Protonation State and Structural Changes of the Tetrapyrrole Chromophore during the $P_r \rightarrow P_{fr}$ Phototransformation of Phytochrome: A Resonance Raman Spectroscopic Study†

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ABSTRACT: The photoconversion of phytochrome (phytochrome A from *Avena satina*) from the inactive (P_r) to the physiologically active form (P_{fr}) was studied by near-infrared Fourier transform resonance Raman spectroscopy at cryogenic temperatures, which allow us to trap the intermediate states. Nondeuterated and deuterated buffer solutions were used to determine the effect of H/D exchange on the resonance Raman spectra. For the first time, reliable spectra of the "bleached" intermediates meta-R_A and meta-R_C were obtained. The vibrational bands in the region 1300−1700 cm^{-1}, which is particularly indicative of structural changes in tetrapyrroles, were assigned on the basis of recent calculations of the Raman spectra of the chromophore in C-phycocyanin and model compounds [Kneip, C., Hildebrandt, P., Németh, K., Mark, F., Schaffner, K. (1999) *Chem. Phys. Lett. 311*, 479−485]. The experimental resonance Raman spectra P_r are compatible with the Raman spectra calculated for the protonated *ZZZasa* configuration, which hence is suggested to be the chromophore structure in this parent state of phytochrome. Furthermore, marker bands could be identified that are of high diagnostic value for monitoring structural changes in individual parts of the chromophore. Specifically, it could be shown that not only in the parent states P_r and P_{fr} but also in all intermediates the chromophore is protonated at the pyrroleninic nitrogen. The spectral changes observed for lumi-R confirm the view that the photoreaction of P_r is a $Z \rightarrow E$ isomerization of the *CD* methine bridge. The subsequent thermal decay reaction to meta-R_A includes relaxations of the *CD* methine bridge double bond, whereas the formation of meta-R_C is accompanied by structural adaptations of the pyrrole rings *B* and *C* in the protein pocket. The far-reaching similarities between the chromophores of meta-R_A and P_{fr} suggest that in the step meta-$R_A \rightarrow P_{fr}$ the ultimate structural changes of the protein matrix occur.

Phytochromes are ubiquitous photoreceptors in plants controlling various photomorphogenic processes (*1*, *2*). The physiological functions are initiated by a photoinduced reaction cycle of the tetrapyrrolic chromophore (phytochromobilin, PΦB;[1] Figure 1), which allows the interconversion between two stable (parent) states, the red-absorbing form P_r and the far-red-absorbing form P_{fr}. The latter is regarded to be the physiologically active state (Figure 2).

A deeper understanding of the functioning of phytochrome on a molecular level requires a detailed knowledge of the chromophore structural changes during the photoinduced reaction cascade. The kinetics of this process has been studied by time-resolved spectroscopies (e.g., refs *3*−*5*). Several intermediate states have been detected from the nano- to millisecond time range. Most of them could be cryogenically trapped and monitored by UV−vis absorption spectroscopy (Figure 2) (*6*). However, no reliable structural information is available about the various states. In the parent state P_r the tetrapyrrole is assumed to exist in an extended conformation with a protonated pyrroleninic nitrogen (*4*, *7*−*9*). Experiments with chromopeptide fragments of phytochrome in the P_r and P_{fr} states have led to the conclusion that the primary photoreaction is a double-bond isomerization at the methine bridge between rings *C* and *D* (*10*, *11*). The subsequent thermal relaxation steps are ascribed to confor-

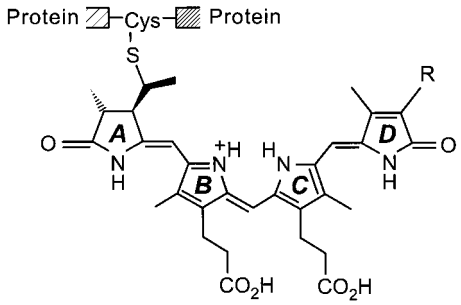

FIGURE 1: Structural formula of phytochromobilin (PΦB; R = vinyl) and phycocyanobilin (PCB; R = ethyl).

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[1] Abbreviations: *A*, *B*, *C*, and *D*, individual tetrapyrrole rings; *AB*, *BC*, and *CD*, methine bridges connecting the corresponding rings (see Figure 1); DFT, density functional theory; FT, Fourier transform; ip, in-plane bending; NIR, near-infrared; phyA, phytochrome from *Avena sativa*; PΦB, phytochromobilin; PCB, phycocyanobilin; phyA-PΦB and phyA-PCB, adducts of the 65-kDa fragment of phyA with PΦB and PCB, respectively; RR, resonance Raman; S/N, signal-to-noise; str, stretching; α-CPC.; α-subunit of C-phycocyanin.

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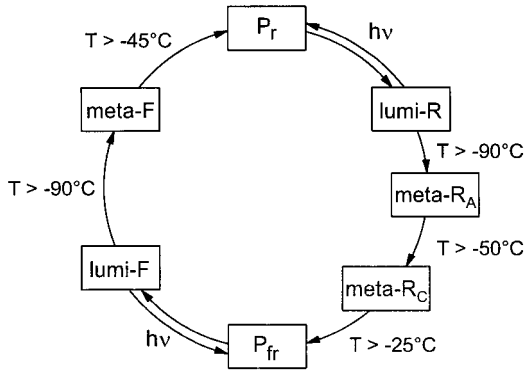

FIGURE 2: Photoinduced $P_r \rightarrow P_{fr}$ reaction cycle of phytochrome.[2] The scheme includes upper temperature limits below which the thermal decay reactions are blocked.

mational rearrangements of the chromophore and the protein. Details of the underlying molecular processes are not known (*4*). Particularly, it remains to be elucidated how the chromophore movement is coupled to the protein structural changes. Altogether, a partial exposure of the chromophore (*12*), the formation of an amphiphilic α-helix (*13*), and eventually, a major structural protein change on the level of the tertiary and quaternary structure have been suggested (*14*).

