Fourier-Transform Infrared Spectroscopy of Phytochrome: Difference Spectra of the Intermediates of the Photoreactions[†]

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ABSTRACT: The photocycle of 124 kDa phytochrome A from *Avena sativa* was studied by Fourier-transform infrared spectroscopy at low temperatures. Difference spectra between the parent state P_r and the intermediates of the $P_r \rightarrow P_{fr}$ pathway, i.e. lumi-R, meta-R_a, and meta-R_c, and between P_{fr} and the intermediates of the $P_{fr} \rightarrow P_r$ pathway, lumi-F and meta-F, were obtained in ^1H_2O and ^2H_2O for the first time. Each spectrum shows characteristic spectral features which allow a clear distinction between the different intermediates. A general feature is that greater changes occur with increasing temperature, i.e. at the later steps of the photoreactions. Nevertheless, the changes in the spectral regions of the protein (amide I and amide II) were found to be surprisingly small, excluding larger conformational changes of the protein. All spectra of the intermediates are characterized by a strong negative band around 1700 cm^{-1}. This band is tentatively assigned to the C═O stretch of ring D of the chromophore. Since it is not observed in the difference spectra between the parent states, it is concluded that ring D is located in a similar molecular environment in P_r and P_{fr}. In the photoproducts lumi-R and lumi-F, this band undergoes an upshift to 1720 cm^{-1}. The high frequencies suggest that the chromophore is protonated in these intermediates as well as in P_r and P_{fr}.

Phytochromes, which are currently the most investigated photosensory pigments of higher plants, constitute a small group of homodimeric chromoproteins, each containing a linear tetrapyrrolic chromophore (Figure 1) (Pratt, 1995). It is characterized by a reversible photoreaction with maximum action in the red and far-red region of the visible spectrum between the two main absorbing forms P_r (λ_{max} = 665 nm) and P_{fr} (λ_{max} = 730 nm) (Rüdiger & Thümmler, 1991). P_r represents the physiologically inactive form which is also directly produced biosynthetically (Terry et al., 1993), while P_{fr} is the physiologically active form which triggers many responses of the plant related to light conditions (e.g. hypocotyl growth inhibition, leaf and chloroplast development) (Kendrick, 1994). The elucidation of the signal transduction pathway in plants is a major field in current phytochrome research (Millar et al., 1994; Quail et al., 1995). Recently it was suggested that activation of heterotrimeric G-proteins is initially involved in the first step of the signal transduction coupled with at least two different pathways which require calcium/calmodulin and cyclic GMP, respectively (Bowler et al., 1994; Neuhaus et al., 1993), but there is also evidence for phytochrome acting as kinases (Algarra et al., 1993; Vierstra, 1993).

The photoreaction has been intensively investigated by optical time-resolved absorption (Büchler et al., 1995; Inoue et al., 1990; Kandori et al., 1992; Lippitsch et al., 1993; Scurlock et al., 1993a,b; Zhang et al., 1992), fluorescence (Sineshchekov & Akhobadze, 1992; Sineshchekov et al., 1995; Wells et al., 1994), and circular dichroism studies (Björling et al., 1992; Chen et al., 1993). However, details of molecular changes of the chromophore and the protein during the phototransformation process are still missing. NMR experiments on phytochrome fragments strongly suggest that during the photoreaction the chromophore undergoes a *Z,E* isomerization around the C_{15}═C_{16} double bond accompanied by rotation around the C_{14}−C_{15} single bond (Rüdiger et al., 1983; Thümmler & Rüdiger, 1983). These primary light reactions are followed by alterations of the conformation of the protein and the chromophore in the dark (Eilfeld et al., 1989; Singh et al., 1988). The comparison of the shape of the absorption spectrum with those of phycobiliproteins of known crystal structures suggests that,

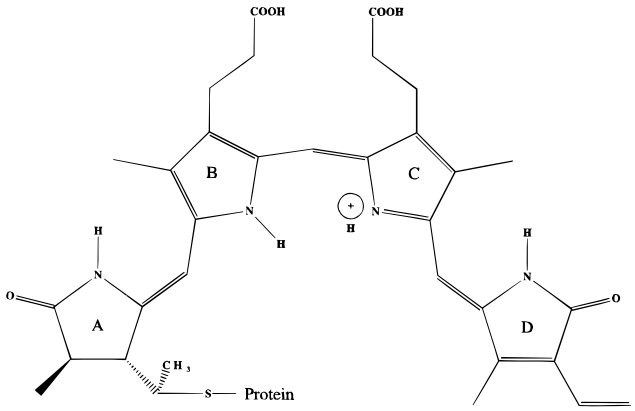

FIGURE 1: Chromophore of phytochrome in the P_r form in an extended and protonated conformation (Fodor et al., 1988; Schaffner et al., 1990).

