Fourier Transform Resonance Raman Spectroscopy of Phytochrome†

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ABSTRACT: The P_r and P_{fr} forms of phytochrome in H_2O and D_2O have been studied by Fourier transform resonance Raman spectroscopy with near-infrared excitation (1064 nm). It is demonstrated that this technique is a powerful method for analyzing the chromophore structures of photosensitive pigments. The high spectral quality allows discussion of vibrational assignments based on an empirical approach using previously published data obtained from model compounds. The reduction in intensity of a high-frequency band assigned to the ring-C/D methine bridge vibration is an indication for the non-coplanarity of the ring D in P_{fr}. The high intensity of a C–H out-of-plane vibration also supports this hypothesis. In P_r, a broad peak at $\sim$1100 cm^{-1} is assigned to an out-of-plane vibration of a strongly hydrogen-bonded pyrrole C=NH^+ group. It is missing in P_{fr}, suggesting deprotonation of the corresponding ring during the transformation from P_r to P_{fr}.

Phytochrome is a photoreceptor in plants which transduces solar energy for the purpose of modification of growth and development (Smith, 1975). The chromophoric group of phytochrome is a linear tetrapyrrole (phytochromobilin) covalently bound to the apoprotein [Figure 1; for recent reviews see Rüdiger and Thümmler (1991) and Schaffner et al. (1990, 1991)]. Upon light absorption, phytochrome is converted from a red-absorbing form, P_r, to a far-red absorbing and physiologically active form, P_{fr}. The underlying molecular structural processes are far from being fully understood.

This study addresses the structural elucidation of the chromophore in each of the two states, P_r and P_{fr}. Resonance Raman (RR)[1] spectroscopy has proven to be an amenable technique for the study of biological materials in general (Carey, 1982), and the results of several RR investigations of phytochrome have already been reported (Fodor et al., 1988, 1990; Tokutomi et al., 1990). This technique offers valuable structural information, since the vibrational pattern of the chromophore sensitively reflects its unique configuration in the protein.

Serious experimental difficulties, however, arise from the photosensitivity of this protein since the RR excitation light triggers the photocycle (Tokutomi et al., 1990). Hence, a flowing sample device has to be employed to avoid accumulation of intermediates in the exciting laser beam (Fodor et al., 1990). In addition, on probing the RR spectra upon excitation into the lowest electronic transition, one encounters interference from fluorescence which is significantly more intense than the RR bands (Fodor et al., 1990).

In order to overcome these difficulties, surface-enhanced RR (SERRS; Farrens et al., 1989; Rospendowski et al., 1989)

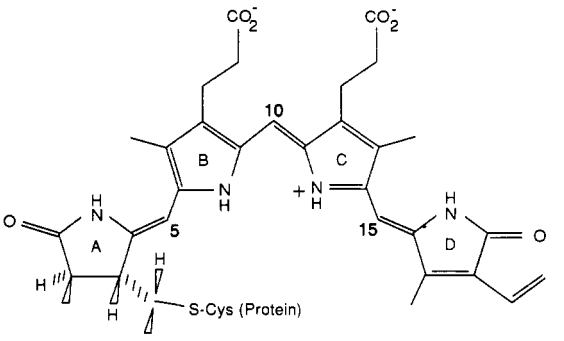

FIGURE 1: Structural formula of the protonated *Z*-anti,*Z*-syn,*Z*-anti isomer of the tetrapyrrole chromophore of phytochrome.

and coherent anti-Stokes Raman spectroscopy (CARS; Hermann et al., 1990) have been employed. However, the poor resemblance of the CARS spectrum with the conventional RR spectra raises some concern about possible denaturation of the protein in these experiments, due to high laser powers. A similar concern is related with the SERRS experiments of phytochrome adsorbed on a silver electrode (Farrens et al., 1989). In the case of silver colloids as the SER-active surface, the spectrum of P_r is quite similar to the RR spectrum although the signal-to-noise (S/N) ratio is rather poor. We have now used the recently developed technique of Fourier transform Raman which offers the advantage of high-frequency accuracy, high S/N ratio, and the simultaneous measurement of a wide spectral range (Hirschfeld & Chase, 1986; Schrader & Simon, 1988; Schrader et al., 1990). This technique has successfully been employed to study chromoproteins such as bacteriorhodopsin, bacterial reaction centers, and phycocyanins, yielding exclusively the vibrational spectra of the prosthetic groups (Fourier transform resonance Raman, FT-RR) (Gerwert et al., 1990; Mattioli et al., 1990, 1991; Savatzki et al., 1990; Johnson & Rubinowitz, 1990). In the present work, it is shown that the NIR excitation line at 1064 nm avoids any interference from chromophore fluorescence and lowers the possibility of a photoinduced conversion of phytochrome. The spectra presented here are of higher quality than those reported previously and provide a sound basis for the discussion of structural aspects of the $P_r \rightarrow P_{fr}$ phototransformation.