Evidently, elucidation of the chromophore structure of the various intermediate states formed during the photoinduced activation of phytochrome is required. In this respect, resonance Raman (RR) spectroscopy is a powerful tool as it selectively probes the vibrational bands of the tetrapyrrole chromophore upon excitation in resonance with its electronic transition (*15*−*24*). However, under rigorous resonance conditions unwanted photophysical and photochemical processes aggravate the RR measurements of phytochrome. These difficulties can be overcome by choosing near-infrared (NIR) excitation (1064 nm), the energy of which is too weak to induce chromophore fluorescence and to initiate the photocycle (*20*, *22*, *24*). The resonance enhancement, albeit weaker than under rigorous resonance conditions, is sufficiently high to permit an effective discrimination of the Raman bands of the apoprotein with respect to the (pre)-resonance-enhanced Raman bands of the chromophore. This approach, NIR Fourier transform (FT) Raman spectroscopy, has been shown to provide high-quality RR spectra of various states of phytochrome (*22*).

In the present work, we have continued these studies focusing on the intermediate states formed during the $P_r \rightarrow P_{fr}$ photoconversion. The extraction of structural information from the RR spectra was guided by recent quantum chemical calculations of the vibrational spectra of open-chain tetrapyrroles (*25*, *26*). In particular, we were interested to identify and characterize the long-lived bleached intermediates (meta-R states)[2] for which no reliable RR spectra could be obtained so far. The formation and the decay of these intermediates are assumed to constitute the linkage between the structural changes of the chromophore and the protein movement, which eventually leads to the activation of the photoreceptor (*4*).

[2] The intermediates of the $P_r \rightarrow P_{fr}$ phototransformation, trapped at low temperature, are denoted according to Eilfeld and Rüdiger (*6*).

MATERIALS AND METHODS

Phytochrome Purification. Phytochrome A (phyA) from etiolated seedlings of *Avena sativa* L. cv. Pirol was purified according to the method described by Grimm and Rüdiger (*27*) with modifications as described by Hildebrandt et al. (*20*). A spectral absorption ratio of $A_{667}/A_{280} = 0.95-1.15$ for the P_r form was routinely achieved.

Sample Preparation. A solution (1−1.5 mL) of phyA in 10 mM phosphate buffer (pH 7.8) containing 5 mM dithiothreitol and 2 mM EDTA, with an optical density of 1 at 660 nm (ca. 8−12 nM phyA), was irradiated with 730-nm light and concentrated through a membrane filter (Centricon 100) to a final volume of ca. 25−40 μL (corresponding to a concentration of 0.2−0.64 mM/L). The viscous sample was placed in the conical bore of a cylindrical aluminum block, shock-frozen in liquid nitrogen, and inserted into a cryostat (precooled to −140 °C) with the sample positioned on the optical axis of the spectrometer. Deuterated samples were prepared in the same way: the sample was concentrated and redissolved in deuterated buffer three times to ensure a complete H/D exchange as verified by characteristic marker bands in the RR spectra (vide infra).

Sample Handling. All manipulations of phytochrome were carried out under dim green light. Prior to shock freezing, phyA was converted into the P_r and P_{fr} states by irradiating the samples with 730- and 667-nm light, respectively. For irradiation a slide projector (250 W) with either a 667-nm band-pass filter (11 nm fwhh) or a 730-nm cutoff filter was used. All spectra were recorded at −140 °C. Enrichment of the individual intermediates was achieved by irradiating the sample at the desired temperature with 667-nm light (lumi-R, −140 °C; meta-R_A, −70 °C; meta-R_C, −30 °C) before a spectrum was recorded at −140 °C. Spectra of the parent state P_r were measured prior to irradiation for every sample. All measurements were repeated at least three times with freshly prepared samples to ensure reproducibility.

FT-RR Measurements. The spectra were measured with a Bio-Rad FT-Raman spectrometer equipped with a Nd:YAG laser (Spectra Physics, FC-106V, $\lambda = 1064$ nm, bandwidth < 1 cm^{-1}) and a liquid nitrogen cooled Ge detector. The Raman scattered light was collected using 180° backscattering geometry. The cryostat (Cryovac) modified for the requirements of Raman measurements allowed temperature control between −190 and +40 °C, achieved with a Pt100 thermocouple placed at the back of the sample holder. Laser power at the sample was 300 mW. Spectra were recorded with a resolution of 4 cm^{-1} and accumulation times between 1024 scans (ca. 40 min) and 3072 scans (120 min). To improve the signal-to-noise (S/N) ratio, spectra of the individual measurements were added. For the apodization of the interferograms, the triangular function was used. The raw spectra were corrected for the instrumental response according to Henderson et al. (*28*) by using a spectrum of the white light standard of the spectrometer and a correction function (*29*) instead of blackbody radiation. The unstructured background was subtracted by using the "multiple points" routine of the program Win-IR. To obtain spectra of the pure intermediates, the contributions of the parent states were subtracted as described in the following section.