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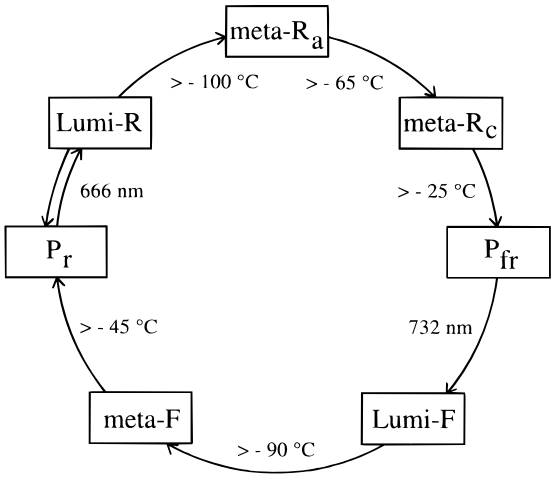

FIGURE 2: Scheme of the intermediates of full length phytochrome found at low temperatures [after Eilfeld and Rüdiger (1985)].

as in these systems, the chromophore adopts extended conformations in the stable states P_r and P_{fr} (Dürring et al., 1990; Scheer et al., 1982). Resonance Raman experiments suggest that in the P_r form the chromophore is protonated at the nitrogen of ring C (Fodor et al., 1988; Matysik et al., 1995; Mizutani et al., 1994). Possibilities of proton transfer during the photoreaction have been controversially discussed (Hildebrandt et al., 1992; Matysik et al., 1995; Mizutani et al., 1991, 1994; Tokutomi et al., 1990). From CD experiments in the UV spectral range, it has been suggested that the protein changes its conformation during the photoreaction by an increase in the α-helical content (Chai et al., 1987; Vierstra et al., 1987), a reaction which may be related to the N-terminal 6 kDa domain (Farrens et al., 1992). The photoreactions of both pathways, i.e. $P_r \rightarrow P_{fr}$ and $P_{fr} \rightarrow P_r$, proceed *via* several intermediates (Eilfeld et al., 1987; Inoue et al., 1990; Scurlock et al., 1993a; Spruit & Kendrick, 1977). For full length phytochrome, three intermediates have been found at low temperatures in the $P_r \rightarrow P_{fr}$ pathway, and another two have been identified in the $P_{fr} \rightarrow P_r$ pathway (Figure 2). They are characterized by their absorbance spectra which were deduced from the difference spectra of the intermediates and the related main absorbing forms P_r and P_{fr}, respectively (Eilfeld & Rüdiger, 1985). However, the structures of the intermediates and details of the kinetic scheme are still unclear.

Fourier-transform infrared (FT-IR) difference spectroscopy has been shown to be a useful tool to study molecular processes during photoreactions of retinoic receptors (e.g. bacteriorhodopsin and rhodopsin) (Rothschild, 1991; Siebert, 1993), because it provides information on changes in both the chromophore and the protein. Due to its large size (≈126 kDa), comparable studies on phytochrome are more complicated and therefore provide a considerable challenge. Earlier studies by Sakai et al. (1990) and our group (Siebert et al., 1990) showed difference spectra between the two stable forms, P_r and P_{fr}, which are in good agreement with each other. Chromophore-derived bands were tentatively assigned by comparison with model compounds (Siebert et al., 1990). The changes in the regions of protein bands were surprisingly small and disagreed with any larger structural changes.

Resonance Raman and FT-Raman spectroscopy, with their spectra mainly representing chromophore bands, have been successfully applied to phytochrome (Fodor et al., 1988, 1990; Hildebrandt et al., 1992; Mizutani et al., 1991) and also to free bile pigments serving as model compounds for vibrational analysis (Margulies & Toporowicz, 1984, 1988; Smit et al., 1993). Nevertheless, it is still difficult to assign the numerous bands in the Raman spectra to specific molecular vibrations of the chromophore.

For an understanding of the molecular events occurring during the photoreactions, a characterization of the intermediates of both pathways is an important prerequisite. Therefore, in this investigation, we present the difference spectra between such intermediates, prepared and stabilized at low temperatures, and the respective initial states. It is observed that, as a general feature, band intensities in the amide I spectral range are lower for the earlier intermediates than for the later ones. In addition, a peculiar band is observed for the early intermediates that is preliminarily assigned to the C═O stretching mode of tetrapyrrole ring D of the chromophore. Thus, although a detailed band analysis cannot yet be made, the spectra and their deuteration (^2H_2O) shifts provide important determinants for the molecular changes occurring during the photoreactions of phytochrome.

MATERIALS AND METHODS

Sample Preparation. Phytochrome A was obtained from etiolated seedlings of *Avena sativa* as previously described (Grimm & Rüdiger, 1986) and was dissolved in a 5 mM phosphate buffer (pH 7.8) containing 2 mM 2-mercaptoethanol and 0.3 vol. % glycerol.

For the infrared measurements, the solution was deposited on a CaF_2 infrared window and the water was evaporated under a gentle stream of nitrogen. The obtained homogeneous film was rehydrated by adding either 1−2 μL of ^1H_2O or ^2H_2O (after six times of ^2H_2O exchange for the latter) into the infrared cuvette which was sealed by another CaF_2 window. 2-Mercaptoethanol decreased aggregation, and glycerol retained photoreversibility after rehydration of the film. The amount of phytochrome of an infrared probe was about 80 and 120 μg for measurements in ^1H_2O and ^2H_2O, respectively.