† Dedicated to Professor Albert Weller on the occasion of his 70th birthday.

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[1] Abbreviations: CARS, coherent anti-Stokes Raman spectroscopy; FT, Fourier transform; i.p., in-plane; NIR, near infrared; o.o.p., out-of-plane; P_r, red absorbing form of phytochrome; P_{fr}, far-red absorbing form of phytochrome; P_{denat}, denatured phytochrome; RR, resonance Raman; SERRS, surface enhanced resonance Raman spectroscopy; S/N, signal-to-noise.

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MATERIALS AND METHODS

Phytochrome Purification. Phytochrome from *Avena sativa* L. cv. Pirol was purified using a modified method of Grimm and Rüdiger (1986). In order to improve the yield of the first steps, the seedlings were not irradiated and phytochrome was eluted from the hydroxyapatite column according to Viestra and Quail (1983) and Brock et al. (1987). The collected fractions were irradiated with red light (667-nm interference filter, Schott) prior to further purification according to Grimm and Rüdiger (1986). A spectral absorption ratio, A_{667}/A_{280}, of 0.95–1.15 in the P_r form was routinely achieved.

Sample Preparation. For all experiments a standard buffer (pH = 7.8) with 20 mM tris(hydroxymethyl)aminomethane chloride, 1 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid was used. All chemicals were of the highest purity grade available and solutions were prepared with Milli-Q-purified water (Millipore Corp.). For deuterated solutions, the pD value was corrected for the deuterium isotope effect (Glasoe & Long, 1960). About 3 mg of phytochrome in the P_r or the P_{fr} form was precipitated with 1.8 M $(NH_4)_2SO_4$ by centrifugation at 48000*g* for 5 min (4 °C). The pellet was resuspended in 50–100 μL of buffer, and the resulting slurry was filled into the sapphire sphere used for the FT-RR experiments (Schrader et al., 1990). A total volume of less than 10 μL phytochrome, corresponding to 0.2–0.5 mg of protein, was required for each experiment.

FT-RR Experiments. FT Raman spectra were recorded with a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module. The spectral resolution was ~4 cm^{-1}. The unfocused 1064-nm line of a diode-pumped cw Nd-YAG laser was used for excitation. Typical laser powers at the sample were 200 mW. The Raman scattered light was collected with a 180° scattering geometry. All spectra were recorded at room temperature. They were based on 1000 interferograms (~1 h) for P_r and 250 interferograms (~15 min) for P_{fr}. In order to improve the S/N ratio, several spectra were added. The reproducibility of the results was checked by comparing the individual spectra. In some cases, slow sedimentation of the protein particles in the sapphire cuvette produced fluctuations of the stray light which led to a sine-function modulation of the spectra. Such spectra were not regarded for the data analysis. In preliminary experiments, it was found that glycerol addition (~20%) did not improve the sample homogeneity, nor did it result in higher stability of the P_{fr} form. In all the spectra displayed in this work, the background was removed by polynomial subtraction.

RESULTS

The FT-RR spectrum of P_r in H_2O is presented in Figure 2A. Sharp lines below 800 cm^{-1}, which originate from the sapphire sphere, are marked by asterisks. In addition, the strong band at 980 cm^{-1} results from the totally symmetric stretching of SO_4^{2-} (marked by S). Unfortunately, these bands cannot be used as intensity standards since, due to the preparation procedure of the samples for the FT-RR measurements, the ratio of sulfate to phytochrome as well as the total amount of protein in the laser beam differed in the various experiments. Besides these lines, all other bands can be attributed to vibrational modes of phytochrome. No bands originating from the buffer components could be detected.