Density functional theory (DFT) calculations of open-chain tetrapyrroles were carried out by following a strategy

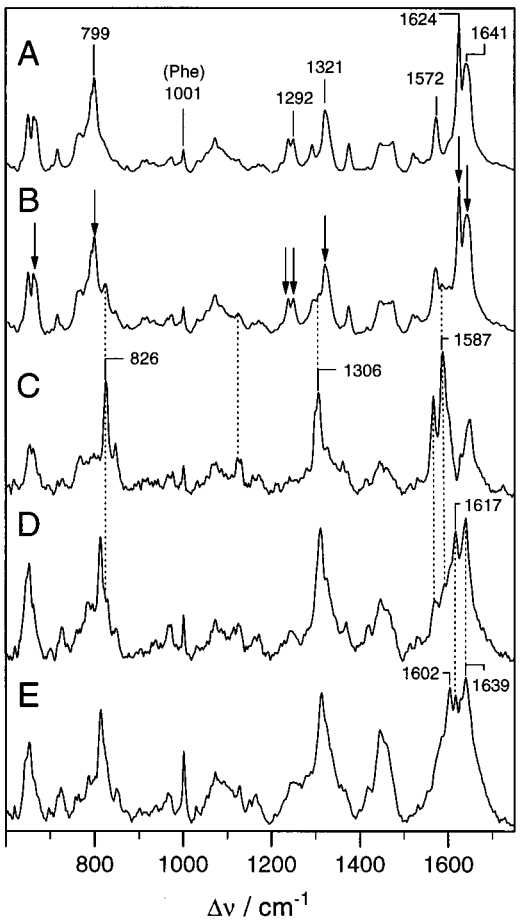

FIGURE 3: RR spectra of phytochrome measured at −140 °C (H_2O) (A) of the parent P_r state and of a photostationary mixture (B) obtained after 667-nm irradiation at −140 °C. Spectrum C is the difference B minus A, i.e., the spectrum of lumi-R. Spectra D and E represent the raw spectra of meta-R_A and meta-R_C, obtained after irradiation at −70 and −30 °C, respectively (the residual contribution of P_r is subtracted from both spectra). The arrows indicate spectral changes in the spectra after irradiation. The dotted lines indicate residual contributions of the respective preceding intermediates. The subtraction procedure is described in the text.

published previously (*30*). The calculated Raman spectra of phycocyanobilin (PCB) in the protonated ($PCBH^+$) and deuterated ($PCBD^+$) *ZZZasa* configuration, which is based on the crystal structure data of PCB in C-phycocyanin (*31*), were obtained as described elsewhere (*25*).

RESULTS

The RR spectrum of the parent state P_r is shown in Figure 3A. It is identical to that reported previously (*20*, *22*). The bands originate from the tetrapyrrole chromophore except for the sharp band at 1001 cm^{-1}, which is due to the totally symmetric stretching of the phenylalanine residues of the apoprotein. In addition, some intensity at ca. 1650 cm^{-1} may result from the amide I mode of the peptide backbone. The spectrum that is obtained after irradiation with 667-nm light at −140 °C is expected to represent a photostationary mixture of P_r and the photoproduct lumi-R (Figure 3B). The spectral changes after irradiation are relatively small, implying that only a small amount of phyA is converted to lumi-R, which, in fact, is in accord with the relatively small quantum yield for the $P_r \rightarrow$ lumi-R photoreaction (*32*). To remove the contribution of the parent state, the spectrum of P_r (recorded before irradiation; Figure 3A) was subtracted from that of the photostationary mixture (Figure 3B). For the subtraction procedure, the phenylalanine band was taken as an internal standard to determine intensity changes of the RR bands of the chromophore and eventually identify the bands of lumi-R and P_r in the spectrum of the photostationary mixture. For instance, the bands at 1587, 1306, and 826 cm^{-1} in the latter spectrum are unambiguously attributed to lumi-R as they are not present in the spectrum obtained prior to irradiation (Figure 3A). Conversely, the bands at 1641, 1624, 1321, 1292, and 799 cm^{-1} have lost intensity (with respect to the internal standard) in spectrum A compared to spectrum B and, hence, can be taken as characteristic marker bands of the parent state P_r. Since it is not known a priori if one of these latter bands accidentally coincides with a band of lumi-R, the subtraction of P_r was carried out to remove all or most of these bands. The difference spectrum which fulfills these requirements at best is shown in Figure 3C. It differs, on one hand, from that reported in our previous study (*22*), in which there was still a substantial residual contribution of the parent state in the difference spectrum due to an insufficient subtraction of P_r. On the other hand, spectrum C agrees very well with that recently reported by Andel et al. (*23*). These authors employed a different approach, i.e., shifted excitation Raman difference spectroscopy, to determine the spectrum of lumi-R from a spectrum measured under rigorous resonance conditions. The agreement between both spectra of lumi-R (as well as P_r and P_{fr}) also demonstrates that NIR excitation does not significantly affect the relative resonance enhancement of the chromophore bands.

To obtain the spectra of the remaining intermediates, which are formed during the photoconversion from P_r to P_{fr}, the samples were irradiated at 667 nm and temperatures between −100 and −20 °C. The residual contributions of P_r were subtracted from the measured spectra following the strategy described above. This procedure yielded crude spectra of two further intermediates from samples irradiated at −70 and −30 °C. On the basis of a previous low-temperature UV−vis absorption study by Eilfeld and Rüdiger (*6*), these intermediates are identified as meta-R_A and meta-R_C, respectively. After subtraction of the P_r contribution, the spectra do not exclusively represent the respective intermediates, as can be seen, for example, in the spectrum of Figure 3D, obtained from a sample irradiated at −70 °C. This spectrum still reveals characteristic bands of the preceding intermediate lumi-R (Figure 3C) as indicated by the connecting dotted lines. Thus, the determination of the spectrum of meta-R_A required the additional subtraction of the lumi-R residuals. This subtraction was monitored by the disappearance of the lumi-R bands at 1587, 1567, and 826 cm^{-1}. In the same way, the spectrum of meta-R_C was obtained from the spectrum measured after irradiation at −30 °C and subtraction of the residual contribution of meta-R_A.

RR spectra were also measured from samples irradiated at different temperatures between −70 and −20 °C. The results reveal no evidence for another intermediate that might be formed in this temperature range either in a thermal decay process or in a secondary photoreaction as suggested by Eilfeld and Rüdiger (*6*). However, it should be noted that species with spectral contributions substantially smaller than that of meta-R_A (or meta-R_C) or with similar RR spectra would be beyond the detection limit.