FT-IR Measurements. The spectra were measured using a Bruker IFS 28 Fourier-transform infrared spectrophotometer equipped with an MCT detector. For the cryogenic experiments, a home-built cryostat was used which allows measurements in the temperature range between −190 and 0 °C. Temperature regulation was within ±0.5 °C. Before insertion into the cryostat, the samples were converted either into the P_r state for measurements of the intermediates of the $P_r \rightarrow P_{fr}$ pathway or into the P_{fr} state for measurements of the reverse reaction. All infrared samples were controlled with respect to any distortions of the photoreaction-induced absorption changes and discarded if deviations had been observed. The degree of rehydration of the protein film was controlled using the broad absorption band of water around 3300 cm^{-1}. It was verified that the sealing CaF_2 window prevented dehydration of the film in the cryostat.

For the conversions from the P_r state, a band-pass filter with peak transmission at 633 nm and a half-width of 9 nm was used for irradiation and a long-pass filter (Schott, RG 715) for conversions from the P_{fr} state. The intermediates were accumulated by irradiation of P_r or P_{fr} at the temperatures given in the figure captions. They were chosen according to Eilfeld and Rüdiger (1985). In contrast to the

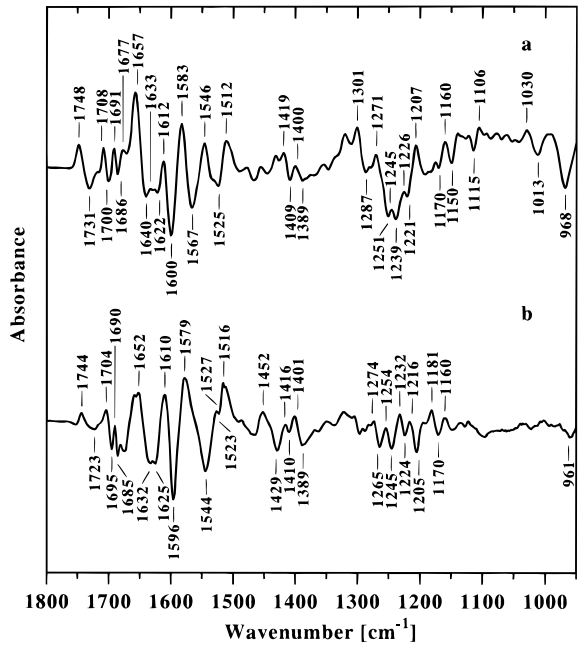

FIGURE 3: FT-IR difference spectra of the two main absorbing forms P_{fr} and P_r. Positive bands represent P_{fr} and negative bands P_r: (a) spectrum in a medium of ^1H_2O ($T = 0$ °C) and (b) spectrum in a medium of ^2H_2O ($T = 0$ °C). The band intensities can be estimated from the difference band around 1590 cm^{-1} in part a which is about 0.0008 absorbance unit. In order to correct in the other spectra the intensities for different photoconversions, the spectra were normalized to the bands around 1400 cm^{-1}.

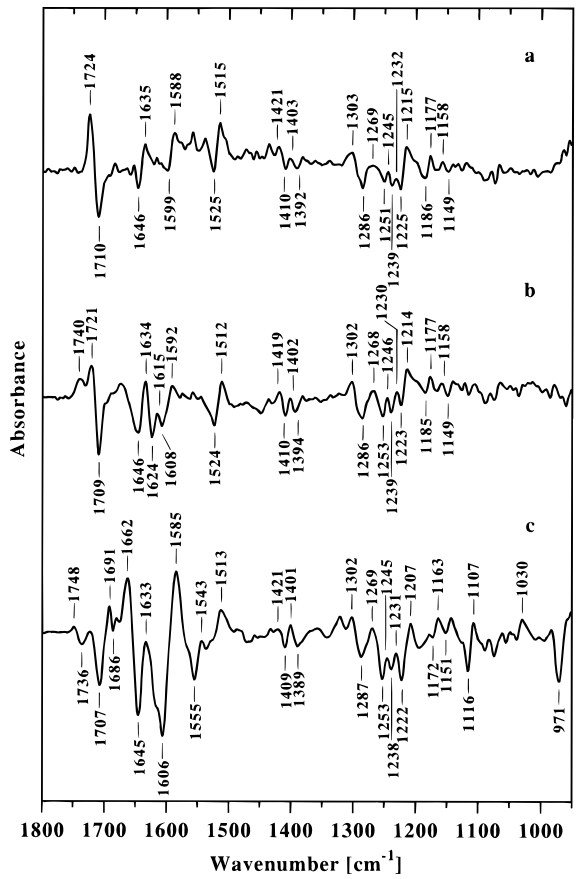

FIGURE 4: FT-IR difference spectra of the intermediates of the $P_r \rightarrow P_{fr}$ pathway in ^1H_2O. Positive bands represent the respective intermediate and negative bands P_r: (a) lumi-R ($T = -140$ °C), (b) meta-R_a ($T = -80$ °C), and (c) meta-R_c ($T = -27$ °C). The band intensities can be estimated from the difference band around 1715 cm^{-1} in part a which is about 0.0002 absorbance unit. In order to correct in the other spectra the intensities for different photoconversions, the spectra were normalized to the bands around 1400 cm^{-1}.