One of the strongest Raman bands of the protein matrix, the amide I, is expected at ~1650 cm^{-1}. However, in this spectral region only a broad hump underneath the 1639- and

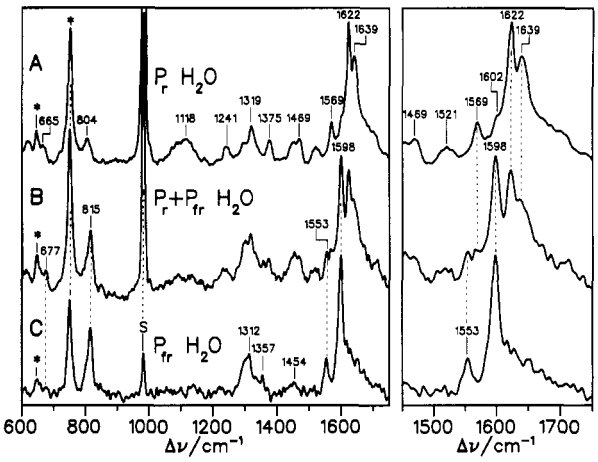

FIGURE 2: FT-RR spectra of the P_r and P_{fr} forms of phytochrome in H_2O from 600 to 1750 cm^{-1}. (A) P_r, sum of individual spectra with a total accumulation time of 6 h. (B) $P_r + P_{fr}$, sum of individual spectra from a P_{fr} sample, each measured for 15 min; total accumulation time was 2 h. (C) P_{fr}, difference spectrum (B) minus (A); details of the subtraction procedure are given in the text. Enlargements show FT-RR spectra of the P_r and P_{fr} forms of phytochrome in H_2O from 1450 to 1750 cm^{-1}.

1622-cm^{-1} bands is observed, which is assigned to the H_2O bending vibration since it vanishes in D_2O. Thus, we can safely conclude that even at 1064-nm excitation the (pre)-resonance enhancement of the bands of the tetrapyrrole chromophore is still sufficiently high to discriminate the Raman bands of the chromophore from those of the protein. This observation is in line with previous results on other chromoproteins studied by this technique (Mattioli et al., 1990, 1991; Savatzki et al., 1990; Johnson & Rubinowitz, 1990).

Figure 2 also displays an enlargement of the region of the C=C stretching vibrations which exhibits the most characteristic signature of the various states of phytochrome (Fodor et al., 1988, 1990). The distinct bands at 1639, 1622, and 1569 cm^{-1} are at similar positions as in the RR spectrum of P_r recently published by Fodor et al. (1988). Furthermore, no time-dependent spectral changes were observed during an accumulation period of 4 h, indicating that the exciting laser line at 1064 nm did not induce conversion of P_r to other intermediate states of phytochrome. This conclusion was confirmed by comparing the visible absorption spectra of the sample before and after the FT-RR experiment.

The behavior of P_{fr} was quite different. Figure 2B shows the spectrum of a sample which was converted to P_{fr} prior to the FT-RR experiment. The spectrum, which was accumulated for 15 min, reveals significant differences as compared to the P_r spectrum (Figure 2A). Upon continuing data accumulation, the P_r bands increased in intensity at the expense of the 1598- and 1553-cm^{-1} bands (see Figure 2B). Hence, irradiation with the 1064-nm laser line causes conversion back to the P_r state. It may be that the probability of the photoinduced conversion is greater for P_{fr} (λ_{max} ~ 730 nm) than for P_r (λ_{max} ~ 660 nm) and/or the thermal conversion from P_{fr} to P_r is accelerated by absorption of NIR light.

Consequently, an improvement of the S/N ratio of the "P_{fr}" spectra without increasing the P_r content could only be achieved by exchanging the sample after a short period of accumulation (i.e., 15 min) and adding the individual spectra. A spectrum of the pure P_{fr} state was thus obtained by subtracting the P_r fraction (i.e., the spectrum in Figure 2B minus that in 2A) using the characteristic P_r bands as markers. Such a difference spectrum is displayed in Figure 2C.

