Fourier-Transform Resonance Raman Spectroscopy of Intermediates of the Phytochrome Photocycle

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ABSTRACT: The parent states of the 124-kDa phytochrome (phy A from *Avena sativa*) and intermediates of its photocycle were studied by low-temperature Fourier-transform resonance Raman spectroscopy. Spectra of the primary photoproducts I_{700} and lumi-F and of the thermal intermediate meta-F have been obtained for the first time. The spectra of the stable photochromic forms of photochrome, P_r and P_{fr}, presented in this work are significantly better in signal-to-noise ratio and resolution than previously published spectra, demonstrating the distinct advantages of our experimental approach. The high spectral quality allows for the identification of subtle details of the vibrational band pattern so that the resonance Raman spectra, which have been measured from samples in H_2O and D_2O, constitute a solid basis for the structural analysis of the various forms of phytochrome. Notwithstanding the current uncertainty in the vibrational assignment of many resonance Raman bands, the spectral changes of the tetrapyrrole chromophore can plausibly be interpreted in terms of conformational changes at two different methine bridges, i.e., torsions around two single bonds and the *E/Z* isomerization of a double bond. Within the framework of this interpretation, which is based on a vibrational analysis of biliverdin dimethyl ester (Smit, K., Matysik, J., Hildebrandt, P., & Mark, F. (1993) *J. Phys. Chem. 97,* 11887−11900), a consistent model is proposed to describe the molecular events in the chromophore during the photocycle. The involvement of a proton transfer in the primary photoprocess of P_r can safely be ruled out. However, previous conclusions concerning the chromophore protonation in the individual states appear premature at the present state of the vibrational assignment. In particular, the attribution of a broad band at 1100 cm^{-1} to the N−H out-of-plane bending of the protonated pyrrolenin nitrogen (Hildebrandt, P., Hoffmann, A., Lindemann, P., Heibel, G., Braslavsky, S. E., Schaffner, K., & Schrader, B. (1992) *Biochemistry 32,* 7957−7962) has now been found to be erroneous.

Phytochrome is a photoreceptor of plants which mediates a number of photomorphogenic processes (Smith, 1975; Rüdiger & Thümmler, 1991; Braslavsky, 1990; Schaffner et al., 1990, 1991). The 124-kDa protein exists in two interconvertible forms, P_r and P_{fr}.[1] However, the physiological action of phytochrome is assumed to be restricted to P_{fr}, which apparently exhibits a specific domain recognized by target proteins (Cordonnier, 1989). The interconversion of P_r and P_{fr} is initiated by light absorption of the phytochromobilin-derived chromophore, a linear tetrapyrrole covalently bound to the apoprotein (Figure 1A). Most likely, the primary photochemical event is a double bond isomerization at the methine bridge between the pyrrole rings C and D (Rüdiger et al., 1983; Rüdiger, 1987; Rüdiger & Thümmler, 1991; Schaffner et al., 1990, 1991). The photoreactions are followed by thermal relaxation processes including conformational changes of the protein which are presumably communicated to the interactive domain of the protein surface. Figure 2 shows a simplified scheme of the phytochrome photocycle which includes the distinct intermediates as detected by time-resolved and low-temperature absorption spectroscopy (Braslavsky et al., 1980; Cordonnier et al., 1981; Eilfeld & Rüdiger, 1985; Ruzsicska et al., 1985;

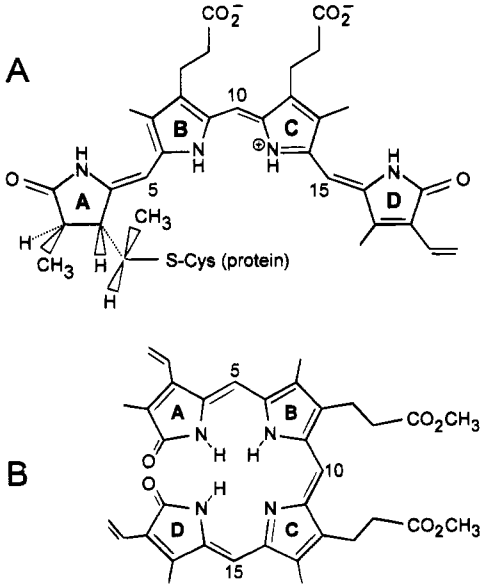

FIGURE 1: Chemical constitutions of the tetrapyrrole chromophore of phytochrome (A) and biliverdin dimethyl ester (BVE; B). The protein-bound phytochrome chromophore is probably in an extended conformation (Brandlmeier et al., 1981; Schaffner et al., 1990, 1991). The *ZZZ,sss* conformation of BVE is encountered in the crystal lattice (Sheldrick, 1976; Lehner et al., 1978).

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[1] Abbreviations: FT, Fourier transform; RR, resonance Raman; P_r and P_{fr}, red and far-red light absorbing forms of phytochrome, respectively; I_{700}, I_{bl}, lumi-F, and meta-F, intermediates of the phytochrome photocycle (see Figure 2); BVE, biliverdin dimethyl ester IXα; Phe, phenylalanine; *s, synperiplanar*; *a, anticlinal*; i.p., in-plane; o.o.p., out-of-plane; PED, potential energy distribution.

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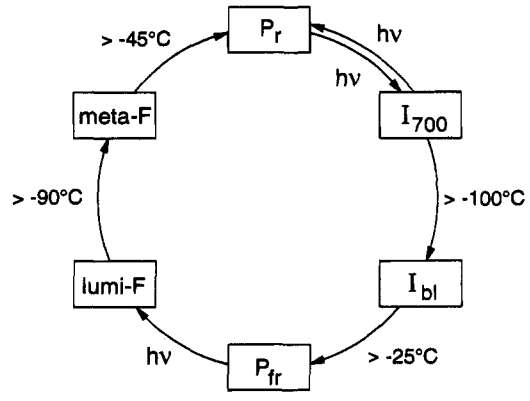

FIGURE 2: Simplified scheme of the photoinduced reaction cycle of phytochrome adapted from Eilfeld and Rüdiger (1985). For greater details on the $P_r \rightarrow P_{fr}$ process, see Scurlock et al. (1993b).

Inoue et al., 1990; Scurlock et al., 1993a,b). Conformational details of the chromophore and its changes during the photocycle are not yet known. Their elucidation should provide a key for understanding the molecular mechanism of light-induced activation and deactivation of phytochrome.

In this respect, resonance Raman (RR) spectroscopic techniques are the most promising tools since excitation in resonance with an electronic transition yields a selective enhancement of the Raman bands of the chromophore (Fodor et al., 1988, 1990; Rospendowski et al., 1989; Farrens et al., 1989; Tokutomi et al., 1990; Hermann et al., 1990; Mizutani et al., 1991, 1994; Hildebrandt et al., 1992). In this way, vibrational spectra can be obtained which include detailed structural data of the active chromophoric site. However, there are serious experimental difficulties associated with the photophysical and photochemical properties of the chromophore. Since the exciting laser light initiates the photocycle, long-lived intermediates may accumulate so that the RR spectra recorded represent a mixture of the various components of the photocycle (Figure 2). Furthermore, excitation close to the lowest and strongest electronic transition induces a relatively strong fluorescence which partially obscures the RR signals.

In our previous study (Hildebrandt et al., 1992), we have employed near-infrared Fourier-transform (FT) Raman spectroscopy (Hirschfeld & Chase, 1986; Schrader et al., 1990) which can effectively overcome these problems. The 1064-nm excitation line is sufficiently red-shifted with respect to the lowest electronic transition of the chromophore to circumvent fluorescence and to lower photochemical activity drastically. On the other hand, the (pre)resonance enhancement of the vibrational bands of the tetrapyrrole is still high enough to dominate the measured spectra (i.e., RR spectra) without interference of the non-resonance-enhanced Raman bands of the apoprotein. In this way, detailed spectra of the chromophore in the P_r and P_{fr} states could be obtained.

