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Infrared spectroscopy of phytochrome and model pigments

Friedrich SIEBERT[1, 2], Rudolf GRIMM[3], Wolfhart RÜDIGER[3], Gabriele SCHMIDT[3] and Hugo SCHEER[3]

[1] Albert-Ludwigs-Universität, Institut für Biophysik und Strahlenbiologie, Freiburg, Federal Republic of Germany
[2] Max-Planck-Institut für Biophysik, Frankfurt-am-Main, Federal Republic of Germany
[3] Botanisches Institut der Universität, München, Federal Republic of Germany

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Fourier-transform infrared difference spectra between the red-absorbing and far-red-absorbing forms of oat phytochrome have been measured in H_2O and ^2H_2O. The difference spectra are compared with infrared spectra of model compounds, i.e. the (5*Z*,10*Z*,15*Z*)- and (5*Z*,10*Z*,15*E*)-isomers of 2,3,7,8,12,13,17,18-octaethyl-bilindion (Et_8-bilindion), 2,3-dihydro-2,3,7,8,12,13,17,18-octaethyl-bilindion (H_2Et_8-bilindion), and protonated H_2Et_8-bilindion in various solvents. The spectra of the model compounds show that only for the protonated forms can clear differences between the two isomers be detected. Since considerable differences are present between the spectra of Et_8-bilindion and H_2Et_8-bilindion, it is concluded that only the latter compound can serve as a model system of phytochrome. The ^2H_2O effect on the difference spectrum of phytochrome supports the view that the chromophore in red-absorbing phytochrome is protonated and suggests, in addition, that it is also protonated in far-red-absorbing phytochrome. The spectra show that protonated carboxyl groups are influenced. The small amplitudes in the difference spectra exclude major changes of protein secondary structure.

Phytochrome, the plant photoreceptor for light-mediated differentiation and development, carries a single 2,3-dihydrobilin chromophore covalently bound to a protein of molecular mass 124 kDa (Shropshire and Mohr, 1983; Lagarias, 1985; Kendrick and Kronenberg, 1986; Furuya, 1987) (Fig. 1a). It is a photoreversibly photochromic pigment. Biosynthesis in the dark leads to the physiologically inactive red-absorbing form (P_r, λ_{max} = 665 nm). In light, a photoequilibrium is established with the physiologically active far-red-absorbing form (P_{fr}, λ_{max} = 730 nm).

Indirect evidence derived from deuterium isotope effects upon fluorescence lifetimes suggested a proton transfer as the primary photoreaction (Sarkar and Song, 1981; Moon et al., 1985). However, this conclusion was later withdrawn (Song and Yamazaki, 1987; Brock et al., 1987). Lack of a deuterium isotope effect for phototransformation of P_r into the first intermediate as detected by low-temperature ultraviolet/visible absorption spectroscopy also argued against tautomerization of the chromophore or proton transfer from chromophore to the protein as the primary photoreaction (Eilfeld and Rüdiger, 1986). Analysis of small chromopeptides obtained by proteolysis of the P_r and P_{fr} forms revealed different configurations for the chromophore at the 15,16-double bond (Rüdiger et al., 1983; Thümmler et al., 1983). Therefore, $Z \rightarrow E$ isomerization at C15/16 was considered as a good candidate for the primary photoreaction (Rüdiger et al., 1985) (Fig. 1b). Comparison of optical spectra with those of suitable model compounds suggested that the tetrapyrrole chromophore might be protonated in native P_r (Lagarias and Rapoport, 1980; Rüdiger et al., 1985). However, neither the state of protonation nor the exact configuration of the chromophore in native phytochrome can unequivocally be deduced from such measurements.

Vibrational spectroscopy has been a powerful tool to study related problems in the retinoic photoreceptors, rhodopsin and bacteriorhodopsin. Resonance Raman spectroscopy (e.g. Oseroff and Callender, 1974; Sulkes et al., 1978; Mathies et al. 1987; Stockburger et al., 1986) and infrared difference spectroscopy (e.g. Siebert et al., 1981; Siebert et al., 1983a; Engelhard et al., 1985; Braiman and Rothschild, 1988; Rothschild and Marrero, 1982; Bagley et al., 1982; Bagley et al., 1985) have been applied. Such studies are more difficult for phytochrome due to its poor solubility. The fluorescence hampers resonance Raman spectroscopy additionally and the large size of the apoprotein further complicates infrared difference spectroscopy. Only a few studies on model compounds, a prerequisite for the interpretation of vibrational spectra of the pigments, have been performed. A free bile pigment, biliverdin (Fig. 1a), has been studied (Margulies and Stockburger, 1979; Margulies and Toporowicz, 1984), but this pigment differs from phytochrome by having a fully unsaturated chromophore system. Another model pigment is phycocyanin, a photosynthetic antenna pigment bearing a chromophore which is very similar to the one of phytochrome, and which has been investigated by resonance Raman (Szalontai et al., 1988; Szalontai et al., 1989; Margulies and Toporowicz, 1988), pre-resonance Raman (Sawatzki et al., 1990) and resonant coherent anti-Stokes Raman spectroscopy (CARRS) (Schneider et al., 1988a, b).

First vibrational studies on phytochrome have only recently been reported using resonance Raman (Fodor et al., 1988) and surface-enhanced resonance Raman (SERRS) (Rospendowski et al., 1989; Farrens et al., 1989) spectroscopy. Here, we wish to present results obtained by Fourier-transform infrared difference spectroscopy on phytochrome. The

Correspondence to F. Siebert, Max-Planck-Institut für Biophysik, Kennedyallee 70, W-6000 Frankfurt am Main, Federal Republic of Germany

Abbreviations. Et_8-bilindion, 2,3,7,8,12,13,17,18-octaethyl-bilindion; H_2Et_8-bilindion, 2,3-dihydro-2,3,7,8,12,13,17,18-octaethyl-bilindion; Me_2-biliverdin, biliverdin 8^3,12^3-dimethyl ester.

