P.H. and Vierstra, R.D. (1984) Plant Physiol. 74, 755–758; (b) Hahn, T.R. and Song, P.-S. (1981) Biochemistry 20, 2602–2609.

[4] Farrens, D., Song, P.-S., Rüdiger, W. and Eilfeld, P. (1989) J. Plant. Physiol., in press.

[5] (a) Fodor, S.P.A., Lagarias, J.C. and Mathies, R.A. (1988) Photochem. Photobiol. 48, 129–136; (b) Fodor, S.P.A., Lagarias, J.C. and Mathies, R.A. (1989) Photochem. Photobiol. 49s, 63s.

[6] Farrens, D.L., Holt, R.E., Rospendowski, B.N., Song, P.-S. and Cotton, T.M. (1989) J. Am. Chem. Soc., submitted.

[7] Vierstra, R.D. and Quail, P.H. (1983) Biochemistry 22, 2498–2505.

[8] Chai, Y.-G., Singh, B.R., Song, P.-S., Lee, J. and Robinson, G.W. (1987) Anal. Biochem. 163, 322–330.

[9] Phytochrome SAR values are defined as the ratio of Pr chromophore absorbance at 660 nm to the (protein) absorbance at 280 nm. Phytochrome with a SAR value of 1 is considered to be essentially pure.

[10] Eilfeld, P. and Rüdiger, W. (1985) Z. Naturforsch. 40c, 109–114.

[11] Kelly, K., Rospendowski, B.N., Smith, W.E. and Wolf, C.R. (1987) FEBS Lett. 222, 120–124.

[12] Scheider, S. et al., to be published.

[13] Margulies, L. and Toporowicz, M. (1984) J. Am. Chem. Soc. 106, 7331–7336.

[14] Margulies, L. and Toporowicz, M. (1988) J. Mol. Struct. 175, 61–66.

[15] (a) Glazer, A.N. (1984) Biochim. Biophys. Acta 768, 29–51; (b) Schmidt, G., Siebzehnrübl, S., Fischer, R. and Scheer, H. (1988) in: Photosynthetic Light-Harvesting Systems (Scheer, H. and Schneider, S. eds) pp. 77–87.

[16] We use here the nomenclature adopted from heme proteins to identify the carbon atoms of the pyrrole ring and the methine bridging carbon atom. α is used to designate the pyrrole carbon atom attached to the N atom. m is used to designate the methine bridging carbon atom. We also use α to designate the carbon atom, opposite to the carbonyl carbon atom, which is attached to the nitrogen atom of the lactam five-member rings.

[17] (a) Falk, H. and Höllbacher, G. (1978) Monatsh. Chem. 109, 1429–1449; (b) Falk, H. and Müller, N. (1983) Tetrahedron 39, 1875–1885.

[18] Wagner, U., Kratky, C. and Falk, H. (1986) Monatsh. Chem. 117, 1413–1422.

[19] Song, P.-S. (1985) in: Optical Properties and Structures of Tetrapyrroles (Blauer, G. and Sund, H. eds) pp. 331–348, Walter de Gruyter, Berlin.

[20] Grimm, R., Kellermann, J., Schäfter, W. and Rüdiger, W. (1988) FEBS Lett. 234, 497–499.