In order to understand the molecular mechanism of the photochemical interconversion of the parent states, the RR spectra of the intermediates are of utmost importance. So far, RR spectroscopic data have only been obtained for the long-lived intermediate I_{bl}. Mizutani et al. (1991, 1994) obtained RR spectra, excited in the near-UV, of quasi-photostationary mixtures of P_r, P_{fr}, and I_{bl}. Based on dual-beam experiments, several RR bands in the spectra were attributed to I_{bl}. The shorter-lived intermediate I_{700} is not accumulated under these conditions. Attempts to obtain the RR spectrum of I_{700} at −120 °C with near-UV excitation were not successful (Mizutani et al., 1994).

In the present work, we have employed near-infrared RT-RR spectroscopy of phytochrome under low-temperature conditions set to trap selectively individual components of the P_r−P_{fr} photocycle. The high quality of the spectra obtained by this technique proved a prerequisite for determining the RR spectra of the intermediates. In this way, it was possible to characterize several intermediates, and thus to provide a sound basis for the analysis of the structural changes of the tetrapyrrole chromophore in the course of phototransformations.

MATERIALS AND METHODS

Phytochrome Purification. Phytochrome from etiolated seedlings of *Avena sativa* L. cv. Pirol (phy A) was purified according to the method by Grimm and Rüdiger (1986) with modifications as described by Hildebrandt et al. (1992). A spectral absorption ratio of $A_{667}/A_{280} = 0.95-1.15$ for the P_r form was routinely achieved.

Sample Preparation. Phytochrome (ca. 7.5 μM) was dissolved in 10 mM phosphate buffer (pH 7.8) including 5 mM dithiothreitol and 2 mM ethylenediaminetetraacetic acid and converted to the P_{fr} form by irradiating with 667-nm light. For low-temperature measurements 1 mL of this solution was concentrated through a membrane filter (Centricon 100, 100 kDa exclusion molecular weight) by centrifugation at 1200*g* for 80 min (4 °C) to yield a final volume of 25 μL. The concentrated viscous sample was inserted into a conical hole of an aluminium block, which in turn was placed into the cryostat in a way that the sample was positioned in the optical axis of the spectrometer (i.e., "horizontally"). In order to prevent the viscous solution to flow out of the metal block, it was rapidly cooled to below 0 °C. Using this geometry, the signal-to-noise ratio was greatly improved as compared to measurements in quartz tubes. Such an arrangement was not applicable for measurements of liquid solutions at room temperature which, therefore, were carried out with precipitates of phytochrome obtained by centrifuging solutions containing 1.6 M $(NH_4)_2SO_4$ at 48000*g* for 5 min (4 °C) (Hildebrandt et al., 1992). Thus, such samples always include a relatively high content of $(NH_4)_2SO_4$ which contributes to the spectra (see below).

Deuterated samples were prepared in the same way by using buffer solutions in D_2O. For these preparations the centrifugation was repeated with samples redissolved in D_2O to ensure a complete H/D exchange at the chromophore binding site as confirmed by the RR spectra.

Sample Handling. Prior to insertion into the cryostat, the samples were irradiated with 667-nm (730-nm) light to convert the protein into the P_{fr} (P_r) state (Colombano et al., 1990). All subsequent manipulations were carried out in the dark. Enrichment of individual intermediates of the phytochrome photocycle was achieved by irradiating the sample with light at 667 nm (P_r) or 730 nm (P_{fr}) for ca. 15 min at the appropriate temperature. The desired intermediate was then formed while subsequent thermal reactions were inhibited. In each case, a FT-RR spectrum of the parent (nonphotolyzed) state (P_r or P_{fr}) was measured at the same temperature. Thus, residual contributions of P_r or P_{fr} could readily be subtracted from the spectra of the photolyzed samples without causing artifacts due to temperature-

dependent variations of spectral parameters. Once the first intermediate (I_{700} or lumi-F) had been formed and measured, a further increase of the temperature allowed for the decay to the subsequent intermediate. In this way, it was possible to run through the entire photocycle (Figure 2) and measure the FT-RR spectra of the samples containing the components of the photocycle. In each case, three spectra were recorded at a given temperature. Comparison of the initial spectrum of the parent state (e.g., P_r) and the spectrum measured after completing the photocycle was a sensitive test for the integrity of the samples during the experiments. No differences were noted within a cycle. The FT-RR measurements of the intermediates of the entire photocycle were repeated four (H_2O) and three (D_2O) times with freshly prepared samples, confirming the reproducibility of the results. The spectra of each intermediate from the individual samples obtained in each series of measurements revealed no differences, so that they were added to improve the signal-to-noise ratio.

FT-RR Measurements. FT-RR spectra were measured using a Bio-Rad FT-Raman spectrometer equipped with a Nd-YAG laser (Spectra Physics, FC-106V) which has a band width of less than 1 cm^{-1} and a maximum output of 2.5 W at 1064 nm. The Raman scattered radiation was collected at 180° backscattering geometry by positioning an aspherical lens directly in front of the entrance window of the cryostat. The aperture of the cryostat was 60°, allowing for an efficient light collection. The liquid-nitrogen evaporation cryostat (CryoVac), which was constructed according to the requirements of the FT-Raman measurements, allowed for experiments in the temperature range between ca. −190 and −40 °C. Temperature measurement and control were achieved via a PT 100 thermocouple placed close to the metal block housing the sample.

The FT Raman spectra were measured with 300 mW (1064 nm) focused onto the sample. The spectral resolution was 4 cm^{-1}, and the accumulation time for each spectrum was 40 min. A triangular function was used for apodization of the interferograms.

Data Handling. The measured FT-RR spectra exhibited a poorly structured background scattering which partially originated from the Al surface. This background was removed by polynomial subtraction. Spectra of the intermediates were obtained upon subtraction of the residual contributions of the parent species as described in detail in the subsequent section. All the spectra were analyzed by a band fitting program using Lorentzian band shapes. It should be noted that the individual bands are artificially broadened due to the apodization, whereas the band shape was evidently not affected. Gaussian−Lorentzian line shapes gave worse fits.

RESULTS

Figure 3 compares the RR spectra of P_r and P_{fr} observed at ambient and low temperatures.[2] The room temperature experiments were carried out with samples precipitated by $(NH_4)_2SO_4$. These samples include considerable amounts of $(NH_4)_2SO_4$ which gives rise to a few Raman bands in the spectra. The most prominent one at 980 cm^{-1} is due to the totally symmetric stretching mode of sulfate. Further bands are only visible as broad and poorly structured humps, partly hidden underneath the RR bands of phytochrome. However, upon lowering the temperature below −50 °C, these features drastically change, leading to a number of extraordinarily strong and sharp bands in the region between 1600 and 1700 cm^{-1} (spectra not shown). These changes, which are ascribed to a phase transition of solid $(NH_4)_2SO_4$ (Schutte & Heyns, 1970), make it impossible to use such phytochrome preparations for low-temperature RR studies.[3] Also the broad hump centered at ca 1100 cm^{-1} originates from $(NH_4)_2SO_4$. In our previous work (Hildebrandt et al., 1992) we have incorrectly assigned this feature to the out-of-plane (o.o.p.) N−H bending of the protonated pyrrolenin ring C (Figure 1A). Thus, for low-temperature experiments the $(NH_4)_2SO_4$-free preparation was used.