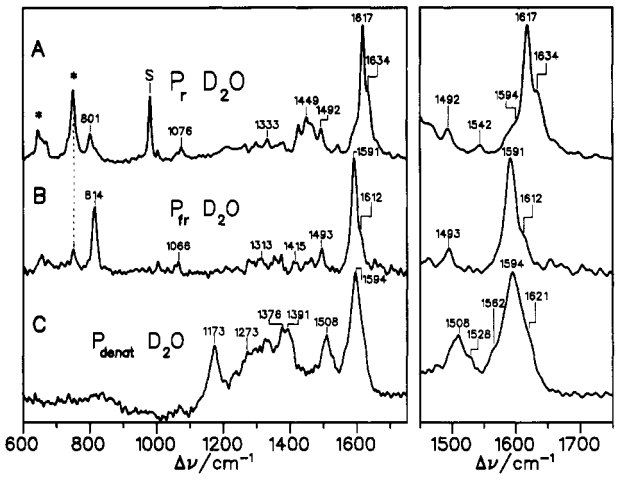

FIGURE 3: FT-RR spectra of phytochrome in D_2O from 600 to 1750 cm^{-1}. (A) P_r, sum of individual spectra with a total accumulation time of 6 h. (B) P_{fr}, obtained as described in Figure 2. (C) A denatured form of phytochrome, P_{denat}, measured from a sample with low D_2O content; further details are given in the text. Enlargements show FT-RR spectra in the region from 1450 to 1750 cm^{-1}.

In the same way as described above, the FT-RR spectra of P_r and P_{fr} were obtained in D_2O (Figure 3A,B). A large number of isotopic shifts reflect the contributions of the N–H bending and C–NH stretching vibrations of the four pyrrole groups to various modes.

The FT-RR spectra of P_r and P_{fr} were fully reproducible using different sample preparations. However, the water content of the samples was a critical parameter. Low H_2O (D_2O) contents led to striking changes for P_r and P_{fr}, yielding essentially identical spectra for both. Evidently, low H_2O (D_2O) contents result in a denatured form of phytochrome (P_{denat}). It may be that the heat absorbed from the NIR excitation line is not efficiently dissipated under these conditions. Such a spectrum, measured from a D_2O-containing sample of P_{fr}, is shown in Figure 3C. For this experiment, the protein was deposited in a glass capillary so that the spectrum is free of sapphire emission lines.

The most pronounced spectral differences compared to the native states are noted in the region between 1100 and 1400 cm^{-1} which exhibits numerous relatively strong and broad bands without counterparts in the spectra of P_r and P_{fr}. In addition, there is only a single poorly resolved peak at 1594 cm^{-1} which has replaced the relatively sharp bands of P_r and P_{fr} between 1590 and 1640 cm^{-1}. It is also noteworthy that there are no bands below 1100 cm^{-1} in the spectrum of P_{denat}.

DISCUSSION

The FT-RR spectra of P_r and P_{fr} are in agreement with the conventional RR spectra reported by Fodor et al. (1988, 1990). These authors have used the 752-nm excitation line which, in comparison to our 1064-nm excitation, is in closer resonance with the lowest electronic transitions of the P_r and P_{fr} states. Thus, the small differences in the relative intensities compared to the FT-RR spectra can readily be attributed to different Raman excitation profiles of the various modes. Furthermore, some of the bands, particularly in the high-frequency region, yield slightly different peak frequencies in the RR spectra. Most likely, these values are less accurate than those obtained from the FT-RR spectra due to the high intrinsic frequency accuracy of the interferometer. The present FT-RR spectra are of a comparable (P_{fr}) or better (P_r) spectral quality than those obtained with the conventional RR technique. Particularly for P_r, the vibrational pattern is well resolved, permitting even the identification of the weaker bands. Thus, the FT-RR spectra provide a solid basis for a detailed discussion of the possible vibrational assignments for both states. However, one must take into account that a molecule as large as a tetrapyrrole exhibits highly complex normal-mode compositions (Curry et al., 1982, 1984; Smith et al., 1987a,b). Thus, it is not possible to assign individual bands to pure group vibrations. Instead, they can only be interpreted in terms of dominant contributions from specific vibrations. Furthermore, replacement of the exchangeable protons by deuterons drastically alters the normal-mode composition so that the interpretation of isotopic shifts must be treated with caution (Smith et al., 1987a,b). Since a detailed and well substantiated normal-mode analysis is not yet available, the assignment of the FT-RR bands of phytochrome which will be discussed in the following sections is largely based on the comparison with previous studies of a variety of model compounds.