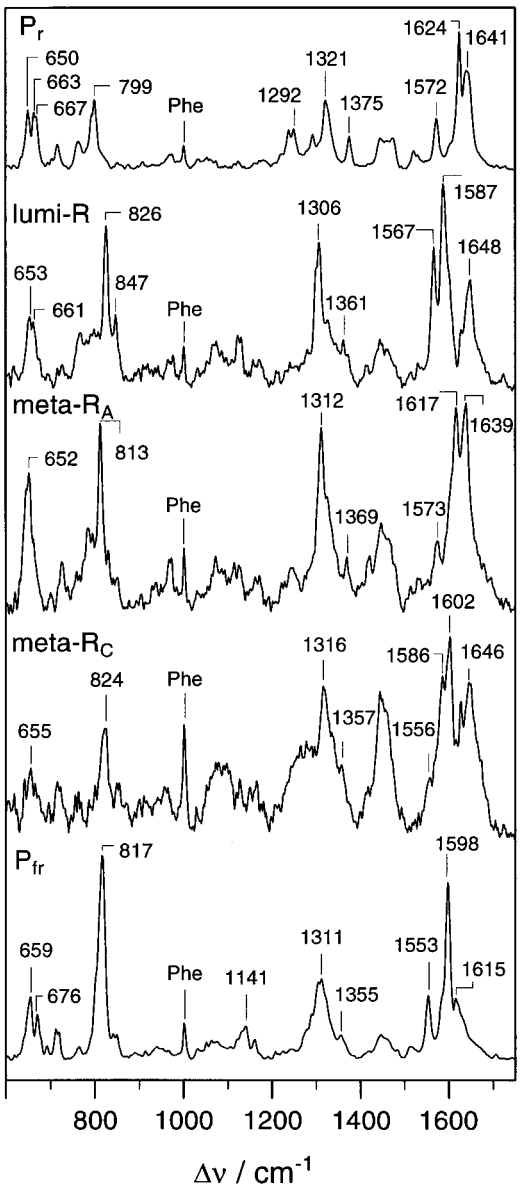

FIGURE 4: RR spectra of the parent and intermediate states of phytochrome measured at −140 °C (H_2O).

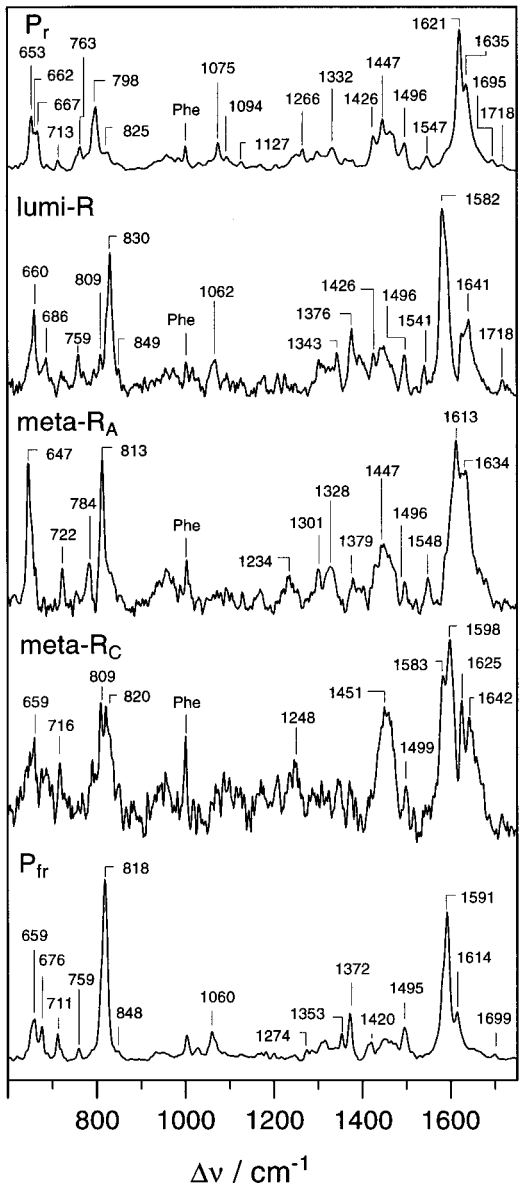

FIGURE 5: RR spectra of the parent and intermediate states of phytochrome measured at −140 °C (D_2O).

RR experiments with samples in deuterated buffer solutions throughout the same temperature range were analyzed as described above. The complete spectra of the parent states and intermediates of the nondeuterated and deuterated species are shown in Figures 4 and 5. Due to the relatively weak oscillator strength of the first electronic transition (*6*), the RR spectra of meta-R_A and meta-R_C could not be obtained in previous studies. In fact, the relative contributions of meta-R_A and meta-R_C to the measured spectra are very small and only the substantial improvement in quality of the raw spectra and the development of a reliable subtraction procedure have now allowed the determination of the "pure" spectra of these species. Note that the intrinsically low resonance enhancement for these "bleached" intermediates, particularly for meta-R_C, is also reflected by the appearance of further (nonresonance) Raman bands of the protein matrix in addition to the phenylalanine band at 1001 cm^{-1}. In the spectrum of meta-R_C the broad humps below the 1316- and 1646-cm^{-1} bands can be attributed to the amide III and amide I modes, respectively, and the peak at ca. 1460 cm^{-1} most likely includes substantial contributions from CH_2 deformation modes of amino acid side chains.