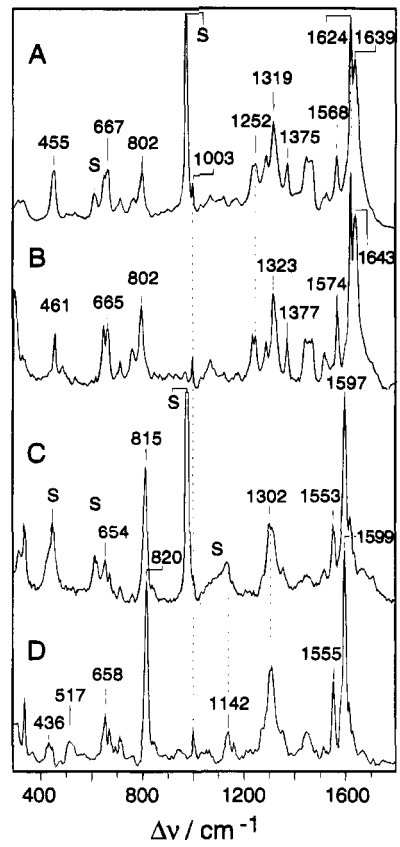

FIGURE 3: RR spectra of P_r and P_{fr} at ambient and low temperatures in H_2O solution (A) P_r, $T = 25$ °C; (B) P_r, $T = -130$ °C; (C) P_{fr}, $T = 25$ °C; (D) P_{fr}, $T = -140$ °C. The measurements at ambient temperature were carried out with samples precipitated by $(NH_4)_2SO_4$. Bands due to $(NH_4)_2SO_4$ are marked by the letter "S". Low-temperature spectra were obtained from samples which were free of $(NH_4)_2SO_4$.

The room-temperature spectra of P_r and P_{fr} are closely related to those published previously (Fodor et al., 1988, 1990; Hildebrandt et al., 1992). However, they exhibit a significantly better spectral quality, allowing for the identification even of weak spectral features. This is also true for the RR spectra measured at low temperature. In both P_r and P_{fr} the overall vibrational band pattern at room and low temperature is very similar, implying that the gross structure

[2] In the following, the FT near-infrared RR spectra will be denoted as RR spectra for the sake of simplicity.

[3] Attempts to subtract the $(NH_4)_2SO_4$ contribution from the low-temperature spectra of these preparations failed due to subtle differences in frequencies and relative intensities as compared to the pure $(NH_4)_2SO_4$.

of the chromophore and the surrounding protein is largely unchanged in the temperature range under investigation. In general, frequency differences are, if at all, not larger than 2 cm^{-1}. In some cases these shifts are the result of the expected band narrowing at low temperature which leads to a significantly better resolution of those band multiplets which at room temperature are only visible as broad envelopes with poorly defined peak maxima. The temperature-dependent changes of the bandwidths also affect the peak heights of some of the bands. A few bands, however, reveal larger frequency shifts which reflect temperature-dependent conformational changes. These are, in particular, in P_r the band at 1568 (1574) cm^{-1} and in P_{fr} the band at 816 (820) cm^{-1} as well as the band pair at 1597 and 1618 cm^{-1}. The latter two bands reveal a peculiar behavior inasmuch as the temperature dependence is different in H_2O and D_2O. In D_2O the band maxima shift up significantly from 1591 and ca. 1610 cm^{-1} at room temperature to 1597 and 1616 cm^{-1} at low temperature (below −75 °C: spectra not shown) while in H_2O the frequencies of these bands are nearly temperature-independent (1597 and 1618 cm^{-1} vs 1599 and 1618 cm^{-1}). Such an effect is not observed in the spectra of P_r, in which the temperature-dependent frequency shifts of the band pair at 1624 and 1639 cm^{-1} are comparable (2−4 cm^{-1}) in H_2O and D_2O. Furthermore, the low-temperature spectrum of P_{fr} exhibits a relatively broad band centered at 517 cm^{-1} which is not present in the room-temperature spectrum and which disappears in D_2O. Further effects are noted for the bands at 436 and 461 cm^{-1} of P_{fr} and P_r, respectively (Figure 3B,D). However, in the room-temperature spectra they partially overlap with the 449-cm^{-1} band of $(NH_4)_2SO_4$. This is particularly relevant in the RR spectrum of P_{fr} which includes higher contributions of $(NH_4)_2SO_4$ than the spectrum of P_r (compare the intensities of the bands at 980 cm^{-1} [$(NH_4)_2SO_4$] and 1003 cm^{-1} [phytochrome]).

On irradiation of the pure P_r form with 667-nm light at −130 °C for 15 min, the only intermediate formed and stabilized under these conditions was I_{700} (see Figure 2). The subsequent measurement of that sample at −130 °C yielded an RR spectrum (Figure 4B) distinctly different from that of P_r, measured at the same temperature (Figure 4A), in particular in the region between 1500 and 1700 cm^{-1} as well as below 900 cm^{-1} (region not shown here). However, it is quite evident that the spectrum in Figure 4B still includes an appreciable contribution from P_r, implying that photoconversion to I_{700} is not complete (Eilfeld & Rüdiger, 1985). Hence, a pure spectrum of I_{700} can only be obtained by subtracting the P_r contribution. A reliable subtraction procedure requires appropriate well-resolved reference bands which are unique features of the parent state. One of these bands is at 1294 cm^{-1} (Figure 4A), which is only detectable as a shoulder in the spectrum of the P_r/I_{700} mixture. This intensity reduction can clearly be verified by comparison with the sharp peak at 1003 cm^{-1} which originates from the non-resonance-enhanced Raman bands of the 44 phenylalanine (Phe) residues of the apoprotein (Hildebrandt et al., 1992). This band, which is apparently the only detectable one of the protein matrix, is unlikely to vary its intensity in the individual states of phytochrome. Thus, it can be regarded as an internal standard to which the RR intensities of the tetrapyrrole bands can be referred to. Referred to this standard, the relative intensity of the 1294-cm^{-1} band as

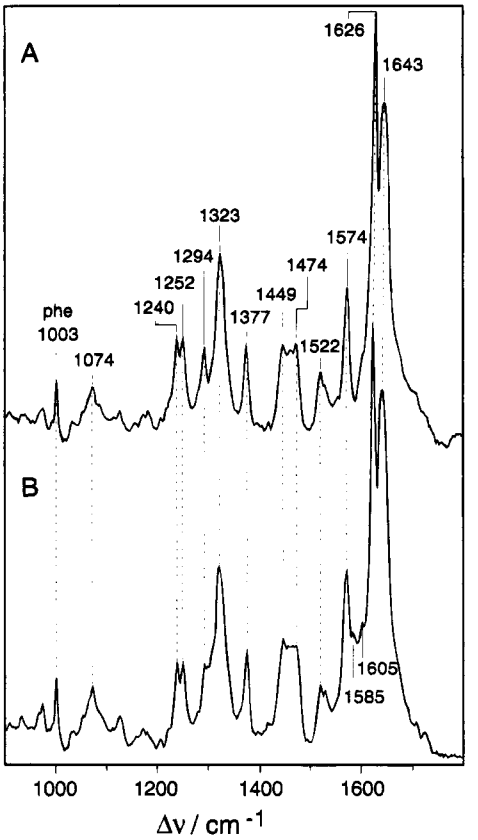

FIGURE 4: RR spectra of P_r (A) and a mixture of P_r and I_{700} (B) measured at $T = -130$ °C in H_2O solution. Spectrum B was obtained after irradiating the P_r sample with 667-nm light. Further details are given in the text.

determined by band fitting drops from 0.94 in P_r to 0.62 in the P_r/I_{700} mixture, corresponding to an I_{700} content of 34% (cf. Eilfeld & Rüdiger, 1985). Consequently, subtraction of the spectrum in Figure 4A from that of Figure 4B was carried out so that the 1294-cm^{-1} band of P_r disappeared in the difference spectrum which, hence, is ascribed to the pure spectrum of I_{700} (Figure 6B). This spectrum contains no negative bands, justifying the underlying assumption of the subtraction procedure that the 1294-cm^{-1} band is exclusively present in P_r. In the deuterated phytochrome, the 1294-cm^{-1} band of P_r disappears. In that case, subtraction of the P_r contribution was guided by the assumption that the relative RR intensities of the H/D-insensitive bands of I_{700} should be similar in H_2O and D_2O (Figures 6B and 7B).