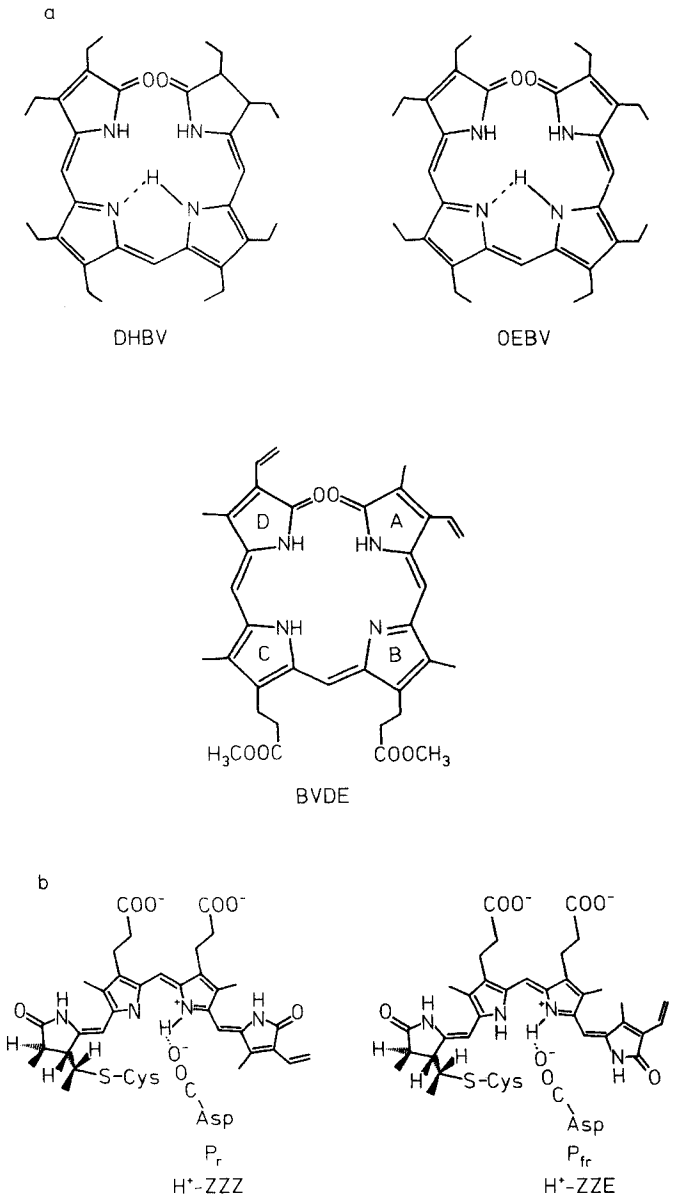

Fig. 1. *Structure of the model chromophores and of the chromophores in P_r and P_{fr}.* (a) DHBV, 2,3-dihydro-2,3,7,8,12,13,17,18-octaethyl-bilindion; OEBV, 2,3,7,8,12,13,17,18-octaethyl-bilindion; BVDE, biliverdin 8^3,12^3-dimethyl ester. (b) Extended conformations of the (5*Z*,10*Z*,15*Z*) and (5*Z*,10*Z*,15*E*) structures of the protonated chromophore in P_r and P_{fr}, respectively

difference spectra will be compared with infrared spectra of a model compound, 2,3-dihydro-octaethyl-bilindion (H_2Et_8-bilindion, Fig. 1a) which has the same conjugation system of the tetrapyrrole as does phytochrome.

MATERIALS AND METHODS

Model pigments

(*ZZZ*)-Octaethyl-bilindion (*Z*-Et_8-bilindion) and (*ZZZ*)-2,3-dihydro-octaethyl-bilindion (*Z*-H_2Et_8-bilindion, Fig. 1a) have been prepared from octaethylporphyrin (Cavaleiro and Smith, 1973). The respective 15*E*-isomers were obtained via the bilirubin isomerization procedure as described previously (Kufer *et al.*, 1982; Scheer, 1984; Schmidt, 1988).

Phytochrome

Phytochrome (124 kDa) was isolated from 3.5-day-old etiolated oat seedlings (*Avena sativa* L., cv. Pirol; Baywa, München, FRG) as previously described (Grimm and Rüdiger, 1986) but omitting the precipitation with polyvinylpyrrolidone. The intactness of the protein was tested by native and SDS gel electrophoresis (Schendel and Rüdiger, 1989). The specific absorbance ratio A_{665}/A_{280} of the preparations used in the present study was between 0.90 – 0.99. For preliminary experiments, the 60-kDa fragment of phytochrome was used which was prepared from 4-day-old etiolated oat seedlings according to Grombein et al. (1975).

Infrared measurements were carried out either on a Bruker IFS 113v Fourier transform spectrophotometer or on a home-built instrument equipped with the interferometer of the Bruker IFS 88 instrument. For the infrared spectra of the model compounds, microcuvettes with a spacing of 50 µm, requiring less than 20 µl solution, were used. The cuvettes were equipped either with ZnSe or with CaF_2 windows. Solvent bands were eliminated by subtracting a spectrum of the respective solvent. Approximately 30 scans were accumulated for the spectra of the model compounds.