intermediates of the $P_r \rightarrow P_{fr}$ pathway for which photoreversibility could be used for all intermediates, for the intermediates of the reverse pathway, lumi-F and meta-F, the phototransformation back to P_{fr} had to be realized at ambient temperatures. Since the maximum photoconversion of P_r into P_{fr} is only about 85% (Lagarias et al., 1987), the illumination of the sample with 633 nm light, inducing the back-reaction from lumi-F or meta-F, at low temperatures would simultaneously cause the formation of photoproducts out of remaining P_r (Eilfeld & Rüdiger, 1985). Irradiation time was 4 min for the generation of all intermediates which was sufficient to saturate the difference signals. A slide projector (150 W lamp) and fiber optics in the spectrometer were used.

Parallel to the infrared studies, difference spectra of all intermediates were recorded in the UV−vis region. As our results were in good agreement with published spectra shown in Eilfeld and Rüdiger (1985), we can assume that the photoconversion of the protein is not seriously influenced by the state of the sample necessary for FT-IR difference spectroscopy (data not shown).

Each FT-IR difference spectrum shown represents a sum of several (approximately 10−30) difference spectra which were obtained from single-beam spectra recorded before and after illumination. For each single-beam spectrum, 256 scans were accumulated with a resolution of 4 cm^{-1}. The noise level can be deduced from the baseline above 1750 cm^{-1}, where no bands could be identified.

RESULTS AND DISCUSSION

Figure 3 shows the $P_r \rightarrow P_{fr}$ difference spectra measured at 0 °C for samples dissolved in ^1H_2O (Figure 3a) and in ^2H_2O (Figure 3b). The positive and negative bands are due to P_{fr} and P_r, respectively. The spectra are in very good agreement with those presented earlier (Siebert et al., 1990) and are shown here again for comparison with the spectra of the intermediates.

The difference spectra for the intermediates of the $P_r \rightarrow P_{fr}$ pathway were obtained by irradiation of P_r at −140 °C (lumi-R), −80 °C (meta-R_a), and −27 °C (meta-R_c). The spectra were taken in ^1H_2O (Figure 4) and ^2H_2O (Figure 5). In each case, the positive bands are due to the respective intermediate, while the negative ones are due to the initial state, P_r. The corresponding spectra for the intermediates of the $P_{fr} \rightarrow P_r$ pathway, which were obtained by irradiation of P_{fr} at −140 °C (lumi-F) and −70 °C (meta-F), are presented in Figure 6 (^1H_2O) and Figure 7 (^2H_2O). Here, the negative bands are due to the P_{fr} state.

It is important to note that the spectra for the different intermediates show clear qualitative differences, corroborating the identification of intermediates obtained by the analysis of low-temperature UV−vis measurements (Eilfeld & Rüdiger, 1985). Since the vibrational analysis of the difference spectra of phytochrome is still at an early stage, only a few bands or a group of bands will be emphasized.

For the quantitative comparison of the difference spectra, it would be helpful to identify a spectral feature that characterizes all spectra pertinent to one direction of the

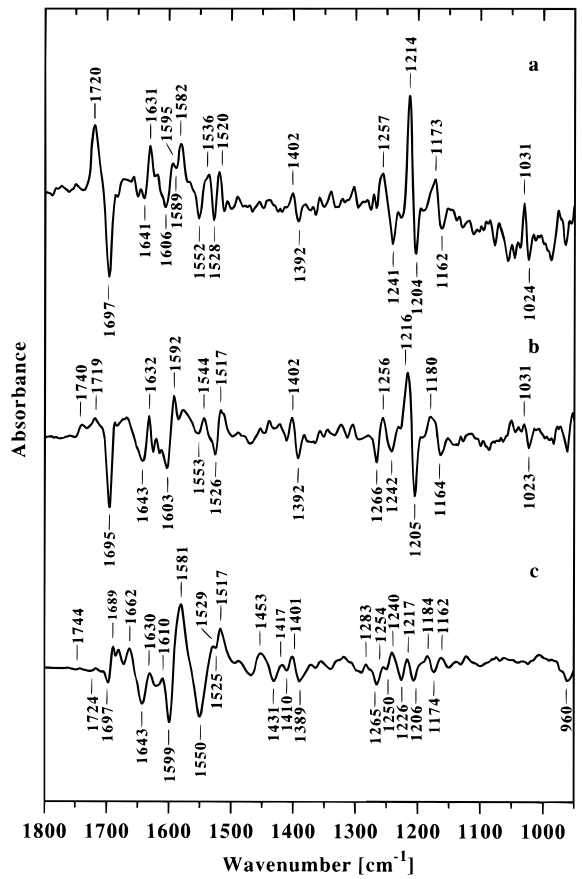

FIGURE 5: FT-IR difference spectra of the intermediates of the $P_r \rightarrow P_{fr}$ pathway in ^2H_2O. Positive bands represent the respective intermediate and negative bands P_r: (a) lumi-R ($T = -140$ °C), (b) meta-R_a ($T = -80$ °C), and (c) meta-R_c ($T = -27$ °C). The band intensities can be estimated from the difference band around 1710 cm^{-1} in part a which is about 0.0003 absorbance unit. In order to correct in the other spectra the intensities for different photoconversions, the spectra were normalized to the bands around 1400 cm^{-1}.