At temperatures above −100 °C, I_{700} converts to the next intermediates meta-R_a, meta-R_c, and possibly meta-R_b (Eilfeld & Rüdiger, 1985), which are commonly denoted as bleached states, I_{bl}, in view of their significantly lower absorption coefficients for the first electronic transition. In fact, the characteristic RR bands of I_{700} disappeared above −100 °C. However, we were unable to obtain reliable RR spectra of the meta-R states in the temperature range of −80 to −40 °C upon subtracting the contributions of P_r. The RR difference spectra obtained in this way (not shown) were very noisy, and one of the few prominent peaks which could unambiguously be identified was the 1003-cm^{-1} band of Phe. The intensity of this band was as high as those of the strongest RR bands of the chromophore (in the region between 1580 and 1650 cm^{-1}). This implies that, due to the weaker electronic transition at ca. 700 nm, the resonance enhancement is much poorer in I_{bl} than in P_r. At a

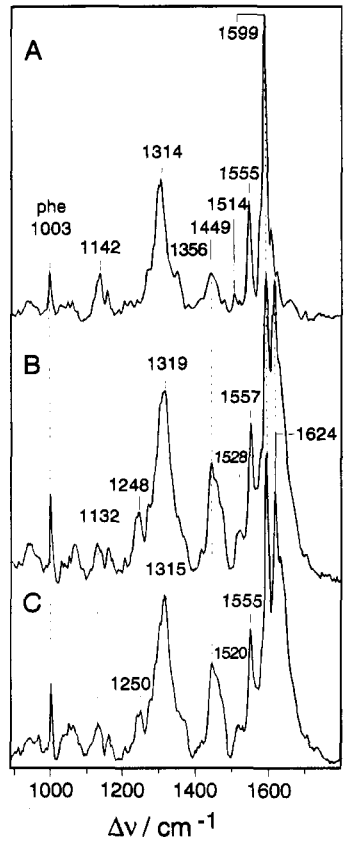

FIGURE 5: RR spectra of (A) P_{fr}, measured at T = −140 °C, (B) a mixture of P_{fr} and lumi-F, measured at T = −140 °C after irradiation with 730-nm light, and (C) a mixture of P_{fr} and meta-F, measured at T = −70 °C subsequent to the measurement of spectrum B. All spectra were obtained from H_2O solution. Further details are given in the text.

temperature higher than −25 °C, P_{fr} was formed as expected from low-temperature absorption data (Eilfeld & Rüdiger, 1985).

The intermediates of the photoinduced reaction sequence from P_{fr} to P_r were obtained in a similar way, irradiating, at 730 nm for 15 min at −140 °C, a sample composed predominantly of P_{fr}. Although P_{fr} samples unavoidably contain up to ca. 20% P_r (Lagarias et al., 1987), the RR spectrum (Figure 5A) exhibits only little contribution of P_r, apparently due to the preferential enhancement of the P_{fr} chromophore, the absorption of which is red-shifted from P_r.

RR spectra which were expected to include the intermediates lumi-F and meta-F were measured at −140 and −70 °C, respectively. Again, residual contributions from the parent P_{fr} state were subtracted. In these cases, an appropriate P_{fr} reference band was at 1599 cm^{-1} which has considerably lost intensity relative to the 1003-cm^{-1} band in the spectra of Figure 5B,C. The resultant difference spectra representing exclusively the intermediates lumi-F and meta-F are shown in Figure 8B,C. The corresponding spectra of the deuterated phytochrome were obtained in a similar way (Figures 9B,C).

DISCUSSION

The detailed chromophore conformation of P_r is not known. NMR studies of the chromopeptide isolated upon hydrolytic degradation of P_r have indicated that the exocyclic double bonds have *ZZZ* configuration in P_r (Figure 1A) and

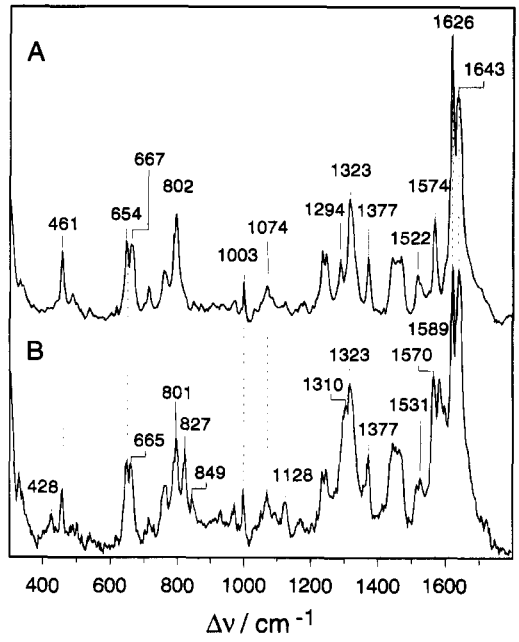

FIGURE 6: Overview RR spectra of P_r (A) and I_{700} (B) in H_2O solution. Spectrum B was obtained by subtracting the P_r spectrum from the composite spectrum shown in Figure 4B. Details of the subtraction procedure are given in the text.

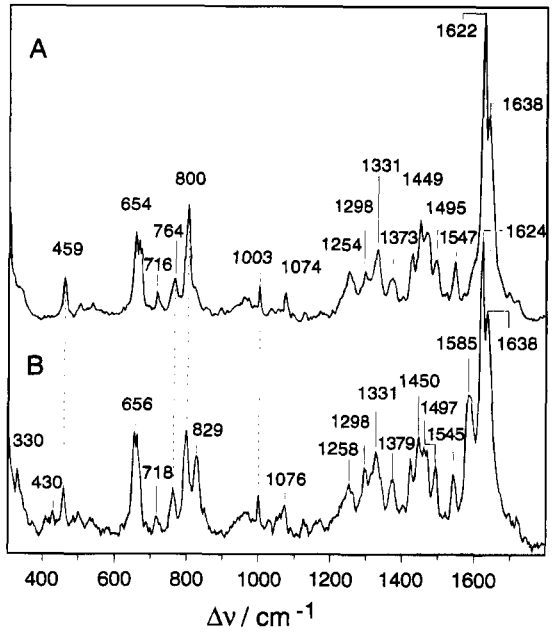

FIGURE 7: Overview RR spectra of P_r (A) and I_{700} (B) in D_2O solution. Spectrum B was obtained by subtracting the P_r spectrum from the composite spectrum (not shown). Details of the subtraction procedure are given in the text.