To prepare relatively homogeneous hydrated films of phytochrome, suitable for infrared spectroscopy, 70 µl of a solution (1 mg/ml phytochrome, 0.3% by vol. glycerol and 2 mM mercaptoethanol) was deposited onto a ZnSe infrared window and the water was evaporated under a gentle stream of nitrogen. The film was rehydrated by placing about 1 µl H_2O or ^2H_2O into the infrared cuvette which was sealed with a special antireflection-coated germanium window. The spacing between the two windows was 2 mm. Mercaptoethanol decreased aggregation and glycerol retained photoreversibility after rehydration of the film. Light-induced infrared difference spectra were obtained in the usual way (Siebert and Mäntele, 1983), by forming the absorbance difference between the single-beam spectra obtained before and after illumination. The samples were illuminated within the spectrophotometer with a slide projector and a focussing lens. To induce the $P_r - P_{fr}$ transition, a band-pass filter with peak transmission at 680 nm and a half-width of 12 nm was used. To evoke the back reaction, a long-pass filter (Schott, RG 715) was employed. For each single-beam spectrum, 512 scans were accumulated. The difference spectra shown represent the sum of 20 measurements, obtained from a single sample, exploiting the good photoreversibility of the phytochrome hydrated film samples.

RESULTS AND DISCUSSION

Model compounds

Fig. 2 shows the infrared spectra of (*Z*)-Et_8-bilindion in CCl_4 (a), (*Z*)-H_2Et_8-bilindion in CCl_4 (b), and of (*Z*)-H_2Et_8-bilindion in C^2HCl_3 (c). It is obvious that the spectrum of the symmetric molecule (a) deviates considerably from that of the asymmetric one. For a comparison of band intensities, the band with shoulder around 1464 cm^{-1}, which is caused by the eight ethyl groups, serves as an internal standard. The comparison of spectra (a) and (b) shows that the band at 1732 cm^{-1} (b) is due to the nonconjugated carbonyl group. Since TLC revealed no dihydro compound present in OEBV, the small band at 1733.5 cm^{-1}, still visible in spectrum a, is not due to impurities. It might be caused by aggregations, which are likely to occur in the solvents used at the concen-

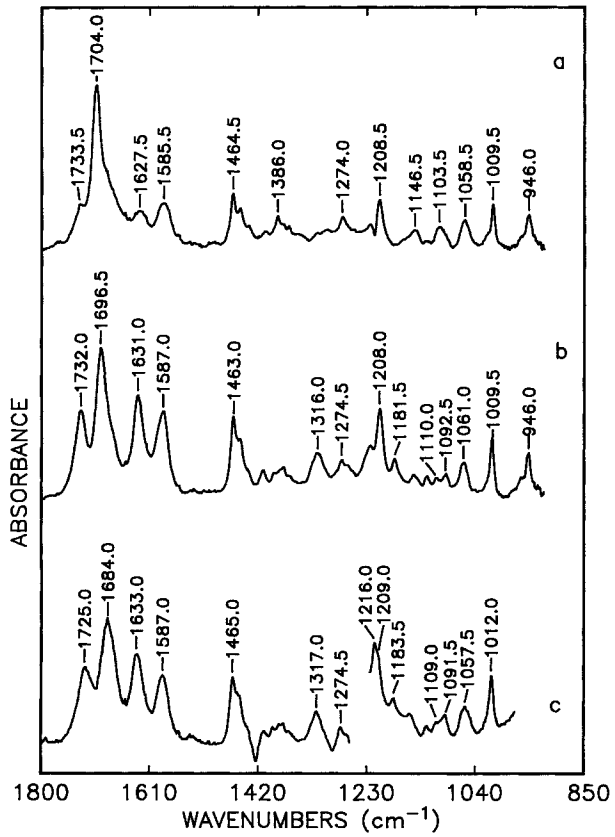

Fig. 2. *Infrared spectra of the ZZZ-isomer of Et_8-bilindion in CCl_4 (a), of H_2Et_8-bilindion in CCl_4 (b), and of Me_2-biliverdin in C^2HCl_3 (c).* The latter solvent obscures the range around 1250 cm^{-1}

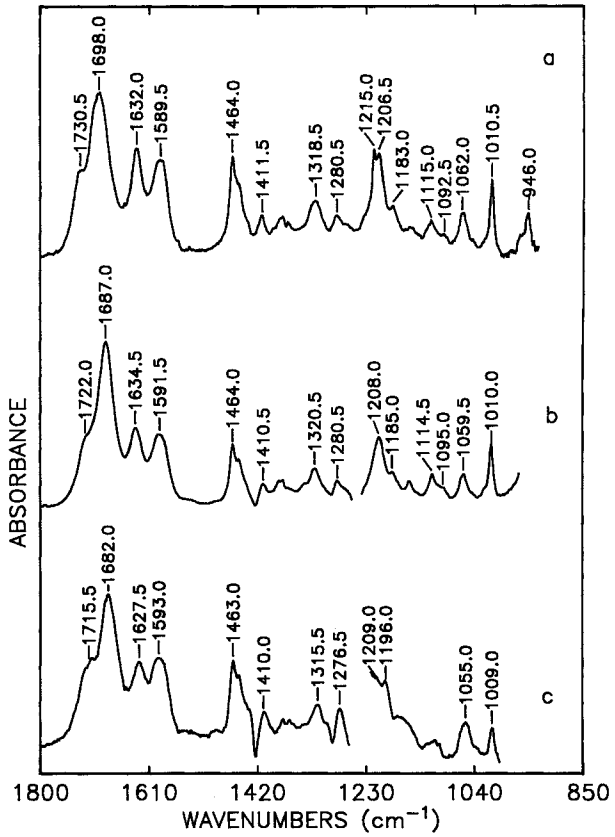

Fig. 3. *Infrared spectra of the ZZE isomer of H_2Et_8-bilindion in CCl_4 (a), in C^2HCl_3 (b), and in C^2HCl_3 saturated with ^2H_2O to cause H/^2H exchange at the nitrogens (c)*