photoreaction. For the $P_r \rightarrow P_{fr}$ pathway, it appears that for measurements in ^1H_2O the spectral features around 1400 cm^{-1} and the difference band at 1286 cm^{-1} (negative) and 1302 cm^{-1} (positive) serve such a purpose. For measurements in ^2H_2O, the 1402(+)/1392(−) cm^{-1} difference band can be used. For the $P_{fr} \rightarrow P_r$ pathway, the spectral features around 1400 cm^{-1} are suitable. Therefore, the spectra have been approximately normalized to those spectral characteristics.

A general observation can be made for the difference spectra of both pathways. Bands often tend to become broader at higher temperatures, e.g. the band around 1590 cm^{-1} in the spectra of the intermediates of the $P_r \rightarrow P_{fr}$ pathway (Figure 4). Whether this is due to an increasing heterogeneity or due to an overlap with additional bands appearing at higher temperatures can presently not been decided.

$P_r \rightarrow P_{fr}$ Pathway. A general feature of the spectra concerns the relative intensities of the bands in the different spectral regions. In the spectral range between 1680 and 1500 cm^{-1}, the bands for the intermediates lumi-R and meta-R_a are relatively small but increase in meta-R_c and P_{fr}. The opposite is true for the region between 1690 and 1750 cm^{-1} where the difference spectra of the early intermediates are characterized by large bands (Figure 4a,b). In the range

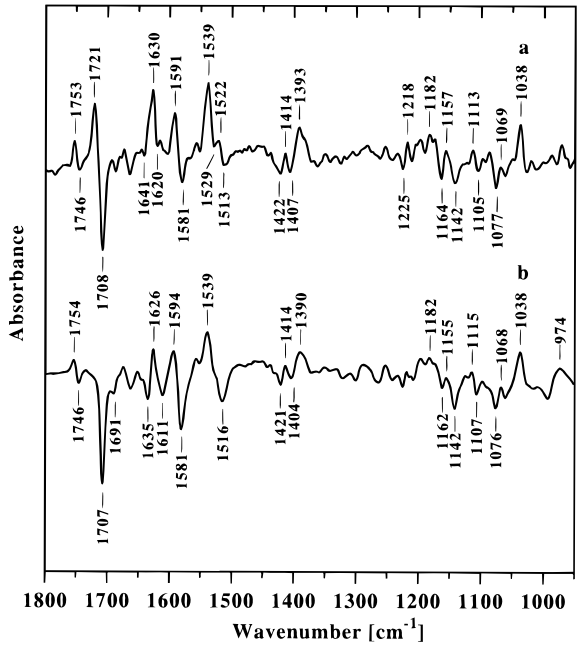

FIGURE 6: FT-IR difference spectra of the intermediates of the $P_{fr} \rightarrow P_r$ pathway in ^1H_2O. Positive bands represent the respective intermediate and negative bands P_{fr}: (a) lumi-F ($T = -140$ °C) and (b) meta-F ($T = -70$ °C). The band intensities can be estimated from the difference band around 1710 cm^{-1} in part a which is about 0.0005 absorbance unit. In order to correct in the other spectra the intensities for different photoconversions, the spectra were normalized to the bands around 1400 cm^{-1}.

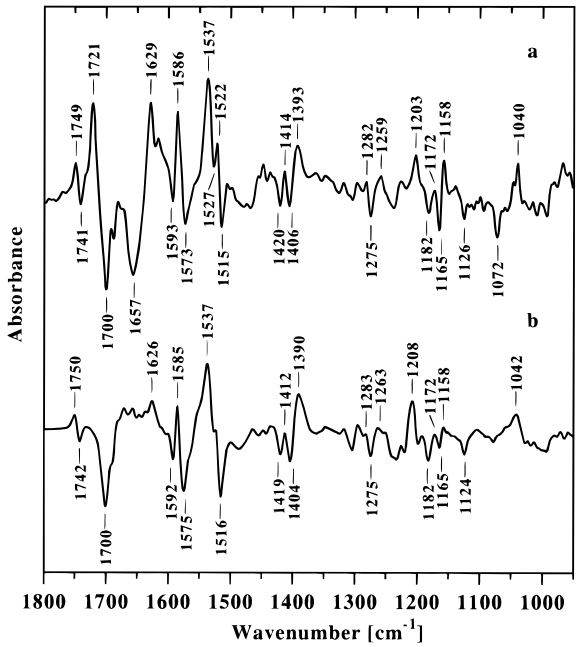

FIGURE 7: FT-IR difference spectra of the intermediates of the $P_{fr} \rightarrow P_r$ pathway in ^2H_2O. Positive bands represent the respective intermediate and negative bands P_{fr}: (a) lumi-F ($T = -140$ °C) and (b) meta-F ($T = -70$ °C). The band intensities can be estimated from the difference band around 1710 cm^{-1} in part a which is about 0.0004 absorbance unit. In order to correct in the other spectra the intensities for different photoconversions, the spectra were normalized to the bands around 1400 cm^{-1}.