ZZE configuration in P_{fr} (Thümmler et al., 1981; Rüdiger et al., 1983), implying that the primary photoprocess involves a $Z \rightarrow E$ isomerization of the methine bridge C−D. In addition, the pyrrolenin nitrogen of ring C has been suggested to be protonated (e.g., Lagarias & Rapoport, 1980; Schaffner et al., 1990, 1991, and references therein), but direct evidence is still missing. Although the RR spectrum contains all the information required for the analysis of the chromophoric structure and protonation state, it is not possible to extract these structural data in detail at present since a safe assignment of all the RR bands is not possible yet. However, the vibrational analysis of BVE leads to conclusions which

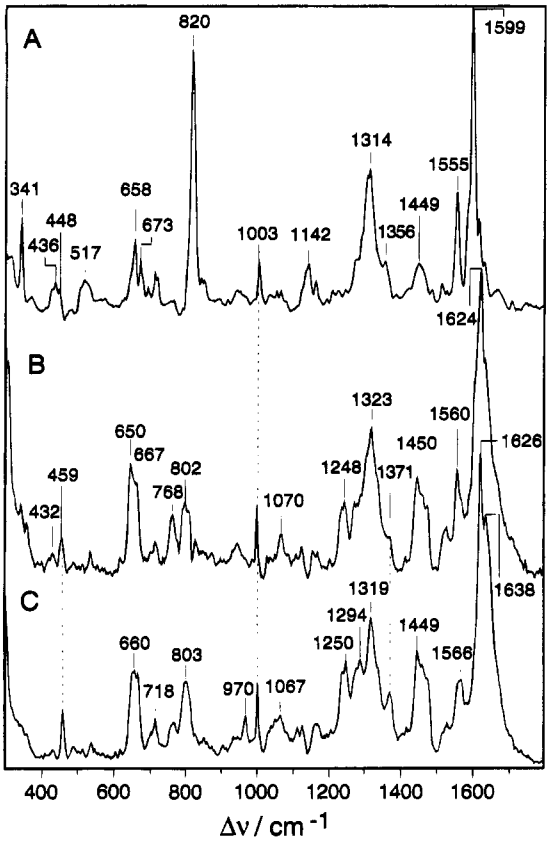

FIGURE 8: Overview RR spectra of P_{fr} (A), lumi-F (B), and meta-F (C) in H_2O solution. Spectra B and C were obtained by substracting the P_{fr} spectrum from the composite spectra shown in Figure 5, panels B and C, respectively. Details of the subtraction procedure are given in the text.

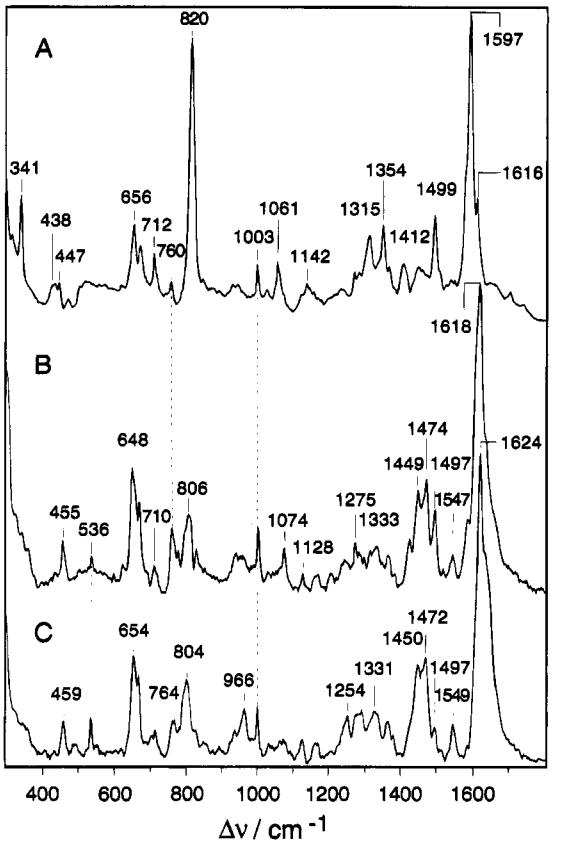

FIGURE 9: Overview RR spectra of P_{fr} (A), lumi-F (B), and meta-F (C) in D_2O solution. Spectra B and C were obtained by subtracting the P_{fr} spectrum from the composite spectra (not shown). Details of the subtraction procedure are given in the text.

are relevant for the interpretation of the spectral changes in the various forms of phytochrome (Smit, 1992; Smit et al., 1993; Matysik et al., 1995).

Generalization of the Vibrational Analysis of BVE. The results obtained for BVE in the *ZZZ,sss* configuration (Figure 1B) have demonstrated that the modes in the C=C stretching region between 1500 and 1650 cm^{-1} are largely loclized [generally 50% or more potential energy distribution (PED)] in individual pyrrole rings and adjacent methine bridges (Smit et al., 1993).[4] The two strongest bands of BVE (at 1593 and 1607 cm^{-1}) originate from modes of the inner pyrrole ring (here denoted as ring B) which is adjacent to the nonprotonated ring. This is apparently a characteristic property of BVE independent of the configuration and conformation of the tetrapyrrole. Quantum chemical calculations have predicted that these two ring B modes, albeit with somewhat different PED, should exhibit the strongest RR intensity in the various possible *E/Z* and *a/s* isomers, as shown in Figure 10 (Smit, 1992).

Moreover, it was found that $s \rightarrow a$ single- and/or $Z \rightarrow E$ double-bond isomerizations around the methine bridge between rings C and D induce only subtle alterations of frequencies, relative intensities, and PED of these modes. On the other hand, substantial frequency shifts are noted for

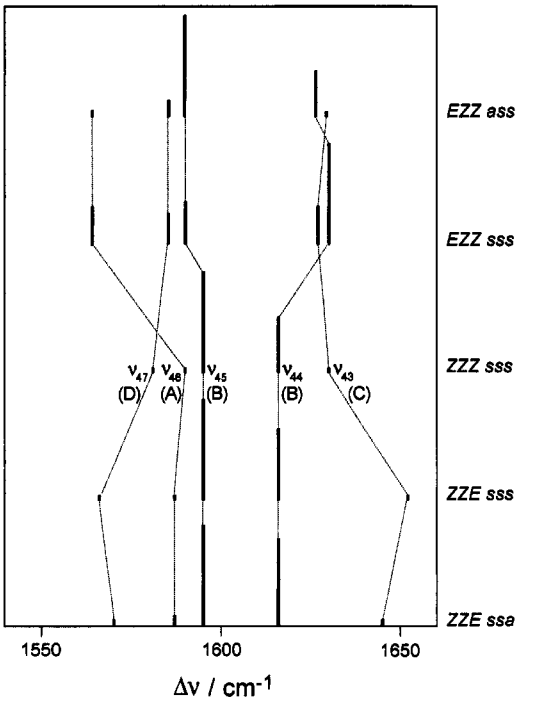

FIGURE 10: Calculated RR spectra of various isomers of BVE (Smith, 1992; Smith et al., 1993; Matysik et al., 1995). The mode numbering follows that by Smit et al. (1993). The notation (A)–(D) refers to the rings of BVE (see Figure 1).

isomerizations around the adjacent methine bridges A–B and B–C. RR spectra of several related tetrapyrroles in the neutral and in the cationic forms exhibit a similar C=C

[4] The region between 1500 and 1650 cm^{-1} will be denoted as "C=C stretching" region although the modes also include other (stretching) vibrations of the chromophore (Smit et al., 1993). Correspondingly, the region between 750 and 850 cm^{-1} will be referred to as the C–H o.o.p. bending region.

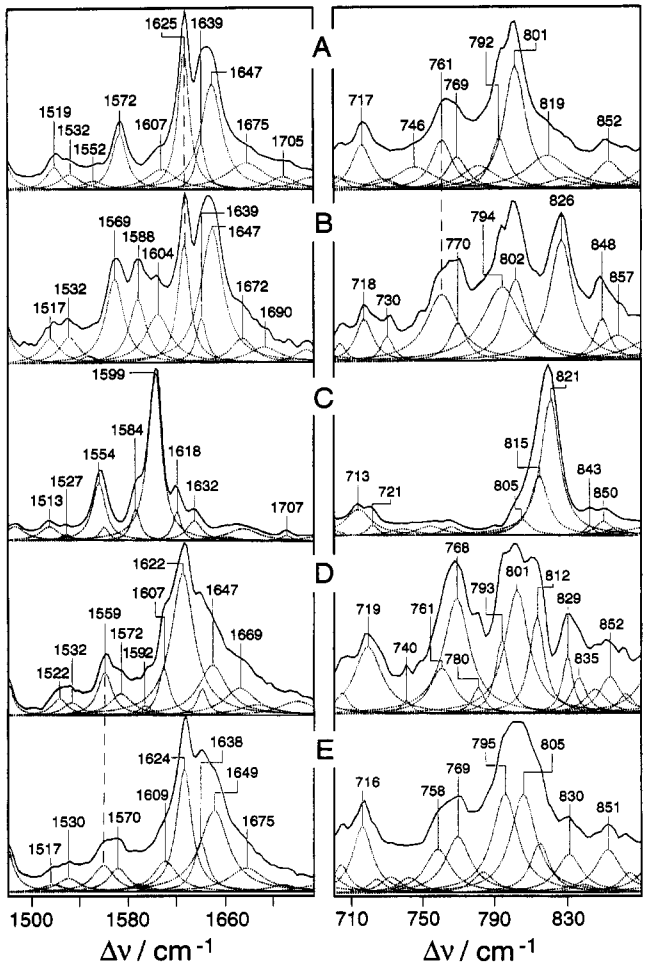

FIGURE 11: RR spectra of P_r (A), I_{700} (B), P_{fr} (C), lumi-F (D), and a meta-F (E) in H_2O in the C=C stretching (left) and C−H o.o.p. bending region (right). The dashed lines represent fitted Lorentzian line shapes.