DISCUSSION

Chromophore Structure in the Parent State P_r. There is strong evidence, albeit no direct proof, that in P_r the chromophore is protonated and adopts an extended structure, which has been suggested to be a *ZZZasa* or a *ZEZsas* configuration (*4*, *7*−*9*, *23*, *33*). RR spectroscopy provides the key to distinguish between both geometries. However, a reliable method for the vibrational analysis of open-chain tetrapyrroles is required, which we tried to develop based on quantum chemical force-field calculations (*25*, *26*). This approach was recently applied to the PCB chromophore of C-phycocyanin, which exhibits a protonated *ZZZasa* structure ($PCBH^+$), as also suggested for P_r (*6*). A good agreement was achieved between the calculated Raman spectra and the experimental RR spectra of the α-subunit of C-phycocyanin (α-CPC) of *Mastigocladus laminosus*.[3] Based on the comparison with the calculated frequencies, intensities, and H/D

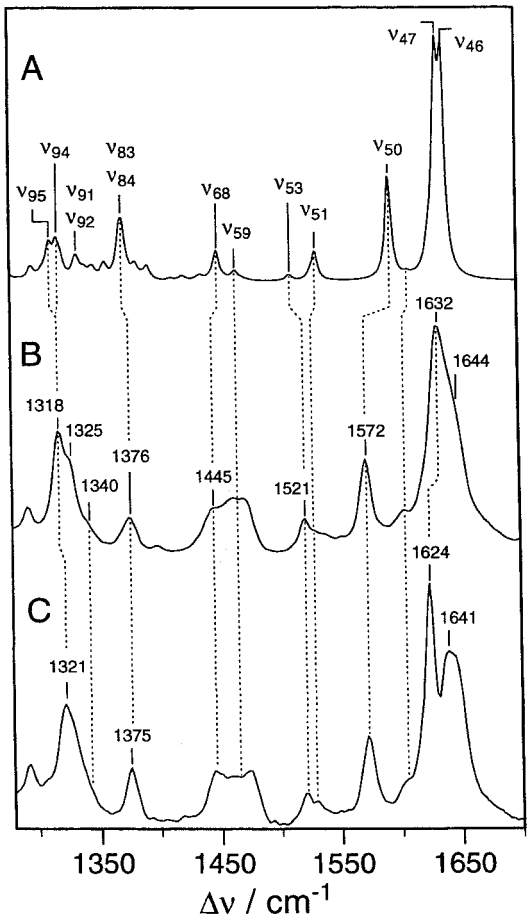

FIGURE 6: Comparison of the calculated Raman spectrum of (A) $PCBH^+$ and the experimental RR spectra of the P_r states of (B) phyA-PCB (*24*) and (C) native phytochrome A, measured in H_2O.

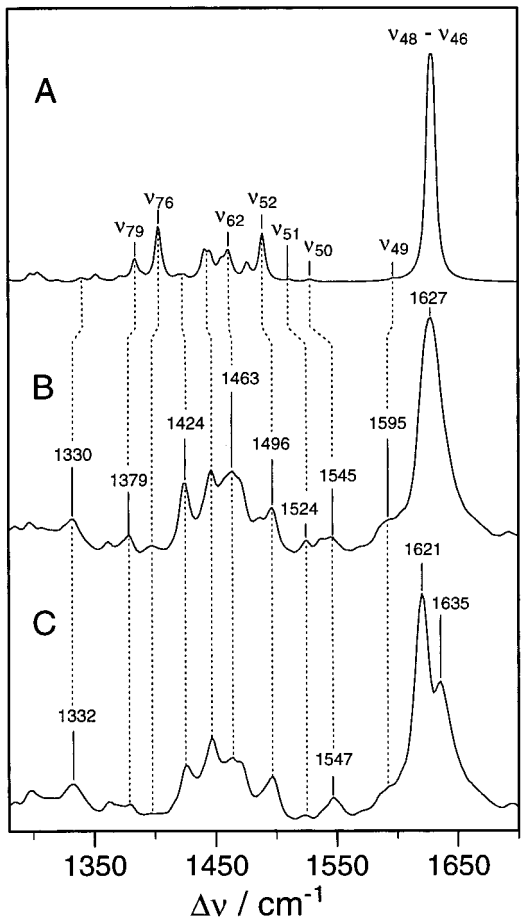

FIGURE 7: Comparison of the calculated Raman spectrum of (A) $PCBD^+$ and the experimental RR spectra of the P_r states of (B) phyA-PCB (*37*, *36*) and (C) native phytochrome A, measured in D_2O.

isotopic effects, a plausible and consistent assignment of the observed bands between 1000 and 1700 cm^{-1} was possible, demonstrating the reliability of this approach. Only in the region between 800 and 900 cm^{-1}, which includes the C−H out-of-plane modes, do the calculated spectra not reproduce the experimental spectra in a satisfactory manner. Presumably, this region reflects most sensitively protein−chromophore interactions that may lead to torsions of the methine bridges. Such interactions cannot be taken into account by the calculations so that at present the analysis of the RR spectra of protein-bound open-chain tetrapyrroles has to be restricted to the region above 900 cm^{-1}.

Figures 6 and 7 display the calculated Raman spectra of the protonated and deuterated cationic PCB (i.e., $PCBH^+$, $PCBD^+$) and the experimental RR spectra not only of P_r from native phytochrome but also from recombinant phytochrome A assembled with the PCB chromophore (phyA-PCB) (*24*). Thus, the spectra of the latter species can be compared directly with the calculated spectra of $PCBH^+$ and $PCBD^+$. Indeed, there are striking similarities in the range between 1000 and 1700 cm^{-1}, and the agreement with the calculated spectra is as good as for α-CPC (*25*).[3] Consequently, the observed RR bands of phyA-PCB can be attributed to individual normal modes in analogy to α-CPC (Table 1). These assignments can readily be extended to the RR spectrum of P_r of phyA-PΦB (the adduct including the natural PΦB chromophore) and native phyA since, despite the different substituents at ring *D* (vinyl vs ethyl), these spectra differ only in a few details from those of phyA-PCB, indicating that the structures of both chromophores are the same in P_r (*24*).