below 1500 cm^{-1}, the overall changes remain fairly similar in all three intermediates. However, for samples dissolved in ^2H_2O, there are certain bands around 1250, 1210, and 1170 cm^{-1} which show significant changes in intensities between the early intermediates lumi-R and meta-R_a and the later

intermediate meta-R_c (Figure 5). Additionally, it is noteworthy that the strong bands in lumi-R have generally higher relative intensities than in meta-R_a.

From our previous investigation of model compounds, bands between 1610 and 1650 cm^{-1} have been assigned to the C═C stretching mode of the methine groups of the chromophore (Siebert et al., 1990), for both unprotonated and singly protonated states. Changes of amide I bands are also expected in this spectral range. It is not yet possible to provide an unequivocal assignment, but a qualitative and still tentative interpretation can be presented. Earlier investigations on dehydrated phytochrome in the presence of high glycerol concentrations (Balangé, 1974), experiments in a nonaqueous medium (freeze-dried tissues in vivo) (Spruit et al., 1975), and laser-flash photolysis of the later steps of P_{fr} formation (Eilfeld et al., 1989) suggest that the protein undergoes larger conformational changes during the transitions to the later intermediates, including P_{fr}. Such structural changes should be reflected by corresponding changes of amide I bands. Therefore, we expect that the spectral features between 1620 and 1660 cm^{-1} in the meta-R_c and P_{fr} difference spectra have considerable contributions from amide I bands. However, since it is suggested that the isomerization of the C_{15}═C_{16} methine bridge already takes place with the formation of lumi-R (Schaffner et al., 1990), corresponding bands in the spectra of the early intermediates lumi-R and meta-R_a may well be mainly caused by the chromophore. Corresponding observations have been made for the infrared difference spectra of the visual pigment rhodopsin (Siebert, 1993). Similarly, as in this case, in order to discriminate between chromophore and protein bands, it is mandatory to perform measurements on modified phytochrome containing isotopically labeled chromophores. Such experiments are in progress.

In the later spectra of meta-R_c, there are strong bands around 1606 cm^{-1} (negative) and 1585 cm^{-1} (positive) in ^1H_2O and around 1599(−)/1581(+) cm^{-1} in ^2H_2O (Figures 4c and 5c). These positions are outside the typical spectral range of amide I or amide II bands. Therefore, these bands are obviously caused by the chromophore, since possible contributions from amino acid side chains are expected to be much smaller. The appearance of such a strong band in meta-R_c indicates that the chromophore still undergoes large changes in the later steps of the $P_r \rightarrow P_{fr}$ pathway.

There are several observations which suggest that the chromophore is protonated in P_r and lumi-R (Fodor et al., 1988; Lagarias & Rapoport, 1980; Mizutani et al., 1994; Schaffner et al., 1990). Our studies of model compounds have shown that the strongest band in the infrared spectrum of an unprotonated dihydrobilindion around 1690 cm^{-1} is the C═O stretching mode of the carbonyl group at ring D, which is part of the conjugated system (Siebert et al., 1990). In the spectra of the protonated pigment, we were not able to identify this band, since it was now probably located under the strong C═O stretching band of the solvent used for protonation (deuterochloroform containing 1% trifluoroacetic acid). This is supported by semiempirical force-field calculations which indicate that this frequency is upshifted by protonation (Margulies & Toporowicz, 1984). Thus, this indirect evidence makes it very likely that the negative band around 1710 cm^{-1} and the positive band around 1720 cm^{-1} in the lumi-R and meta-R_a spectra (^1H_2O) are due to this group. In meta-R_c, the upshifted band around 1720 cm^{-1} disappears, and instead, a new band is observed at 1691 cm^{-1} (Figure 4c). In the $P_r \rightarrow P_{fr}$ difference spectrum (Figure 3a), there are only small bands in this region. If it can be confirmed that protonation of the chromophore induces an upshift of the C═O stretching mode and that the chromophore is protonated in P_r, the absence of great changes in the $P_r \rightarrow P_{fr}$ difference spectrum at 1710 cm^{-1} would suggest that the chromophore is also protonated in P_{fr}.