stretching band pattern, with the strongest bands more or less closely spaced between 1590 and 1630 cm^{-1} (Matysik, and Hildebrandt, unpublished results). It appears justified, therefore, to extend this particular aspect of the vibrational analysis of BVE—i.e., the two dominant C=C stretching modes being localized in ring B—also to the tetrapyrrole chromophore of phytochrome, despite the different chemical constitution of ring A and the possibly protonated ring C. Accordingly, the dominant bands in P_r at 1625 and 1647 cm^{-1} (Figure 11A) can be attributed to modes originating from the pyrrole ring which in Figure 1A is denoted as ring B. It should be emphasized that the frequencies of these ring B modes (as well as those of the remaining bands in this region) cannot be used to determine the configuration and conformation of the phytochromobilin chromophore in P_r by comparison with the quantum chemical results for BVE isomers. However, changes of these bands in the various forms of phytochrome can be taken as an indicator for structural changes associated with ring B and the adjacent methine bridges (A−B, B−C).

Structural Changes in I_{700}. The dominant RR bands in the C=C stretching region of $P_r(H_2O)$ at 1625 and 1647 cm^{-1} are at the same positions albeit with slightly different RR intensities in I_{700} (Figure 11B). Also the frequency shifts upon H/D exchange are the same (Figure 7), implying that this agreement is not accidental. Hence, we conclude that these bands originate from the same modes (i.e., ring B modes), possessing the same PED in both species. Consequently, the photoinduced configurational changes should be confined to the methine bridge remote from ring B, i.e., to the C−D bridge, provided that ring C is the pyrroleninic ring (as depicted in Figure 1A). Alternatively, isomerization would occur at the A−B bridge provided that ring B is pyrroleninic. Isomerization around the A−B bridge has been suggested by a molecular modeling study (Gabriel & Hoober, 1991) but appears unlikely taking into account the NMR evidence in favor of the *ZZE* configuration in P_{fr} (Rüdiger et al., 1983).

The RR spectra of P_r and I_{700} (in both H_2O and D_2O) reveal striking similarities. The most pronounced differences in the RR spectrum of I_{700}, compared to that of P_r, are the relatively strong bands at 1588 (1583) cm^{-1} (C=C stretching region, Figures 6, 7, and 11A,B) and at 826 (827) cm^{-1} in H_2O (D_2O), which have no counterparts in the spectra of P_r. These bands evidently reflect the specific structure of the tetrapyrrole chromophore in I_{700}. The 826-cm^{-1} band is located in the region of C−H o.o.p. bending modes of the methine bridges. The RR intensities of such modes depend on the differences of the C−H o.o.p. coordinates in the ground and electronically excited state (Eyring et al., 1980). If there are substantial torsions of the methine bridges (i.e., the C(5)−C(6), C(9)−C(10), and C(14)−C(15) single bonds) in the ground state, such differences can be large, resulting in strong RR intensities for those modes involving the C−H o.o.p. coordinates. In BVE isomers, these modes are expected between 820 and 750 cm^{-1}, depending on the configuration and conformation of the tetrapyrrole (Smit, 1992), and the assignment of some of these modes has been confirmed by isotopic labeling. Their frequencies are insensitive toward H/D exchange at the pyrrole nitrogens, which is in fact observed for the bands at 826 cm^{-1} (I_{700}) and at 801 and 792 cm^{-1} (P_r and I_{700}).

Within the framework of our interpretation of the RR spectra, the new 826-cm^{-1} band in I_{700} is assigned to the o.o.p. bending of the C(15)−H (i.e., the isomerization site), and the intensity increase of this mode relative to P_r then implies a substantial torsion around the C(14)−C(15) bond, accompanying the $Z \rightarrow E$ isomerization. Consequently, the nearby bands at 801 and 792 cm^{-1} which are present in both P_r and I_{700} are assigned to the C−H o.o.p. modes of the remaining methine bridges (Figures 11A,B). Their intensities relative to the bands in the C=C stretching region are clearly higher than in BVE (Smit et al., 1993), indicating that already in the stable parent chromophore the structure is more strongly twisted than in the helical structure of BVE crystals (ca. 20 °C; cf. Sheldrick, 1976). Evidently, interactions with the immediate protein environment prevent the tetrapyrrole from adopting an essentially planar structure, and these torsions are largely the same in P_r and I_{700}.

In the region between 1250 and 1400 cm^{-1}, which includes the modes involving the N−H i.p. bending coordinate, the only distinct spectral difference between both forms is that the band at 1294 cm^{-1} of P_r is missing in I_{700}, which in turn exhibits a new peak at 1310 cm^{-1} (Figure 6). Both the 1294- and the 1310-cm^{-1} bands disappear in D_2O (Figure 7). It is not very likely to relate the P_r band at 1294-cm^{-1} in H_2O to the band at 1298 cm^{-1} in D_2O since the latter is also found, at the same frequency and with quite similar relative intensity, in the RR spectrum of I_{700} in D_2O. The remaining bands in this region reveal very similar frequencies, relative

intensities, and H/D isotopic effects in P_r and I_{700}, implying that the PED's of the modes involved are largely the same. Consequently, the bands at 1294 and 1310 cm^{-1} of P_r and I_{700}, respectively, must be due to a mode including an N−H i.p. bending coordinate which is not significantly admixed to other modes in this region. Then, the 16-cm^{-1} frequency shift apparently reflects modifications of the hydrogen bonding interactions of this particular N−H group with the protein environment.

Structural Changes in P_{fr}. While the overall vibrational band patterns of P_r and I_{700} are still similar, the RR spectrum of P_{fr} reveals quite a different picture (cf. Figures 8A, 9A, 6, and 8). P_{fr} has a characteristic vibrational signature differing from that of all other phytochrome forms studied in this work. In particular, all the strongest RR bands in the C=C stretching region are downshifted (Figure 11). The bands at 1625 and 1647 cm^{-1}, which are a typical signature of P_r and I_{700}, are not present in P_{fr}. This strongly suggests that the structural changes occurring during the conversion of I_{700} (via I_{bl}) to P_{fr} must also involve ring B and the adjacent methine bridges which are not directly affected by the primary photochemical process. Support for this interpretation is found in the C−H o.o.p. bending region where the P_r- and I_{700}-characteristic bands at 800 cm^{-1} have disappeared in P_{fr} (Figure 11). Instead, an unusually strong peak at ca. 820 cm^{-1} is observed which includes two components at 815 and 821 cm^{-1}. Both bands are assigned to modes involving a methine C−H o.o.p. bending, and the strong RR intensities indicate a significant twisting around the corresponding methine bridge (cf. Fodor et al., 1990; Hildebrandt et al., 1992). Most likely, both components are associated with the same methine bridge as judged from the similar temperature dependence of their frequencies. Upon warming up from −140 to 25 °C, the 815- and 821-cm^{-1} bands gradually shift down to 807 and 815 cm^{-1}, respectively, while the intensity ratio of both bands remains unchanged. In addition, the relative intensities of both bands are ca. 25% higher at low temperature than those of the bands in the C=C stretching region. These spectral changes are likely to arise from a subtle temperature-dependent conformational change of the protein which in turn induces a further torsion of this particular methine bridge. Thus, we conclude that the underlying conformational change of the protein is confined to the immediate vicinity of the methine bridge involved. The comparison with the C−H o.o.p. modes of I_{700} (826 cm^{-1}; Figure 11B) suggests that it is the methine bridge at the isomerization site (C−D) and not the one (A−B or B−C) which is somewhat twisted in P_r (801 cm^{-1}; Figure 11A). This assignment is consistent with the observation that the 800-cm^{-1} bands of P_r reveal no temperature dependence, implying that the protein environment of the methine bridge A−B or B−C is less flexible than at the isomerization site.