Vibrational Assignments: C=C Stretching Vibrations. The strongest bands of P_r in H_2O are at 1639 and 1622 cm^{-1} [Figures 2A (*) and 4A]. They reveal only small isotopic shifts upon H/D exchange. A doublet of this kind appears to be a general property of linear tetrapyrroles since it is found also in a variety of related compounds (Siebert et al., 1990; Margulies & Stockburger, 1979; Holt et al., 1989; Yang et al., 1991). For example, despite the replacement of the C(10) methine bridge (Figure 1) by methylene, bilirubin IXα still displays such a pair of bands (Hsieh et al., 1987; Yang et al., 1991). This implies that these bands do not originate from modes delocalized over the entire tetrapyrrole chain. Instead, they must be localized in the A–B and C–D dipyrrole moieties of the chromophore. This conclusion was recently confirmed by Yang et al. (1991), who, on the basis of a RR study of various bilirubin derivatives, attributed the low- and high-frequency components of this band pair to the dipyrrole A–B and C–D units, respectively. The authors tentatively assigned these bands to lactam ring modes including C=O and C=C stretching vibrations. However, the frequencies are largely independent of the endo-pyrrole C=C double bond since they are observed at quite similar positions in the FT-IR spectra of both octaethylbilindione and 2,3-dihydrooctaethylbilindione (Siebert et al., 1990). Consequently, the bands must be assigned to normal modes including significant contributions from the C=C stretching vibrations of the methine bridges adjacent to the rings A and D. We therefore assign the bands of P_r at 1639 and 1622 cm^{-1} to the corresponding modes of rings D and A, respectively.

In biliverdin dimethyl ester, the vibrational structure in this region has been found to be pH dependent. In neutral solution, in addition to the doublet at 1611 and 1586 cm^{-1}, a band at 1691 cm^{-1} was observed, which has been assigned to the C=O stretching vibrations of rings A and D (Margulies & Stockburger, 1979; Margulies & Toporowicz, 1984). This band disappears in acidic solution and the doublet shifts up to 1630 and 1618 cm^{-1}. Fodor et al. (1988) argued that, under these conditions, the pyrrolenic nitrogen of ring C is protonated. In the protonated form, the RR spectrum of biliverdin dimethyl ester is very similar to the FT-RR spectrum of P_r, as revealed by the agreement of the C=C stretching frequencies and the lack of a C=O stretching band. This implies that the P_r chromophore is protonated at ring C (Fodor et al., 1988; Braslavsky, 1990; Schaffner et al., 1990, 1991).

In P_{fr} we note a frequency shift to 1598 cm^{-1} and a pronounced intensity redistribution of the C=C stretching vibrations as compared to P_r. In H_2O, the high-frequency

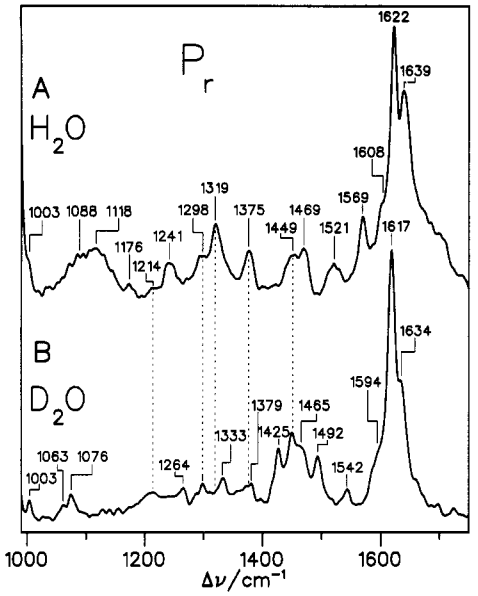

FIGURE 4: FT-RR spectra of the P_r form of phytochrome from 990 to 1750 cm^{-1}: (A) H_2O; (B) D_2O.

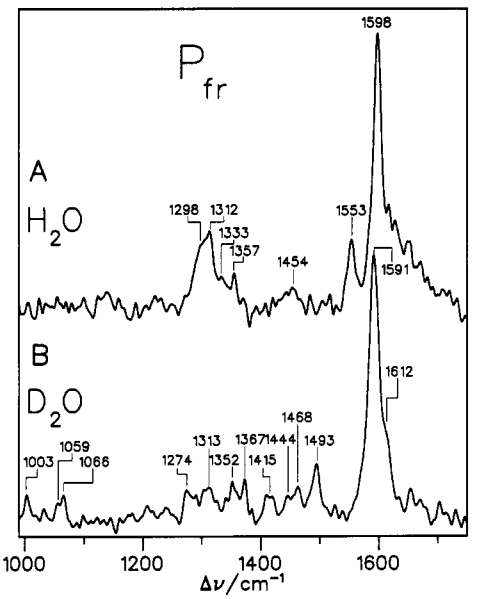

FIGURE 5: FT-RR spectra of the P_{fr} form of phytochrome from 990 to 1750 cm^{-1}: (A) H_2O; (B) D_2O.

component can hardly be detected, and in D_2O it is present only as a small shoulder at ~1612 cm^{-1}, while the low-frequency component prevails either at 1598 cm^{-1} or at 1591 cm^{-1} in D_2O (Figures 2C, 3B, and 5A,B).