trations necessary for infrared spectroscopy (≈ 1 mg/ml). Such aggregations may introduce asymmetries in the conjugated system and reduce the conjugation of one carbonyl group. The conjugated carbonyls show up with higher intensity at 1696.5 cm^{-1} for H_2Et_8-bilindion (b) and at 1704 cm^{-1} for Et_8-bilindion (a). The band causing the shoulder in spectrum 2a may also be present in spectrum 2b. The intensity of the band at 1631 cm^{-1} of H_2Et_8-bilindion is higher than that of the comparable band at 1627.5 cm^{-1} of Et_8-bilindion. In model calculations on biliverdin dimethyl ester (Margulies and Toporowicz, 1984), two modes, one at 1623 cm^{-1} and the other at 1606 cm^{-1}, have been assigned to the two methine C = C stretches adjacent to the two lactam rings. The lower frequency was assigned to the methine group near ring D. In the resonance Raman spectra of Me_2-biliverdin (Margulies and Stockburger, 1979; Margulies and Toporowicz, 1984), the latter vibration shows up with high intensity, whereas the former is not resolved. In resonance Raman spectra of bilirubin (Hsieh and Morris, 1988), the higher frequency mode can be seen as a small shoulder. Et_8-bilindion can best be compared with Me_2-biliverdin. Thus, Fig. 2a shows that the higher-frequency mode is stronger in the infrared and that the lower-frequency mode cannot be resolved. It can be expected that hydrogenation of ring A will exert a stronger influence on the adjacent methine group as compared to the group at ring D. Thus, the intensity increase of the band at higher frequency in H_2Et_8-bilindion together with an upshift of 4 cm^{-1} to 1631 cm^{-1} would be in agreement with the assignment made by Margulies and Toporowicz (1984). However, it may also be that the two modes mix and that the mixing behaviour is influenced by hydrogenation of ring A. The band at 1318 cm^{-1} seems to be characteristic of the dihydro compound. Fig. 2c shows the influence of a hydrogen-bonding solvent such as C^2HCl_3 on the spectrum of H_2Et_2-bilindion. The main influence is the downshift of the carbonyl bands. It should be noted that the carbonyl bands show up in the resonance Raman spectra, if at all, with very low intensity. An additional difference is the large band at 1244 cm^{-1} in the resonance Raman spectrum of Me_2-biliverdin which is not seen in the spectrum of Fig. 2a.

Fig. 3a shows the spectrum of the (*ZZE*)-isomer of H_2Et_2-bilindion in CCl_4 and Fig. 3b in C^2HCl_3. Fig. 3c shows the corresponding spectrum in C^2HCl_3 saturated with ^2H_2O, i.e. the exchangeable hydrogens at the nitrogens are replaced by deuterium. The different geometries cause only very small spectral changes. Surprisingly, the carbonyl bands are mostly influenced. The band caused by the conjugated group is increased and broadened, that of the nonconjugated group decreased. In addition, a small upshift of the former band is observed. In the spectrum of the (*ZZE*)-isomer, the band around 1208 cm^{-1} is split into two bands at 1215 and 1206.5 cm^{-1} (Fig. 2b vs Fig. 3a). Both spectra of H_2Et_2-bilindion in CCl_4 and in C^2HCl_3 show an intensity exchange of two small bands at 1094 cm^{-1} and 1115 cm^{-1}, the latter being larger for the (*ZZE*)-isomer. As expected, ^2H_2O causes small downshifts of the carbonyl bands, due to the coupling to the NH bending vibrations of the neighbouring nitrogens. The band at 1635 cm^{-1} shifts down upon deuteration. This is in agreement with the assignment to the methine C = C stretches made above, which also couple to the NH bending vibrations. Since the band around 1590 cm^{-1} does not shift upon nitrogen deuteration, it could be due either to the C = N stretch of ring

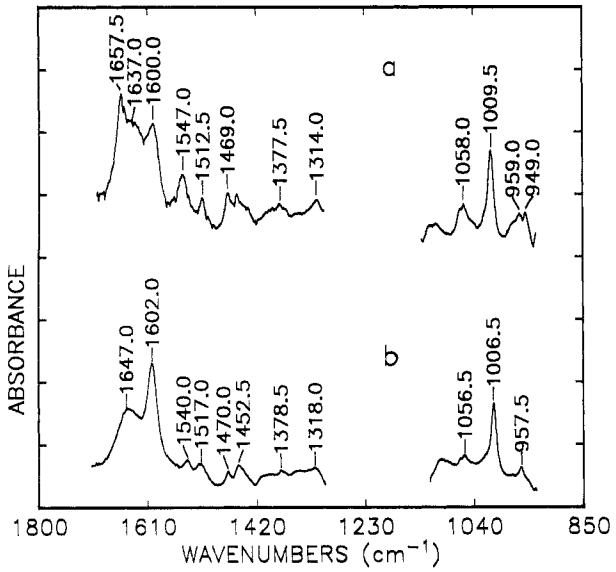

Fig. 4. *Infrared spectra of the ZZZ (a) and ZZE (b) isomers of protonated H_2Et_8-biliverdin.* Protonation was achieved by adding 1% trifluoroacetic acid to the solvent C^2HCl_3

C or to C = C stretches of the endocyclic carbons of ring C and D. The spectra show no evidence for the NH bending modes.