Structural Changes in Lumi-F. The first intermediate which is detected in the course of the photoinduced conversion from P_{fr} to P_r is lumi-F. The RR spectrum of this species has significantly changed compared to that of the P_{fr} (Figures 8 and 9). For instance, the strongest band in the high-frequency region is significantly upshifted from 1599 to 1622 cm^{-1}, and the characteristic 821-cm^{-1} band of P_{fr} in the C−H o.o.p. bending region is missing in the spectrum of lumi-F (Figure 11D). The primary photochemical process in P_{fr} most likely is the double-bond reisomerization at the methine bridge C−D, i.e., the reversal of the photoreaction of P_r. It is interesting, therefore, to compare the RR spectra of lumi-F and P_r which should exhibit the same C(15)=C(16) double-bond configuration.

At the first sight, there are many similar features. Several bands in the C=C stretching region of lumi-F (e.g., 1622, 1647, 1572 cm^{-1}) are at frequencies close to those in P_r. However, most of these bands reveal different relative intensities and isotopic effects (Figures 8, 9, and 11). This comparison indicates that the double-bond isomerization in lumi-F does not lead to the same tetrapyrrole geometry as in P_r, i.e., the C−D moiety is not yet relaxed. This interpretation is supported by the analysis of the C−H o.o.p. bending region (Figure 11), which includes numerous relatively intense bands. Among them is a band at 829 cm^{-1} which may have the same origin as the bands at 826 and 821 cm^{-1} of I_{700} and P_{fr}, respectively, i.e., the C−H o.o.p. bending of the twisted methine bridge C−D. On the other hand, this region also reveals similarities with P_r. In particular, the band at 801 cm^{-1} and its low-frequency shoulder at 793 cm^{-1} are at essentially the same positions as in P_r although they are much less intense (ca. 1/3) than the 1003-cm^{-1} reference band. This means that the methine bridge which gives rise to this band (A−B or B−C; see above) is already somewhat twisted in lumi-F but not as strongly as in P_r.

Structural Changes in Meta-F. In the subsequent intermediate, meta-F, the geometry of the methine bridge A−B (or B−C) apparently does not change very much since the intensities of the 793- and 805-cm^{-1} bands (relative to the 1003-cm^{-1} band) are still comparable in magnitude to that of lumi-F (cf. Figures 8, 9, and 11). However, the RR intensity at 830 cm^{-1}, which may be related to the C−H o.o.p. of the methine bridge C−D, is further reduced, implying a more relaxed geometry of this dipyrrole moiety. The bands at 1649 cm^{-1} (and its counterpart in D_2O) and 1570 cm^{-1}, which both are characteristic features of P_r, are stronger than in lumi-F (Figure 11). Alternatively, the band at 1559 cm^{-1}, which may correspond to the 1554-cm^{-1} in P_{fr} but is not present in P_r, is clearly weaker in meta-F than in lumi-F.

Protonation State Changes during the Photocycle. There is a general agreement that in P_r the pyrrole nitrogen of ring C is protonated (cf. Lagarias & Rapoport, 1980; Fodor et al., 1988, 1990; Siebert et al., 1990; Schaffner et al., 1991; Mizutani et al., 1991, 1994). This conclusion is drawn, inter alia, from a comparison of the absorption spectra of P_r and model compounds (Lagarias & Rapoport, 1980; Rüdiger et al., 1983; Schaffner et al., 1991, 1993). In particular, the bathochromic shift and concomitant intensity increase of the red absorption band which are induced upon protonation of tetrapyrrole model compounds in organic solvents and in proteic matrix have been taken as evidence for a cationic chromophore in P_r. Accordingly, the absorption spectra of all other forms of phytochrome (possibly I_{bl} excepted) should arise from a protonated chromophore. However, such arguments should be taken with caution since other factors such as the specific geometry of the chromophore and variable interactions with surrounding amino acid residues can strongly affect the energy and the strength of the electronic transitions as well (Falk et al., 1978; Krois, 1991; see also the discussion on retinal proteins, e.g., Kakitani &

Table 1: RR Intensities of the 800- and 820-cm^{-1} C−H o.o.p. Modes of the Various Forms of Phytochrome[a]

form	C(10)−H o.o.p. (800 cm^{-1})	C(15)−H o.o.p. (820 cm^{-1})	form	C(10)−H o.o.p. (800 cm^{-1})	C(15)−H o.o.p. (820 cm^{-1})
P_r	69	13	lumi-F	35	20
I_{700}	66	41	meta-F	32	13
P_{fr}	<10	166			

[a] The data are relative integrated band intensities (with respect to the 1003-cm^{-1} band), determined by a band fitting analysis (Figure 11). The data are average values from the spectra in H_2O and D_2O. The errors are less than 5% for the strong bands of P_r and P_{fr} and up to 20% for the weaker bands in the intermediates.

Kakitani, 1979; Grossjean & Tavan, 1988; Sheves & Nakanishi, 1983).

At present, the RR spectra do not allow for an unambiguous determination of the protonation state of the chromophore since those modes which predominantly include the C=N stretching (or N−H) vibrations of (the possibly protonated) ring C (cf. Figure 1) have not yet been identified. In all phytochrome forms studied in this work, H/D exchange leads to numerous changes in the spectra. This implies that in general the internal coordinates involving the pyrrole and pyrroleninic nitrogens are widely distributed among many normal modes and that their PEDs differ strongly in the deuterated states (cf. Smit et al., 1993). Thus, the assignment of the 1572-cm^{-1} band of P_r to the C=N stretching of the protonated ring C, as proposed by Fodor et al. (1988), remains to be verified.

In our previous work, a broad band in the P_r spectrum was assigned to the o.o.p. bending of a strongly hydrogen-bonded N−H group of ring C (Hildebrandt et al., 1992). Since this band was not detected in the RR spectrum of P_{fr}, we concluded that the $P_r \rightarrow P_{fr}$ conversion is associated with a deprotonation or a drastic change of the hydrogen-bonding interactions at this ring. However, this argument is not valid as the present study has shown that the 1100-cm^{-1} band is due to $(NH_4)_2SO_4$.