As expected from model compound studies, these bands are shifted down by deuteration of the exchangeable hydrogens, bound to the nitrogens of the lactam rings. The spectra demonstrate that the downshift of the positive band is only 4 (lumi-R) or 2 cm^{-1} (meta-R_a and meta-R_c), whereas that of the negative one, due to P_r, amounts to approximately 13 cm^{-1} (Figure 5). This is explained by the large downshift of the NH bending mode induced by $^1H/^2H$ exchange which removes the coupling of the C═O stretching vibration with the neighboring NH bending vibration. The extent of coupling will largely be determined by the frequency of the NH bending mode; the closer it is to the C═O stretching frequency, the larger will be the coupling. Because of the small downshift, the coupling must be smaller in the intermediate states than in P_r, showing a large isotope effect. Therefore, the upshift from 1710 to 1724 cm^{-1} or 1721 cm^{-1} and the downshift from 1707 to 1691 cm^{-1} induced by the formation of the intermediates in ^1H_2O must have other causes than increased coupling (Figure 4). Changes in hydrogen bonding of the carbonyl to groups of the protein or changes of the electronic structure of the conjugated system caused by electrostatic interactions or geometrical changes could explain this upshift. Our studies of model compounds have indicated that the intensity is strongly influenced by the degree of conjugation which could be modified by twists of the methine bridge. If these qualitative considerations are correct, one could infer that the methine bridge connecting rings C and D is strongly twisted in lumi-R and meta-R_a. We are aware that this interpretation is in conflict with recent resonance Raman investigations which suggest a strong twist of the C−D−methine bridge in P_{fr} only (Fodor et al., 1990; Matysik et al., 1995). In the $P_r \rightarrow P_{fr}$ difference spectrum, both the negative and the positive bands cannot be unequivocally identified, for measurements either in ^1H_2O or in ^2H_2O (Figure 3a,b). This indicates that the molecular alterations modifying the C═O stretch in the intermediates are reversed in P_{fr}.

In the spectrum of meta-R_a, there is a positive band at 1740 cm^{-1}, which is also observed, although with reduced intensity, in the spectrum measured in ^2H_2O (Figures 4b and 5b). If the formation of meta-R_c is followed at −65 °C according to the scheme of Figure 2, the positive band around 1720 cm^{-1} disappears as expected while a band with decreasing intensity around 1740 cm^{-1} is still observed as long as the temperature is below −50 °C. In the temperature range between − 50 and − 30 °C, no bands are seen above 1710 cm^{-1} (data not shown). The difference band around 1740 cm^{-1} in the spectra of meta-R_c is only observed when the temperature is higher than −30 °C (Figure 4c). This difference band can hardly be identified if the spectrum is measured in ^2H_2O (the small wiggles around 1740 cm^{-1} clearly larger than the noise level indicate the presence of bands with very small intensities) (Figure 5c). In the $P_r \rightarrow P_{fr}$ difference spectrum, a somewhat larger difference band is observed at a similar position for measurements in both

^1H_2O and ^2H_2O (Figure 3). It is not clear which molecular groups cause these bands around 1740 cm^{-1}. Since meta-R_c is formed between −65 and −25 °C (Figure 2) and there is a temperature range between −50 and −30 °C where no such bands can be observed, it is probable that the features observed in the spectra of meta-R_a, meta-R_c, and P_{fr} must be assigned to different molecular groups whose bands cancel in the intermediate temperature range. The intensities, frequencies, and deuteration-induced shifts would be compatible either with a protonated carboxyl group or with the C═O stretching mode of ring A of the chromophore. It is important to note that the transitions between the intermediates refer to UV−vis experiments mainly monitoring conformational changes of the chromophore (Figure 2). Therefore, the temperature dependence of these bands deviating from that of formation and decay of meta-R_a and meta-R_c suggests an assignment to protein bands. Again, the use of isotope labels will help to discriminate between the various possibilities. The varying intensities for the different measuring conditions (intermediate monitored, ^1H_2O, ^2H_2O) have to be explained by varying overlap of the respective positive and negative bands.

In the spectra measured in ^2H_2O, the intensity of the band at 1214 cm^{-1} of the lumi-R state is considerably reduced in meta-R_a. It cannot be identified in meta-R_c (Figure 5). A similar behavior is observed for the difference band around 1027 cm^{-1}. In the spectrum measured in ^1H_2O, these characteristic bands are missing (Figure 4). It is, however, noteworthy to mention that all the spectral features appearing between 1150 and 1300 cm^{-1} in the lumi-R difference spectrum in ^1H_2O are also observed for the later intermediates, including P_{fr}. The only difference is a broad negative band around 1240 cm^{-1} first showing up in the meta-R_c difference spectrum and gaining intensity in the $P_r \rightarrow P_{fr}$ difference spectrum (Figures 3a and 4c). An assignment of the bands to normal modes of the chromophore is not yet available.

$P_{fr} \rightarrow P_r$ Pathway. The negative bands at 1708 cm^{-1} (^1H_2O) and 1700 cm^{-1} (^2H_2O) in the lumi-F and meta-F difference spectra confirm the conclusion that the frequency and intensity of the C═O stretch of ring D in the P_{fr} state are very similar to those in P_r, in both ^1H_2O and ^2H_2O (Figures 6 and 7). The small differences observed for the measurements in ^2H_2O could explain the 1695/1704 cm^{-1} difference band in the corresponding $P_r \rightarrow P_{fr}$ difference spectrum (Figure 3b).