Fig. 4 shows the influence of protonation of the *ZZZ* (a) and *ZZE* (b) isomers of H_2Et_8-bilindion DHBV by 1% trifluoroacetic acid in C^2HCl_3. The total amount of trifluoroacetic acid in both the sample and reference cuvettes is the same. However, due to the protonation of the chromophore, the amount of protonated trifluoroacetic acid is reduced in the sample cuvette. This causes the strong negative bands above 1700 cm^{-1}. Therefore, it is not clear whether the carbonyl band above 1700 cm^{-1} is still present. The strong band at 1600 cm^{-1} in both spectra may partially be due to the asymmetric CO_2^- stretching vibration of deprotonated trifluoroacetic acid. The difference in intensity upon $Z \rightarrow E$ isomerization, however, shows that its largest part is caused by the chromophore. If the two bands around 1460 cm^{-1} are taken as an approximate internal standard, it is apparent that, as compared to the unprotonated chromophore, the protonated one exhibits much larger bands in the region between 1580 – 1670 cm^{-1}. Also the band around 1010 cm^{-1} shows a larger intensity. There are, in principle, several possibilities for the protonation of the chromophore. In the resonance Raman investigation of Me_2-biliverdin (Margulies and Stockburger, 1979; Margulies and Toporowicz, 1984), it was suggested that two protonation stages can be discriminated; in the first, the nitrogen at ring C is protonated, whereas in the second the lactam carbonyl of ring A is protonated. However, H_2Et_8-bilindion shows only a single protonation step (Scheer, 1976) and the second step is probably related to traces of metal complexes (H. Scheer, unpublished results). Similarly, only one proton is taken up by a modified H_2Et_8-bilindion, protected to avoid unwanted side-reactions, even in concentrated sulfuric acid (Falk and Zrunek, 1983). In addition, for H_2Et_8-bilindion bound to a protein (denatured phytochrome and phycocyanin), only one protonation step was observed between pH 1 and pH 10 (Grombein et al., 1975). Thus, it is safe to assume that for H_2Et_8-bilindion only the nitrogen of ring C can be protonated. If the intensity of the non-conjugated carbonyl band does not change upon protonation, it may be hidden under the strong negative bands caused by protonated trifluoroacetic acid. In unprotonated H_2Et_8-bilindion the conjugated carbonyl shows the strongest intensity (Figs 2 and 3). Thus, the question arises which of the bands can be identified with this mode. Upon $Z \rightarrow E$ isomerization the band at 1657.5 cm^{-1} disappears and the band around 1600 cm^{-1} increases. Since this isomerization is not expected to alter drastically the carbonyl frequency, the band at 1657.5 cm^{-1} must be caused by another mode. According to calculations performed by Margulies and Toporowicz, protonation of the nitrogen tends to increase the frequency of the conjugated carbonyl (Margulies and Toporowicz, 1984). Thus, it may be that the conjugated carbonyl band is also shifted under the strong negative bands of protonated trifluoroacetic acid. In addition, because of the large increase in intensity of the C = C (and C = N) stretches, the conjugated carbonyl group will now appear as a relatively weak band. Therefore, it is unlikely that one of the bands of Fig. 4 can be assigned to this vibration.

A tentative explanation for the effects observed upon isomerization can be derived from the following arguments. Since protonation tends to render rings B and C more equivalent, the distribution of the positive charge between rings B and C is influenced by the local environment. Therefore, vibrational modes from this part of the molecule will highly depend on solvent and local interactions, e.g. position of the charge-compensating counter-ion. If rings B and C are completely equivalent, the electrons are mostly delocalized, decreasing double bonds and increasing single bonds. This would shift down the C = N and C = C stretches of the B-C part of the molecule to lower frequencies. Thus, it could be that, due to special interactions with the carboxyl groups of trifluoroacetic acid, in the *Z*-isomer the positive charge is more localized at one ring than in the *E*-isomer, thereby favoring localized double bonds. It is interesting to note that a comparable band pattern is also observed in the spectra of the *E*- and *Z*-isomers of protonated Et_8-bilindion. Here, the high-frequency band of the *Z* isomer is located at 1675 cm^{-1}. In the *E*-isomer, this band disappears and the intensity increases around 1600 cm^{-1} (data not shown). In the resonance Raman spectra of protonated Me_2-biliverdin, which is best comparable to Et_8-bilindion, the highest frequency mode is at 1630 cm^{-1}. This contrasts with the high position of the band at 1675 cm^{-1} of Et_8-bilindion. For the resonance Raman spectra, protonation was obtained by HCl. Thus, the different counter-ions and their effect on the charge distribution could explain the different band positions. A small but distinct feature is observed both for H_2Et_8-bilindion and Et_8-bilindion: the band at 1009.5 cm^{-1} for the *ZZZ* conformation is shifted to 1006.5 cm^{-1} for the *ZZE* geometry.

For protonated H_2Et_8-bilindion as well as Et_8-bilindion, the spectral differences between the two isomers could be reproduced by illuminating the *E*-isomer in the infrared cuvette (data not shown). The visible controls showed that a species very similar to the *Z*-isomer was obtained (Scheer, 1976). Although the infrared spectra of protonated Et_8-bilindion are similar to those of H_2Et_8-bilindion for both the *Z*- and *E*-isomers, distinct differences are still present. In contrast to the nonprotonated species, at least three double bond stretches (C = C and C = N) can now be identified for the *Z*-isomer, the third causing the broad band around 1637 cm^{-1}. For the *E*-isomer, two bands probably overlap, increasing the band at 1602 cm^{-1}.