At present, the good agreement with the spectra calculated for the *ZZZasa* form does not definitely rule out other configurations such as the *ZEZsas* configuration inasmuch as vibrational spectra have not yet been calculated on the same level for both geometries. However, semiempirical (QCFF) calculations for (truncated) protonated tetrapyrroles that differ by the conformation at the *CD* methine bridge (anti vs syn) predicted substantial differences for the RR spectra (*18*, *23*), particularly in those regions (1500−1700 and 1280−1350 cm^{-1}) where the present (DFT-) calculated spectra for the *ZZZasa* geometry nicely reproduce the RR spectra of P_r. Furthermore, in that region where the calculations fail to predict frequencies and intensities adequately (800−900 cm^{-1}),[4] the experimental RR spectra of P_r

[3] As shown by comparison with the experimental spectra, calculated Raman intensities provide a good approximation for the observed RR intensities obtained under preresonance conditions, at least in the region between 1000 and 1700 cm^{-1} (*25*).

[4] Since the band assignment in this region is yet ambiguous, proposed assignments for the C−H out-of-plane modes cannot be confirmed at present. Consequently, the previously suggested model (*22*) in which RR intensities of these modes are related with torsional angles of the methine bridges has to be regarded as speculative.

Table 1: Vibrational Assignments of Selected Modes for the Various States of Phytochrome[a]

	phycocyanobilin		phytochromobilin				
mode	*ZZZasa*, calcd[b]	P_r(phyA-PCB)[c]	P_r	lumi-R	meta-R_A	meta-R_C	P_{fr}
ν_{46}, [C=C str; *AB*]	1640 (−9), vs	1645 (−13), s	1641 (−6), s	1648 (−7), s	1639 (−5), s	1627 (−2), m	1615 (−1), m
ν_{47}, [C=C str; *CD*]	1635 (−6), vs	1632 (−5), vs	1624 (−3), vs	1587 (−5), vs	1617 (−4), vs	1602 (−4), vs	1598 (−7), vs
ν_{48}, [C=C str; *D*]	1628 (−1), w			1628 (−3), w			
ν_{49}, [C−C str; *BC*]	1610 (−12), vw	1605 (−13), w	1607 (−13), w	1600 (−10), w		1586 (−3), s	1583 (−1), w
ν_{50}, [N−H ip; *B, C*][d]	1595, s	1572, s	1572, s	1567, s	1573, m	1556, w	1553, s
ν_{51}, [N−H ip; *B, C*][d]	1534, m	1521, w	1521, w	1528, w			1513, w
ν_{95} [N−H ip; *D*][d]	1313, m	1318, s	1321, s	1306, s	1312, vs	1316, s	1311, s

[a] Frequencies are given in reciprocal centimeters; values in parentheses are the frequency shifts due to H/D exchange. [b] Calculated normal-mode frequencies for the protonated *ZZZasa* configuration; the main character is given in brackets. Qualitative measures for Raman intensities are given by the abbreviations vs (very strong), s (strong), m (medium), w (weak), and vw (very weak). For a quantitative description of the calculated Raman intensities as well as further details of the calculations, see Kneip et al. (*25*). [c] Data from refs *24*, *36*, and *37*. [d] For the N−H ip modes the H/D shifts cannot be quantified as the normal mode composition is not comparable for the deuterated species. The only mode involving an N−D ip coordinate (rings B and C) to a significant extent is calculated at 1073 cm^{-1} (ν_{121}) and predicted to be of medium Raman intensity.

resemble those of the *ZZZasa* chromophore of α-CPC (see Supporting Information). Thus, we conclude that this configuration is the most probable chromophore structure in P_r.

Spectral Markers for the Protonation State of the Chromophore. Many normal modes were found to be largely localized in individual rings and adjacent methine bridges (*25*, *26*). Thus, these modes may constitute spectral markers for structural changes in specific parts of the tetrapyrrole. This is particularly true for the mode ν_{50}, which is a characteristic marker for the protonation state of the tetrapyrrole, i.e., it is missing in the nonprotonated neutral forms (*25*, *26*). This mode, which in $P_r(H_2O)$ is assigned to the 1572-cm^{-1} band, is nearly a pure N−H in-plane bending (ip) of the rings *B* and *C*, so that it may sensitively reflect hydrogen-bonding interactions of the inner pyrrole rings with the protein environment. Such interactions are evidently different in the various tetrapyrrole proteins so that the frequency variation of this mode between 1590 and 1567 cm^{-1} in α-CPC and in the P_r states of phyA and the phytochrome-like protein Cph1 from *Synechocystis* can readily be understood (*24*, *34*, *35*). The corresponding N−D ip mode ν_{121} at 1073 cm^{-1}, which is predicted to be the only mode of substantial Raman intensity in the range between 1000 and 1100 cm^{-1}, can be assigned to the prominent band at 1075 cm^{-1} (Figure 5). Thus, it also appears to be an appropriate marker for hydrogen-bonding interactions of protonated tetrapyrroles. Further modes involving N−H ip coordinates are ν_{51} (rings *B* and *C*), which can be assigned to the weak 1521-cm^{-1} band, ν_{91} (ring *A*), ν_{92}, and ν_{95} (ring *D*) (Figure 6). The latter mode and an adjacent methylene wagging mode (ν_{94}) give rise to the strong and asymmetric peaks at 1318 and 1321 cm^{-1} in the experimental RR spectra of phyA-PCB and phyA-PΦB, respectively (Table 1).