The comparison of the spectra shows that there are many similarities in lumi-F and meta-F in the spectral region between 1700 and 1550 cm^{-1}. This is in contrast to lumi-R and meta-R_a, the spectra of which were recorded at nearly the same respective temperatures. The main difference is the lack of the positive band due to the C═O stretch in meta-F which is, however, similar to meta-R_c. Since the corresponding negative band has nearly the same intensity in the two spectra of the $P_{fr} \rightarrow P_r$ pathway, the absence cannot be explained by an overlap (Figure 6b). Thus, this mode must have a very low infrared intensity. A considerable variation in intensities has already been mentioned for the $P_r \rightarrow P_{fr}$ pathway.

Another peculiarity has to be emphasized. Whereas the negative band exhibits a clear isotope shift upon $^1H/^2H$ exchange, the positive band in lumi-F remains at the same position (Figures 6a and 7a). Whether this is due to a special coupling behavior between the C═O stretch and NH bending modes or due to a special electronic structure of the conjugated π-electronic system is not clear at present. In this respect, it is interesting to note that the strong positive band at 1213 cm^{-1} observed in the lumi-R spectrum (^2H_2O) is not present in the corresponding lumi-F intermediate. In line with the similarity of the two spectra of the $P_{fr} \rightarrow P_r$ pathway, the difference band attributed either to a protonated carboxyl group or to the C═O stretch of ring A is already present in lumi-F around 1750 cm^{-1}. It exhibits a clear deuteration-induced isotope shift.

One clear difference between lumi-F and meta-F should, however, be mentioned. In the spectra measured in ^2H_2O, the narrow difference band at 1165 (negative) and 1158 (positive) cm^{-1} observed for the transition to lumi-F has negligible intensity in the meta-F difference spectrum (Figure 7). Its dependence on ^2H_2O could indicate that it has a similar molecular cause as the band at 1213 cm^{-1} in the spectra of lumi-R and meta-R_a. The clear differences observed in the spectra of the intermediates justify also for the back-reaction at low temperatures the classification of distinct intermediates as deduced from UV−vis spectroscopy. Since the positive bands of the meta-F spectrum deviate considerably from the negative bands of the $P_r \rightarrow P_{fr}$ difference spectrum, large molecular changes must still take place in the transition from meta-F to P_r.

CONCLUDING REMARKS

We have shown that reliable difference spectra of high quality can be obtained for the intermediates of the forward and backward photoreactions of phytochrome. In this way, the identification of low-temperature intermediates as derived from UV−vis spectroscopy could be confirmed.

In our previous publication (Siebert et al., 1990) we tried to correlate infrared bands of the $P_r \rightarrow P_{fr}$ difference spectra with bands in the resonance Raman spectra. Especially in the region between 1550 and 1650 cm^{-1}, where also strong protein bands are expected, we have identified the 1567/1546 cm^{-1} difference band in the $P_r \rightarrow P_{fr}$ difference spectrum with resonance Raman bands of P_r at 1568 cm^{-1} and of P_{fr} around 1550 cm^{-1}, and we have taken this band, since it disappears in spectra measured in ^2H_2O, as an indication that the chromophore is protonated. It must be emphasized that a correlation between resonance Raman and infrared spectra remains to be more or less speculative; there is for example little if any correspondence of the resonance Raman and infrared spectra in the spectral range <1500 cm^{-1}, which is generally free of strong protein bands. From the present work, it appears therefore questionable whether the identification based on resonance Raman spectra was correct, especially since we do not observe a corresponding difference band in the meta-F difference spectrum. Such a band should be present from recent resonance Raman investigations (Matysik et al., 1995).

There is, however, different evidence for a protonated state of the chromophore. The identified C═O stretch of ring D is at a frequency too high for such a group being included in the conjugated π-electronic system of an unprotonated chromophore. According to quantum chemical calculations, such a high position is only compatible with a singly protonated chromophore (Margulies & Toporowicz, 1984).

Therefore, we conclude that in P_r, P_{fr}, lumi-R, and lumi-F the chromophore in phytochrome is protonated. Due to the reduced intensities observed for meta-R_a and meta-F, and due to the strong downshift deduced for meta-R_c, statements on the protonation states in these intermediates cannot be made.

The evident lack of congruence of infrared and resonance Raman bands makes it mandatory to perform experiments on phytochrome containing isotopically labeled chromophores. Since it has been shown that the apoprotein can be reconstituted with chromophores (Deforce et al., 1993; Kunkel et al., 1993; Lagarias & Lagarias, 1989; Li & Lagarias, 1992), such experiments now appear feasible. Together with the possibility to obtain reliable spectra for the intermediates of the photoreaction demonstrated here, it is obvious that similar success can be achieved for phytochrome in deriving the molecular mechanism from vibrational spectroscopy as it was possible in the case of retinal proteins.

In contrast to resonance Raman spectra, in which the C=O stretching vibrations could hardly be identified (Fodor et al., 1990; Hildebrandt et al., 1992; Matysik et al., 1995; Mizutani et al., 1991, 1994), we provided clear evidence for the presence of such bands. Their spectral development in the course of the photoreaction will be important to determine the respective molecular changes. Furthermore, if molecular changes of the protein can clearly be identified, such observations will be helpful for an understanding of the active state of phytochrome.

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