The bands at 1569 and 1553 cm^{-1} of P_r and P_{fr} disappear in D_2O. This points to a substantial contribution of the C–N stretching and N–H bending vibrations, as suggested by a classical normal mode analysis of simple model compounds (Hermann et al., 1990).

Vibrational Assignments: N–H Vibrations. The N–H bending vibrations are expected between 1300 and 1400 cm^{-1} (Dollish et al., 1974; McDermott, 1986; Hermann et al., 1990; Fodor et al., 1990; Margulies & Toporowicz, 1984). However, it is well established that these vibrations can be strongly coupled to C–C or C–N stretching and C–H bending vibrations (Alshuth & Stockburger, 1981; Smith et al., 1987a,b), so that modes including contributions of the N–H bending may appear even between 1100 and 1650 cm^{-1}. In fact, in this part of the P_r spectrum there are four bands, at 1375, 1319, 1241, and 1176 cm^{-1}, which presumably include significant contributions from the N–H i.p. bending. Upon H/D exchange, they are replaced by a set of bands at 1379, 1333, 1264, 1076, and 1063 cm^{-1}. No band between 1000 and 900 cm^{-1} was observed in D_2O indicating that the N–D bending is also strongly coupled to other vibrations of the tetrapyrrole chain. We should like to point out that the apparent frequency upshift of some of the bands in D_2O (e.g., 1379 vs 1375 cm^{-1} and 1333 vs 1319 cm^{-1}) originates from the different normal compositions in both molecules so that in D_2O some modes can accidentally appear at higher frequencies than in H_2O (Smith et al., 1987a,b). According to calculations by Fodor et al. (1990), those modes with the largest N–H(N–D) i.p. bending character are attributed to the bands at 1375 and 1319 cm^{-1} (1076 and 1063 cm^{-1}). The corresponding modes of P_{fr} in H_2O (D_2O) are found at 1357, 1312, and 1298 (1367, 1352, 1274, 1066, and 1059) cm^{-1}.

A striking feature in the FT-RR spectrum of P_r (H_2O) is the broad and relatively intense hump at ~1100 cm^{-1} which is missing in the spectra of P_r (D_2O) and of P_{fr}. A similar observation was made by Fodor et al. (1988) in the RR spectrum of P_r at low temperature. It should be noted that this band does not result from the buffer components, nor can it be attributed to a Raman band of the protein matrix, so that it must originate from a normal mode of the chromophore including substantial contribution from an N–H vibration. The unusually broad structure of the band can be interpreted in terms of strong hydrogen-bonding interactions which, in addition, produce frequency upshifts of both N–H out-of-plane (o.o.p.) and N–H in-plane (i.p.) vibrations (Pimentel & McClellan, 1960; Hadzi & Bratos, 1975). Therefore, the frequency of this peak appears to be too low to be assigned to an N–H i.p. bending vibration. On the other hand, for strongly hydrogen-bonded protonated C=NH^+ systems, such as the pyridinium cation (Glazunov & Odinokov, 1982) or the protonated Schiff base (Baron, unpublished results), the relatively pure N–H o.o.p. vibration is observed at ~1100 cm^{-1}, while such vibrations for the corresponding neutral species (pyrrole) are not found at frequencies higher than 650 cm^{-1} (Campos-Vallette, 1988). Therefore, we tentatively assign the broad 1100-cm^{-1} band to the N–H o.o.p. vibration of a positively charged system involved in a strong hydrogen bond, i.e., ring C (or B), as opposed to A or D (see Figure 1). The lack of any corresponding band in D_2O can readily be understood in terms of weaker deuterium bond strengths which, along with the intrinsic H/D shift (~ –300 cm^{-1}), may reduce the corresponding frequency to below 600 cm^{-1}. The observation of RR activity in an o.o.p. vibration could be explained by a strongly hydrogen-bonded system in which the acceptor lies out of the pyrrole ring plane. This vibration is expected to be almost pure, and the width of the corresponding i.p. vibration may be masked through its coupling to the many C–N vibrations.