Some of these modes can also be identified in the RR spectra of P_{fr} (Figures 4 and 5). The relatively strong band at 1553 cm^{-1} is assigned to the mode ν_{50}, i.e., the N−H ip of rings *B* and *C*, since it vanishes in the spectrum of the deuterated sample, which in turn displays a band at 1060 cm^{-1}, i.e., close to the expected frequency for the corresponding N−D ip. Correspondingly, the second N−H ip of the rings *B* and *C* (ν_{51}) is attributed to the weak 1513-band of $P_{fr}(H_2O)$, whereas the relatively strong band at 1311 cm^{-1} may result from the N−H ip of ring *D* (ν_{95}) and the adjacent mode ν_{94}. Thus, there is no doubt that P_{fr} also exhibits a protonated tetrapyrrole. Hence, a previous suggestion by Mizutani et al. (*21*) that the formation of P_{fr} involves a deprotonation of the tetrapyrrole can be ruled out.

Protonation State of the Photocycle Intermediates. The comparison of the RR spectra of P_r and P_{fr} indicates that, regardless of the chromophore structure in both states, the N−H ip modes appear in the same frequency range and exhibit similar RR intensities and H/D isotopic shifts. Thus, these assignment criteria can be used to identify these modes also in the intermediate states of the photocycle, thereby determining the protonation state of the chromophore. In the RR spectrum of lumi-R, the N−H ip of rings *B* and *C* (ν_{50}) is readily assigned to the 1567-cm^{-1} band since it is the only one in this region that disappears upon H/D exchange (Figures 4 and 5). This assignment is consistent with the appearance of a new band at 1062 cm^{-1} in the RR spectrum of the deuterated sample, attributable to the corresponding N−D ip mode (ν_{121}). The assignments of these modes are more difficult for the "bleached" intermediates meta-R_A and meta-R_C due to the relatively low resonance enhancement for the vibrational bands of the chromophore. As a consequence, the S/N ratio of these spectra is lowered and the relative contribution of the (nonresonance) Raman bands of the apoprotein is increased as indicated by the broad and poorly resolved humps at ca. 1630, 1440, 1300, and 1080 cm^{-1}, which overlap with the RR bands of the chromophore. Nevertheless, the identification of ν_{50} in the RR spectrum of meta-$R_A(H_2O)$ is straightforward since the distinct 1573-cm^{-1} band disappears upon H/D exchange. However, the corresponding N−D ip mode cannot be identified due to relatively high noise. This is also true for the meta-R_C intermediate. A probable candidate for ν_{50} in the RR spectrum of meta-R_C in H_2O is the weak band at 1556 cm^{-1}. Although the S/N ratio is poorer in the RR spectrum of the deuterated sample, a band of an intensity comparable to that of the 1556-cm^{-1} band should be detectable in this region, if it had not disappeared upon H/D exchange. Note that in the RR spectrum of the meta-R_C-like intermediate of phyA-(PCB), the protonation marker band ν_{50} is more clearly detectable (*36*, *37*). Additional strong support for the conclusion that this intermediate also exhibits a protonated chromophore is obtained from the far-reaching spectral similarities with P_{fr}, which include the entire frequency range. Further assignments of N−H ip modes are listed in Table 1.

It should be noted that the N−H(N−D) ip modes ν_{50} (ν_{121}) can also be identified in the RR spectra of lumi-F and meta-

F, i.e., the intermediates of the $P_{fr} \rightarrow P_r$ back transformation, implying that in all states of phytochrome detected so far the chromophores are protonated (*22*).

Spectral Marker for Structural Changes at the Methine Bridge CD. The most pronounced spectral difference in the P_r state of phyA-PCB compared to that of phyA-PΦB refers to the prominent peak at 1632 cm^{-1}, which exhibits a shoulder at 1644 cm^{-1} (Figure 6). These two bands are assigned to the modes ν_{47} and ν_{46}, respectively. Whereas the latter component remains largely unchanged in native phytochrome (1641 cm^{-1}), the 1632-cm^{-1} band shifts down by 8 cm^{-1}. These findings can readily be understood in terms of the normal mode compositions since ν_{47} predominantly includes the C═C stretching coordinates of the *CD* methine bridge and, hence, is likely to respond more sensitively to an alteration of the ring *D* substituents than ν_{46}, which includes the C═C stretching of the *AB* methine bridge. The same downshift of the mode ν_{47} is found for P_r in D_2O, in agreement with the calculations (Figure 7) (*26*).

A similar behavior is noted for the prominent 1598-cm^{-1} band of P_{fr} (Figure 4), which is the only band in that region to show a substantial upshift (5 cm^{-1}) upon replacement of PΦB by PCB (*24*). Thus, the underlying mode of this band must possess a similar composition as mode ν_{47} of P_r, i.e., the C═C stretching of the *CD* methine bridge. These findings also imply that for protonated tetrapyrroles such a mode gives rise to the strongest RR band in the C═C stretching region, largely independent of the chromophore configuration. In the RR spectra of unprotonated tetrapyrroles, the strongest band in this region originates from the C═C stretching of the *BC* methine bridge, which is predicted and found at essentially the same frequency in the dimethyl esters of PCB and PΦB (*25*, *26*).

Since lumi-R, meta-R_A, and also meta-R_C have a protonated tetrapyrrole chromophore, it is now possible to extend the assignment of the mode ν_{47} (as well as the remaining modes in the C═C stretching region) from the parent to the intermediate states, guided by the intensity pattern and the characteristic H/D shifts. In the RR spectrum of lumi-R the most likely candidate for the mode ν_{47} is the band at 1587 cm^{-1}, which is the strongest band in this region (Figure 4). The band at 1648 cm^{-1} corresponds to the 1641-cm^{-1} band of P_r attributable to C═C stretching of the *AB* methine bridge (ν_{46}). In meta-R_A, this latter mode gives rise to the 1639-cm^{-1} band so that the assignment of ν_{47} to the 1617-cm^{-1} band is straightforward even though the relative RR intensities differ somewhat with respect to P_r and lumi-R.