Our results confirm that Et_8-bilindion is not an appropriate model for the chromophore of phytochrome. Further-

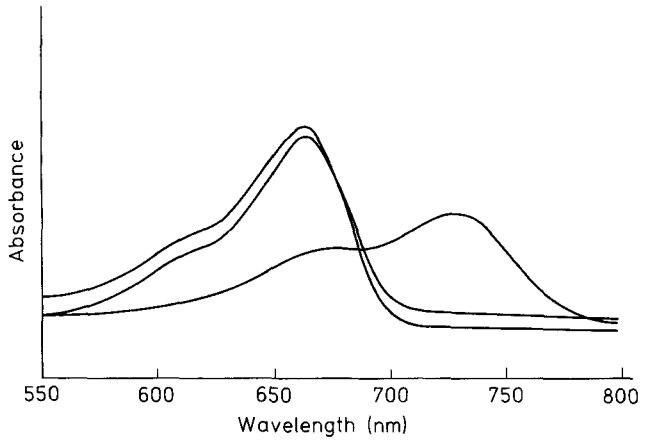

Fig. 5. *Visible absorption spectra of the hydrated film samples of phytochrome to demonstrate the good photoreversibility.* The lower trace of the phytochrome spectrum was obtained by illuminating the P_r-P_{fr} mixture with far-red light

more, the spectral changes observed for the two geometries of H_2Et_8-bilindion, which reflect mostly shifts of the carbonyl bands, suggest that the spectra are largely determined by intermolecular interaction or even aggregation caused by the high concentration necessary for infrared spectroscopy. In addition, many subconformations are probably present, differing in single-bond twists of the methine bridges (Braslavsky et al., 1983). In phycocyanin, the chromophore is in a semi-extended conformation (Schirmer et al., 1987) and it is generally assumed that this also holds for phytochrome (Rüdiger et al., 1985) (Fig. 1b). However, in the solvents used, the chromophore adopts a helical geometry (Scheer, 1981; Braslavsky et al., 1983). This probably explains the observed influence on the carbonyl bands upon $Z \rightarrow E$ isomerization. Therefore, to sum up, the spectral differences between the two geometries of H_2Et_8-bilindion are only of limited relevance for the deduction of the structure of the chromophore in P_r and P_{fr}. The protonated chromophores exhibit much better resolved spectra and better defined differences between the *Z*- and *E*-isomers, indicating less aggregation and better defined geometries. Since the chromophore in phytochrome is protonated (Fodor et al., 1988), the spectra of these model compounds could serve as a guide for the elucidation of the structure both in terms of protonation and isomerization state. However, the strong dependence of the modes on the local environment, as indicated by the large spectral changes caused by the isomerization, requires better defined model compounds.

Phytochrome

If no glycerol is added to the phytochrome solution, drying of the sample onto the infrared window causes an irreversible denaturation such that after rehydrating the sample, no photoreversibility can be obtained (data not shown). However, Fig. 5 demonstrates that the addition of glycerol preserves phytochrome, since after rehydration complete photoreversibility is obtained. (The small apparent absorbance decrease at 680 nm after illumination with light of 730 nm is due to the baseline drop observed above 700 nm, since, for illumination, the hydrated film sample had to be taken out of the spectrophotometer.) The photoreversibility lasted for more than 20 illumination cycles. Therefore, to increase the signal/noise ratio in the infrared difference