A small band at 1003 cm^{-1} is detectable in both P_r and P_{fr}, although partly obscured by the adjacent sulfate band. This band is insensitive to H/D exchange. It may be that it results from the phenylalanine mode ν_1 which, although not resonance enhanced, may contribute to the FT-RR spectrum due to the large number (44) of these amino acid residues in phytochrome (Hershey et al., 1985).

Vibrational Assignments: C–H Out-of-Plane Vibrations. The most striking features below 900 cm^{-1} are the bands at ~800 cm^{-1} which in P_{fr} possess an extraordinarily high intensity, comparable to that of the C=C stretching modes (Figures 2C and 3B). In agreement with Fodor et al. (1990),

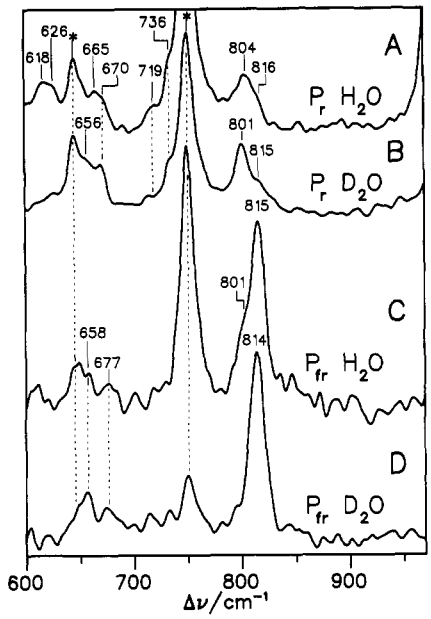

FIGURE 6: FT-RR spectra of the P_r and P_{fr} forms of phytochrome from 600 to 970 cm^{-1}: (A) P_r in H_2O; (B) P_r in D_2O; (C) P_{fr} in H_2O; (D) P_{fr} in D_2O.

we assign these bands to C–H o.o.p. vibrations of the methine bridges since only subtle frequency shifts are noted in D_2O. A careful inspection of these bands in Figure 6 reveals that, in both states, there are two strongly overlapping bands, suggesting that at least two of three possible C–H o.o.p. vibrations are resonance enhanced. At present, we cannot definitely assign these bands to individual C-H groups. However, Fodor et al. (1990) calculated the C(5)–H o.o.p. mode to be at ~890 cm^{-1} and the C(10)–H and C(15)–H o.o.p. modes to be closely spaced at ~850 cm^{-1}. Thus, it is tempting to assign the latter two modes to the bands at 804 (801) and 816 (815) cm^{-1} in P_r (P_{fr}) in H_2O.

Implications for the Chromophore Structure in P_r and P_{fr}. The most striking spectral difference between the two states of phytochrome is the pronounced intensity lowering of the high-frequency C=C stretching mode in P_{fr} (1639 cm^{-1} in P_r) attributed to the stretching of the methine bridge to ring D. This reduction of the resonance enhancement can be taken as an indication for the non-coplanarity of ring D with respect to the remaining pyrrole rings. Such a conformational distortion would require a rotation around the C(14)–C(15) and/or C(15)–C(16) bonds. It is consistent with the strong C–H o.o.p. RR activity since distortions around single or double bonds of conjugated polyene systems can significantly increase the RR intensity of such modes (Fodor et al., 1990; Eyring et al., 1980). Hence, we tentatively assign the 815-cm^{-1} band of P_{fr} (H_2O) to the C(15)–H o.o.p. mode, in agreement with the suggestion by Fodor et al. (1990). The FT-RR spectra provide further indications for a configuraftional change at the methine bridge between the rings C and D. In a SERR study of isomeric biliverdin dimethyl esters, Holt et al. (1989) found a drastic intensity decrease of a band at 1245 cm^{-1} upon conversion from the *Z,Z,Z* to the *Z,Z,E* configuration, suggesting that the intensity of this band is sensitive to the geometry of the linkage between rings C and D. A similar conclusion was drawn from a CARS study on C-phycocyanin (Schneider et al., 1987). In fact, the FT-RR spectrum of P_r displays a distinct band at 1241 cm^{-1} (Figure 4) which cannot be detected in the spectrum of P_{fr} (Figure 5).