Mizutani et al. (1991, 1994) strongly favor the idea of a deprotonated ring C in P_{fr} based on RR spectra excited in resonance with the second electronic transition. The authors found no isotopic shifts upon H/D exchange for the bands in the C=C stretching region, which is in contrast to the results obtained upon excitation in resonance with the first electronic transition in this and in previous studies (Fodor et al., 1990; Hildebrandt et al., 1992). This contradiction was suggested to reflect the selective enhancement of the ring C modes at resonance with the second electronic transition. The same explanation has been stressed by Szalontai et al. (1994) based on an RR study of phycocyanins. However, our own studies with BVE cast some doubt on this idea since we have found that the RR spectra of BVE in the C=C stretching region include exactly the same modes with only slightly different relative intensities whether the excitation line is close to the first or to the second electronic transition (Matysik and Hildebrandt, unpublished results). Furthermore, the number of bands detected in the C=C stretching region of the FT-RR spectra is about the same for P_{fr} (as well as for all other species of phytochrome and BVE) and is not smaller than the number of modes calculated for BVE (cf. Figure 11; Smit et al., 1993), implying that all normal modes of the tetrapyrrole in P_{fr}, which are expected in this frequency range, contribute to the FT-RR spectrum. Hence, it is very unlikely that the prominent 1599-cm^{-1} band of P_{fr} in the UV-excited RR spectrum (Mizutani et al., 1991, 1994) does not originate from the same mode as the strong 1599-cm^{-1} band found with red or near-infrared excitation (see Figures 8, 9, and 11; Fodor et al., 1990; Hildebrandt et al., 1992).

Summarizing these considerations, it is necessary to point out that at the present state of vibrational analysis the RR data of all phytochrome forms studied in this work are compatible with both a protonated and a nonprotonated chromophore. Furthermore, since the RR spectra of P_r and I_{700} in both H_2O and D_2O show far-reaching similarities in the region of the modes including the N−H i.p. bending vibrations (1250−1400 cm^{-1}), changes of the protonation state during the transition from P_r to I_{700} can be ruled out; i.e., both forms are either protonated or deprotonated. This conclusion is in line with the observation that the primary photochemical process of P_r does not reveal any kinetic H/D effect (Aramendiá et al., 1987; Brock et al., 1987).

Configurational and Conformational Changes during the Photocycle. The RR spectra of the various forms of phytochrome indicate structural changes at two sites of the tetrapyrrole. At the methine bridge C−D, these are the isomerization of the C(15)=C(16) double bond and rotational changes around the C(14)−C(15) single bond which are reflected by the C−H o.o.p. modes at ca. 820 cm^{-1}. Torsional changes also occur at another methine bridge. Presumably, this is the adjacent B−C bridge since its conformation should be more sensitive to the *E/Z* isomerization than the more remote A−B bridge.[5] Hence, the marker bands for these changes at ca. 800 cm^{-1} are attributed to the hydrogen o.o.p. modes of the C(10)−H group. The RR intensities of these o.o.p. modes, which are listed in Table 1, can be taken as a crude measure of the degree of rotation around the C(9)−C(10) and C(14)−C(15) bonds.

Based on these assignments a simplified model for the structural changes in the phytochrome photocycle is proposed (Figure 12). In P_r, the tetrapyrrole chromophore is in a *ZZZ* configuration with a twisted C(9)−C(10) bond. *E/Z* isomerization of the C(15)=C(16) double bond in the primary photoprocess is accompanied by a substantial torsion around the C(14)−C(15) single bond. These concomitant conformational and configurational changes may be necessary to fit the *ZZE* tetrapyrrole into the protein pocket since significant conformational changes of the protein environment itself are not expected at this stage. The methine bridge B−C does not reveal any conformational change as indicated by the unaltered intensity of the 800-cm^{-1} band and, further, by the overall similarity of wide regions of the P_r and I_{700} spectra. Based on temperature-dependent RR measurements,

[5] At present, we cannot rule out the assignment to the C(5)−H o.o.p. mode, which would imply that the intensity changes of the 800-cm^{-1} bands reflect torsional changes of the C(4)−C(5) instead of the C(9)−C(10) single bond as discussed in the following and in Figure 12.

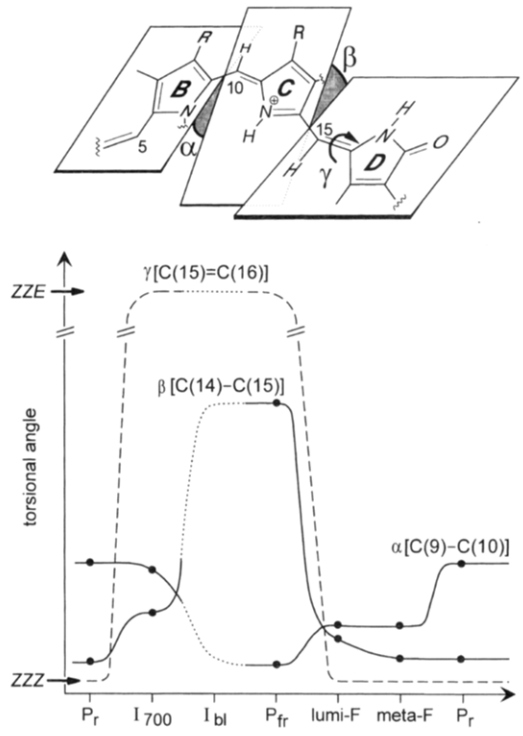

FIGURE 12: Changes of the bond distortions along the reaction coordinate of the phytochrome photocycle. The integrated RR intensities of the 800- and 820-cm^{-1} C−H o.o.p. modes (adopted from Table 1) were taken as a measure for the torsional angles α and β of the single bonds C(9)−C(10) and C(14)−C(15), respectively. Since there is certainly no simple proportionality between the RR intensities and the torsional angles, the *Y* axis is in arbitrary units and not necessarily linear. The angle γ refers to the C(15)=C(16) double bond and is taken to be 0° and 180° for the *ZZZ* and *ZZE* configurations, respectively.

the immediate surrounding of this part of the chromophore has been suggested to be even more rigid. The further transformation to P_{fr} is accompanied by a drastically increasing twist around the C(14)−C(15) bond while the geometry of the methine bridge B−C is fully relaxed. These conformational changes of the chromophore must be paralleled by matching structural changes of the immediate protein environment. This is most likely the crucial step of the conformational coupling between the chromophore and the protein which initiates the physiologically relevant structural changes in the interaction domain of phytochrome. It is not clear whether these changes have already occurred in I_{bl}. The RR data in the C=C stretching region of the I_{bl} species reported by Mizutani et al. (1994) suggest far-reaching similarities with P_{fr}. However, our own preliminary and rather noisy spectra of I_{bl} indicate that, at least, in the C−H o.o.p. region, the spectra of both species are different.

The photoinduced transition from P_{fr} → lumi-F does not only involve the *Z*/*E* isomerization of the C(15)=C(16) double bond and a concomitant decrease of the torsion around the C(14)−C(15) single bond but also a torsion around the C(9)−C(10) single bond. This is in contrast to the photoreaction of P_r where the molecular changes were confined to the isomerization site. Evidently, the primary photoreaction of P_{fr} lays the foundation for a different reaction pathway of the phototransformation P_{fr} → P_r as compared to the reversed process. In meta-F, the C(9)−C(10) bond remains largely unchanged while the torsional angle of the C(14)−C(15) bond is further flattened to yield a largely planar partial structure as in P_r. This suggests that the accompanying relaxation process of the protein are restricted to the vicinity of the methine bridge C−D. In the final step, i.e., the formation of P_r, protein conformational changes presumably also occur in the environment of the methine bridge B−C, enforcing the C(9)−C(10) bond into a more twisted structure as compared to meta-F.

CONCLUSIONS

The experimental approach of this work, i.e., low-temperature FT-RR spectroscopy, to elucidate the structures of the individual components of the phytochrome photocycle, has been shown to be most appropriate to provide high quality RR spectra. These spectra contain all necessary information required for a comprehensive analysis of the chromophore configuration and conformation, its protonation state, and its interactions with the protein environment. Unfortunately, this information cannot be extracted in detail since the vibrational assignment of linear tetrapyrroles is still not yet far enough developed. Currently, only preliminary conclusions concerning the structural changes during the photocycle can be drawn. The model we have derived from the RR data is plausible and self-consistent but needs to be tested by further experimental work using phytochrome reconstituted with isotopically labeled chromophores.

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