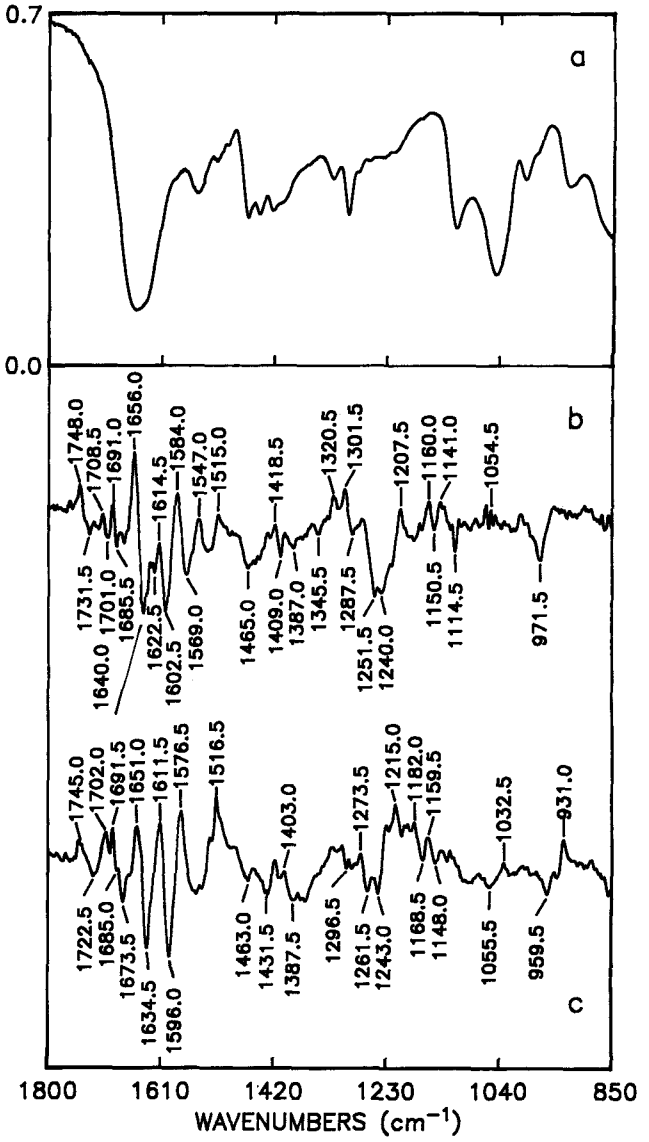

Fig. 6. *Infrared spectra of phytochrome.* (a) Single-beam spectrum of a hydrated film sample of phytochrome with intensity on the ordinate. (b, c) $P_r - P_{fr}$ infrared difference spectra of a hydrated film sample of phytochrome hydrated with ^1H_2O (a) and ^2H_2O (b) with absorbance on the ordinate. Each difference spectrum represents the sum of 20 individual difference spectra obtained from 10 illumination cycles as described in Materials and Methods

spectra, spectra from 10 illumination cycles were added. In addition, since it was found that the $P_r - P_{fr}$ difference spectra are just the negative of the $P_{fr} - P_r$ difference spectra, these two spectra could be subtracted from each other, increasing the signal/noise further.

Fig. 6a shows the infrared single-beam spectrum of a hydrated film sample of phytochrome. The spectrum is dominated by the amide-I band at 1655 cm^{-1} and the band around 1500 cm^{-1} caused by glycerol. As compared to the spectra of membrane proteins, the amide-I band is much larger than the amide-II band at 1550 cm^{-1}. This is due to the random orientation of the phytochrome molecule, whereas membrane proteins are oriented, favoring the amide-II band and reducing the amide-I absorbance. In order to keep the amide-I band on scale, only 70 μg phytochrome could be used for the hydrated films.

Fig. 6b and c show the $P_r - P_{fr}$ difference spectra in H_2O and ^2H_2O. As mentioned, the difference spectra are the sum of 20 individual difference spectra each. Negative bands are due to P_r and positive bands due to P_{fr}. The amplitude of the largest bands of one difference spectrum is approximately 2×10^{-3}. Deuteration of the medium causes a general downshift of bands between 1660 – 1580 cm^{-1}, where the most intense bands are observed. Amide-I bands of the protein span the region 1690 – 1610 cm^{-1}. Due to the NH in-plane bending coupling, the amide-I mode generally undergoes a downshift upon deuteration of the nitrogens. Since the single-beam spectrum of phytochrome in ^2H_2O shows that the amide-II band disappears almost completely (data not shown), considerable $^1H/^2H$ exchange must have taken place. Therefore, if structural changes of phytochrome during the $P_r - P_{fr}$ transition are reflected in changes of the amide-I modes, one would expect that the corresponding bands in the difference spectrum undergo a downshift of 5 – 15 cm^{-1}. However, since the most intense bands of the chromophore are also observed in this spectral range (Figs 2 – 4) and since the carbonyl bands and methine C = C stretches undergo a deuteration-induced isotope shift of similar size, $^1H/^2H$ effects observed in the $P_r - P_{fr}$ difference spectra do not allow one to discriminate between chromophore and protein bands. But the strong difference band at 1602.5/1584 cm^{-1} (1596/1576.5 cm^{-1}, ^2H_2O) is outside the range of amide-I modes. Furthermore, from our Fourier-transform infrared investigations on retinal proteins, we know that amino-acid side chains cause much smaller bands. The higher intensity of chromophore bands can be explained by the charge alternations in the conjugated system of the chromophore, caused by the delocalized π-electrons. These residual charges give rise to strong infrared transition moments for molecular vibrations involving the corresponding atoms. Therefore, the strong difference band must be caused by the chromophore. From this it is expected that chromophore bands of similar size contribute to the difference spectrum between 1660 – 1620 cm^{-1}.

The similarity of the spectral appearance suggests that the bands of protonated *Z*-DHBV at 1657.5, 1637 and 1600 cm^{-1} correspond to the negative bands of the phytochrome difference spectrum at 1640, 1622.5 and 1602.5 cm^{-1}, respectively. But since contributions from the protein have also to be taken into consideration, this identification is not unequivocal. In the resonance Raman spectrum, a band is observed at 1644 cm^{-1} which shifts down upon deuteration of the medium to 1633 cm^{-1}. This suggests that at least the negative band at 1640 cm^{-1} (1634.5 cm^{-1}, ^2H_2O) in the difference spectrum is due to the chromophore. The deviations in band positions can be explained by the overlap of photoproduct bands with bands of the initial state.

Comparing the resonance Raman spectra of phytochrome (Fodor et al., 1988) and of Me_2-biliverdin (Margulies and Stockburger, 1979; Margulies and Toporowicz, 1984), a band located at 1327 cm^{-1}, which disappears in ^2H_2O, was interpreted as the corresponding NH bending vibration of the protonated chromophore. There may be two reasons why this band is not observed in the difference spectrum: (a) if P_{fr} also had a protonated chromophore, the NH bends could just cancel in the difference spectrum; (b) if we take the band at 1318 cm^{-1} as the corresponding NH bend of protonated H_2Et_8-bilindion, its small intensity could also explain why it is not detected in the phytochrome difference spectrum.