In meta-R_C, ν_{47} is attributed to the 1602-cm^{-1} band, whereas the shoulder at 1586 cm^{-1} most likely originates from ν_{49}, localized mainly on methine bridge *BC*. For the mode ν_{46}, there are two candidates, i.e., at 1627 and 1646 cm^{-1}, which in D_2O shift down to 1625 and 1642 cm^{-1}, respectively. In view of the low resonance enhancement and the relatively high contributions by nonresonance Raman bands of the apoprotein, we attribute the lower-frequency band to ν_{46}. The higher-frequency band most likely originates from the amide I mode of the polypeptide backbone. Further assignments of the modes in the C═C stretching region are listed in Table 1 (see also Supporting Information).

Structural Changes of the Chromophore during the $P_r \rightarrow P_{fr}$ Transformation. In the RR spectrum of lumi-R the most pronounced spectral change is due to the 37-cm^{-1} downshift of ν_{47}. This suggests that the structural changes associated with the photoreaction $P_r \rightarrow$ lumi-R involve the *CD* methine bridge. This conclusion is in line with the view that the primary photoprocess is a $Z \rightarrow E$ isomerization of the *CD* double bond. In addition, the other bands in the C═C stretching region reveal much smaller changes, suggesting that the primary photoinduced structural changes are largely confined to rings *C* and *D*. It may be that the rigid protein matrix hinders the rotation around the *CD* double bond so that the tetrapyrrole has to adopt a strained configuration in lumi-R. This interpretation is consistent with the observation that the formation of lumi-R requires thermal activation since the yield of lumi-R formation decreases when the temperature is lowered (*23*). Furthermore, the view of a hindered $Z \rightarrow E$ photoisomerization is also supported by the finding that the quantum yield for this process is smaller by a factor of 1.4 than the yield for the back photoisomerization (*37*).

As a result of the photoisomerization, ring *D* must undergo the most pronounced movement in the chromophore pocket. This is likely to alter the hydrogen-bonding interactions of the ring *D* N−H group with the protein environment, which is reflected by the significant 15-cm^{-1} downshift of the ring *D* N−H ip mode to 1306 cm^{-1}. Since the N−H ip (ν_{50}) and N−D ip (ν_{121}) modes shift down as well by 5 and 13 cm^{-1}, respectively, the hydrogen-bonding interactions of ring *C* may also be affected.

It is reasonable to assume that the subsequent transition to the intermediate meta-R_A primarily involves the relaxation of the strained chromophore structure. In fact, the mode ν_{47} is shifted from the unusually low frequency in lumi-R up to 1617 cm^{-1}. Furthermore, the frequency of mode ν_{50} is the same as in P_r (1573 cm^{-1}), suggesting a relaxation of rings *B* and *C* into a P_r-like conformation.

The most pronounced spectral changes in meta-R_C appear in the C═C stretching region, where all the bands shift down substantially. In addition, the C═C stretching mode of the methine bridge *BC* (ν_{49}) at 1586 cm^{-1} now becomes clearly detectable. Evidently, the composition of this mode is altered compared to that of the preceding states since the H/D-dependent downshift and the RR intensity are different compared to P_r and lumi-R, respectively (Table 1). However, the frequencies of the modes in this region and presumably also the normal mode composition of ν_{49} are very similar to those in P_{fr}. This is also true for the unusually low frequency of ν_{50} at 1556 cm^{-1}, which may either be attributed to a conformational change associated with the rings *B* and *C* or to an alteration of the hydrogen-bonding interaction of their N−H groups. In any case, the transition from meta-R_A to meta-R_C presumably involves a structural adjustment of the inner tetrapyrrole rings in the binding pocket and/or adjacent hydrogen-bonded amino acids. Whereas local conformational changes of the protein environment cannot be ruled out, major structural changes of the protein are not likely to occur during this step since the coexistence of meta-R_A and meta-R_C in a relatively narrow temperature range (between −70 and −30 °C) indicates that the enthalpy difference between the two species is small.

The far-reaching similarities between meta-R_C and P_{fr} suggest that the chromophore relaxation is essentially completed in meta-R_C. One may conclude, therefore, that the ultimate protein relaxation sets in only at the decay of meta-R_C to P_{fr}, inducing the changes in the UV−vis absorption spectra.

CONCLUSIONS

The present RR study provides insight into the molecular processes of the chromophore during the phototransformation from P_r to P_{fr}. The analysis of the RR spectra strongly suggests that in the parent state P_r the chromophore is in a protonated *ZZZasa* configuration and it remains protonated in all intermediate and stable states of the photocycle. In the primary photoprocess, the tetrapyrrole undergoes a *Z*→*E* isomerization of the *CD* methine bridge. The rotation around the double bond is sterically hindered, leading to a strained configuration in lumi-R so that relaxation of the *CD* double bond occurs in the subsequent decay to meta-R_A above −100 °C. The activation energy associated with the formation of meta-R_A is obviously required for a structural rearrangement in the binding pocket, allowing the sterically demanding movement of ring *D*. The final relaxation of the chromophore upon formation of meta-R_C also includes structural changes associated with the pyrrole rings *B* and *C* leading to a chromophore structure very similar to that of P_{fr}. The functionally relevant structural changes of the protein eventually occur during the formation of P_{fr}.

Although the determination of structural details of the chromophore in the various phytochrome states is not yet possible, the present study indicates that the interpretation of the RR spectra, supported by quantum chemical calculations, is a powerful tool for the structural analysis. Extending the DFT calculations to different tetrapyrrole configurations and conformations promises to contribute substantially to the elucidation of the molecular changes of the chromophore during the photoinduced reaction cycle.

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SUPPORTING INFORMATION AVAILABLE

Four figures, comparing the RR spectra of α-CPC, phyA-PCB, and native phyA in H_2O and D_2O in the frequency range from 600 to 1750 cm^{-1} and showing an expanded view of the RR spectra of the various states of native phyA in the region from 1480 to 1750 cm^{-1}. This material is available free of charge via the Internet at http://pubs.acs.org.

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