In P_r, the strong intensity of the C(15)=C(16) stretching (1639 cm^{-1}) rules out a substantial o.o.p. distortion of ring D. On the other hand, a full coplanarity of the tetrapyrrole chain cannot be reconciled with the RR activity of the 804-cm^{-1} band in the C–H o.o.p. region. It should be noted that a band close to this position may also be present in the FT-RR spectrum of P_{fr} as indicated by the small shoulder at 801 cm^{-1} in Figure 6C. We therefore conclude that the 804-cm^{-1} band in P_r does not originate from the C(15)–H o.o.p.. Instead, it is tentatively assigned to the C(10)–H o.o.p. bending. This would imply a slight torsion around the C(10) methine bridge which would not affect the resonance enhancement of the C=C stretching bands of the C(5) and C(15) methine bridges. It may be that the steric constraints of the immediate protein environment may induce such a distortion at the linkage between rings B and C.

Independent of the exact assignment of the 804(801)-cm^{-1} band, the present FT-RR data imply that the photoinduced conversion from P_r to P_{fr} primarily involves a rotation around the C(15) methine bridge. Previous NMR studies of chromopeptides isolated from P_r and P_{fr} have suggested that the chromophore is in a *Z,Z,Z* and *Z,Z,E* configuration, respectively (Lagarias & Rapoport, 1980; Rüdiger, 1987; Rüdiger et al., 1983). In addition, Fodor et al. (1990) have suggested that the conversion of P_r to P_{fr} includes the simultaneous rotation around the C(15)=C(16) double and the C(14)—C(15) single bonds. Both conclusions are compatible with the present FT-RR spectra, since a full coplanarity of ring D with the remaining pyrrole rings can only be achieved for a *Z,Z,Z* but not for a *Z,Z,E* configuration (K. Smit, unpublished results). Since also in P_r the resonance enhancement of the C–H o.o.p. modes suggests some deviation from the planar chromophoric structure, it is evident that molecular details of the chromophore geometry depend on the specific interactions with the protein matrix. Thus, it may also be that in P_{fr} rotation around the C(15) methine bridge is not sufficiently complete to form a relaxed *Z,Z,E* configuration.

The pronounced influence of the protein environment on the chromophore structure can be assessed by the FT-RR spectrum of P_{denat} (Figure 3C). The lack of any C–H o.o.p. bending vibrations (600–1100 cm^{-1}) suggests a relaxed chromophore structure, free of steric constraints imposed by the protein matrix. In this context it is interesting to note that this spectrum is quite similar to the RR spectrum of α-phycocyanin at pH 1.5 which has been ascribed to a nonnative chromophore conformation (Debreczeny et al., 1991).

In addition to the configurational changes, the transition from P_r to P_{fr} may also involve a weakening of hydrogen-bonding interactions of the ring C (or B) pyrrole nitrogen as indicated by the disappearance of the broad peak at ~1100 cm^{-1} (N–H o.o.p.). A possible explanation may be that the rotation of ring D ruptures a strong hydrogen bond of the N–H group with a nearby amino acid residue. The loss of this hydrogen bond as well as the distorted chromophore geometry may provide the driving force for the thermal back-reaction of P_{fr} to P_r.

With respect to the possible deprotonation of P_{fr}, we refer to a recent RR study by Mizutani et al. (1991). These authors have used near-UV excitation lines which apparently enhance different normal modes than does excitation in the first allowed electronic transition. For the bands above 1550 cm^{-1}, frequency shifts upon H/D exchange were only observed for P_r but not for P_{fr}, indicating a deprotonation of ring C in the latter.

CONCLUSIONS

FT-RR has proven to be a useful technique for the study of P_r and P_{fr} forms of phytochrome at room temperature,

requiring small sample volumes and quantities. The spectral quality is at least as good as that obtained from conventional RR experiments. The use of NIR excitation reduces fluorescence to negligible levels, and it minimizes photoconversion.

An empirical approach to the vibrational assignment has been developed, on the basis of comparison of the present FT-RR spectra to RR and IR spectra of appropriate model compounds. Structural implications of such an analysis support a previously suggested aspect of the phototransformation, viz., the C(15)–C(16) double bond isomerization, additionally implicating some degree of distortion about this or the C(14)–C(15) linkage in the P_{fr} form. There are also indications for a slight distortion about the central C(10) bridge in both forms. The disappearance of a postulated N–H o.o.p. vibration in P_{fr} is interpreted in terms of a deprotonation of the pyrrole ring C during the photoinduced transformation.

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