In the difference spectrum of phytochrome, the 1569/1547-cm^{-1} difference band disappears upon deuteration. Thus, in comparison to the other large bands, it must exhibit a large isotope shift. This is similar to the effect observed for a band located at 1576 cm^{-1} in the resonance Raman spectrum P_r (Fodor et al., 1988). There, the band was interpreted as the C = NH stretching mode of the protonated chromophore, which is shifted to 1498 cm^{-1} upon deuteration because of the large coupling with the NH rocking mode. Again, the deviating band position may be caused by the overlap with the strong positive band at 1584 cm^{-1}. Unfortunately, we are unable to detect the shifted band in the difference spectrum. It may be hidden under the broad structure around 1500 cm^{-1}. If the interpretation of the band is correct, the fact that the complete difference band disappears indicates that P_{fr} is also protonated. Our investigations on the model compounds as well as the resonance Raman data on Me_2-biliverdin (Margulies and Toporowicz, 1984) show that protonation of the chromophore causes a considerable upshift of the two non-carbonyl bands with highest frequency. Since the band around 1640 cm^{-1} of phytochrome observed in our difference spectrum, as well as in the resonance Raman spectrum, is at a much higher position than the non-carbonyl bands of the unprotonated model compounds (Figs 2 and 3) (Margulies and Toporowicz, 1984), further support for a protonated chromophore is obtained. In principle, the positive band at 1547 cm^{-1} could also be caused by an amide-II band, which disappears upon deuteration. However, relating this band to the small amide-II absorbance of phytochrome, much larger changes would then be expected to occur in the amide-I region.

Upon isomerization of the chromophore from the *Z* to the *E* geometry, one would expect that the interaction of the carbonyl group at ring D undergoes an environmental change. Thus, the corresponding band should be seen in the difference spectrum. Our model compound studies indicate that, for the protonated chromophore, the carbonyl bands have much lower intensities than the C = C stretches. In the phytochrome difference spectrum we only observe small bands above 1660 cm^{-1}. This observation additionally supports a protonated chromophore in both P_r and P_{fr}. Many of the bands above 1660 cm^{-1} are influenced by ^2H_2O. They may partially be due to amide bands of the protein or to the carbonyl bands of the chromophore. The 10-cm^{-1} downshift of the negative band at 1731.5 cm^{-1} suggests that it may be due to a carboxyl group protonated in P_r. Schendel and Rüdiger (1989) have recently identified a peptide region of native oat phytochrome (residues 323 – 360) which is exposed at the protein surface only in the P_{fr} form. This region carries seven glutamic acid and two aspartic acid residues. Its differential exposure was considered as a reason for the higher negative surface charge in P_{fr} (pI = 5.80 – 5.85). The present results agree with these data if we assume that the carboxylic group is ionized only at the surface (in P_{fr}) but protonated in the interior of the protein (in P_r).

Chai et al. (1987) calculated from photoreversible circular dichroic spectra in the far-ultraviolet region an α-helix content of 52.2% for P_r, which increases to 55.3% for P_{fr} at the expense of β-turns. The positive band at 1656 cm^{-1} in Fig. 6b could indicate an increase in α-helical content. However, if the area of this band is related to the total area of the amide-I absorbance band derived from Fig. 6a, the α-helical increase would be at most 2×10^{-3}. The reported 3% difference in helix content derived from CD data disappeared either by binding of a monoclonal antibody to the N-terminus which also blue-shifted the 730-nm band of the P_{fr} chromophore or by chemical oxidation or reduction of the chromophore in P_r and P_{fr} (Chai et al., 1987). Therefore, it may be that the

photoreversible changes of the CD spectrum are due to different chromophore structure or interaction with the protein rather than due to real changes in the helix content. One has to remember that tetrapyrrole chromophores absorb light also in the far-ultraviolet region (Scheer, 1981).

Recently, a surface-enhanced resonance Raman (SERRS) spectrum of phytochrome was published. It was obtained by excitation at 406.7 nm into the ultraviolet absorption band (Rospendowski et al., 1989). The spectrum has little resemblance to the resonance Raman spectrum as published by Fodor et al. (1988) or with our data. However, the SERRS spectrum of Me_2-biliverdin (Holt et al., 1989) with excitation into the ultraviolet band also deviates considerably from the resonance Raman spectrum published by Margulies and Stockburger. Apparently, different modes are enhanced by the SERRS effect and by the ultraviolet excitation. Comparing the resonance Raman spectrum of phycocyanin with excitation in the visible band (Margulies and Toporowicz, 1988) with that obtained by excitation into the ultraviolet band (Szalontai et al., 1989), large deviations are apparent. Thus it appears that, for bilin pigments, the two types of resonance Raman spectra cannot be directly compared. However, the former spectrum agrees reasonably well with the Fourier-transform Raman spectrum obtained under pre-resonance conditions (Sawatzki et al., 1990).

Our data show that reliable Fourier-transform infrared difference spectra can be obtained from a protein as large as phytochrome. Since, as compared to a membrane protein, the amide-I band of a soluble protein appears much stronger, less sample can be deposited onto the infrared window. Together with the molecular mass of 124 kDa, this explains the larger 'noise' in the phytochrome difference spectra in comparison to the difference spectra of retinal proteins. Our data can be related to the resonance Raman spectrum obtained by excitation into the visible absorption band. Based on somewhat different arguments, we also arrive at the conclusion that the chromophore is protonated in P_r (Fodor et al., 1988) and, in addition, in P_{fr}. The infrared technique offers the advantage that protein molecular changes can be detected, as well as changes of the chromophore. To discriminate between the two possibilities, the method of recombining isotopically labelled chromophores with the apoprotein was highly successfull in the case of retinal proteins (e.g. Ganter et al., 1988). In the last year much progress has been made in reconstituting phytochrome (Lagarias and Lagarias, 1989). Thus, the method of using labelled chromophores may become applicable also for this pigment. This is an essential condition for a molecular interpretation of the chromophore bands.

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