FTIR Studies of Phytochrome Photoreactions Reveal the C═O Bands of the Chromophore: Consequences for Its Protonation States, Conformation, and Protein Interaction†

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Received August 15, 2001; Revised Manuscript Received October 18, 2001

ABSTRACT: The molecular changes of phytochrome during red → far-red and reverse photoreactions have been monitored by static infrared difference spectroscopy using the recombinant 65 kDa N-terminal fragment assembled with a chromophore chemically modified at ring D or with a chromophore isotopically labeled with ^{18}O at the carbonyl group of ring A. This allows the identification of the C═O stretching vibrations of rings D and A. We exclude the formation of an iminoether in Pfr. The positions of both these modes show that the chromophore always remains protonated. The upshift of the C═O stretch of ring D in the first photoproducts is explained by a twisted methine bridge connecting rings C and D. The changes in the vibrational pattern during the red → far-red conversion show that the backreaction is not just the reversal of the forward reaction. The infrared difference spectra of the fragment deviate very little from those of the full-length protein. The differences which are related to the lack of the C-terminal half of the protein constituting the signaling domain are possibly important for the understanding of the signaling mechanism.

Among the sensory photoreceptors of plants (*1*), phytochromes (Phy)[1] represent the best-characterized protein family (*2*). The active molecule is a soluble homodimer of two polypeptides of $M_w \sim 125$ kDa, each containing a covalently linked linear tetrapyrrole chromophore (Figure 1a, PΦB). It shows a characteristic photoreversible photoreaction between two forms, which according to their maximum absorption in the red and far-red light spectral regions were termed Pr ($\lambda_{max} = 666$ nm) and Pfr ($\lambda_{max} = 730$ nm) (*3*). The quality of the incident light determines the Pr/Pfr ratio and thereby initiates many responses of plants via largely unknown signal transduction pathways (*1*, *4*−*6*). Phytochromes possess kinase activity (*7*, *8*), and upon phototransformation, Pfr is translocated from the cytoplasm into the nucleus (*9*, *10*). The discovery of homologous prokaryotic proteins in *Synechocystis* and other cyanobacteria (cyanochromes) (*11*) and, very recently, in nonphotosynthetic bacteria (*12*) has considerably enlarged this family of photoreceptors, and their comparison may help to unravel the signaling mechanism.

Phytochrome photoreactions have been the focus of many spectroscopic studies, but there is still little known on the details of the molecular events occurring during the photoconversions (*3*, *13*, *14*). Time-resolved experiments have revealed several intermediates in both the Pr → Pfr and the Pfr → Pr conversions (*15*, *16*), which can also be trapped at low temperature (*17*). The molecular structure of the chromophore changes from 15*Z* in Pr to 15*E* in Pfr, and it has been concluded that the isomerization of the chromophore takes place in the first step(s) of both photoreactions, within the picosecond time range (*16*−*22*). For native phytochrome A from oat (PhyA), three intermediates were found in the Pr → Pfr pathway (lumi-R, meta-R_a, and meta-R_c), and at least two intermediates (lumi-F and meta-F) during the reverse reaction (Figure 1b). Similar photoreactions have also been found in recombinant phytochrome N-terminal fragments (65 kDa, phyA65) comprising the photoreceptor domain (*23*). Photochemistry is also maintained if a modified chromophore, phycocyanobilin (PCB, Figure 1a), is introduced into the proteins, but the modification at ring D of this pigment causes differences in the time course of the photoreaction (*24*).

† This work was supported by the Deutsche Forschungsgemeinschaft, SFB533 (to C.B.) and Grant SI 287/20-1 (to F.S.), and by Fonds der Chemischen Industrie (to F.S.).

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[1] Abbreviations: FTIR: Fourier transform infrared; PCB: phycocyanobilin; PΦB: phytochromobilin; PhyA: phytochrome species A; phyA65: the 65 kD N-terminal fragment of PhyA; phyA65-PCB, phyA65-PΦB: phyA65 reconstituted with PCB resp. PΦB; Pr, Pfr: red, resp. far-red absorbing forms of PhyA, phyA65-PCB, or phyA65-PΦB.

10.1021/bi0156916 CCC: $20.00
Published on Web 11/08/2001

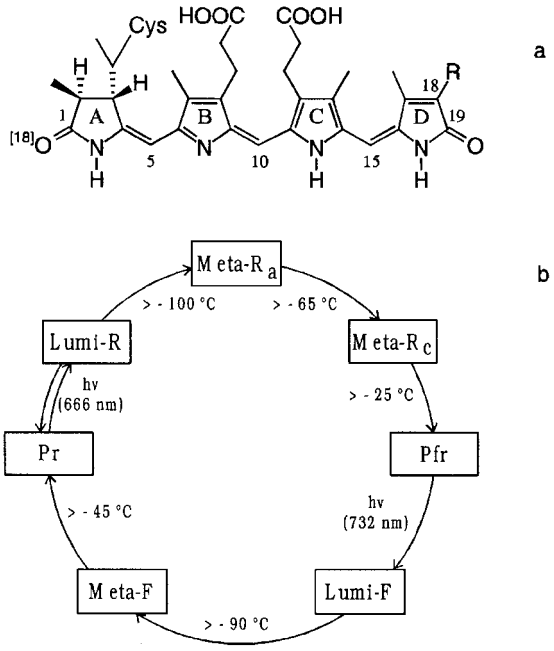

FIGURE 1: Chemical structure of the tetrapyrrole chromophores bound to the phytochrome apoprotein via a thioether bond to Cys-321. (a) R = C_2H_3 (vinyl): phytochromobilin (PΦB); R = C_2H_5 (ethyl): phycocyanobilin (PCB) and, as indicated at ring A, selectively ^{18}O-labeled PCB. (b) Scheme of the photoreactions of native PhyA at low-temperature adapted from Eilfeld and Rüdiger (*17*). The intermediates and the approximate transition temperatures are indicated.

Because of the similarity in the absorption bands, it is assumed that the chromophore maintains a predominantly extended conformation throughout the photoreactions, comparable to that of PCB in the phycobiliprotein phycocyanin (*25*). The configuration of the chromophore in Pr − 15*Z*, *syn* vs 15*Z*, *anti*, based on resonance Raman studies, is controversially discussed (*26*−*30*). Further studies have indicated that in the low-temperature intermediate, the chromophore is considerably twisted about the C_{15}−methine bridge (*29*, *30*). 15*Z* → 15*E* isomerizations of bile pigments are generally accompanied by blue-shifts of their visible absorption bands (*31*). Such a shift is also seen in the photoactive phycobiliprotein, phycoerythrocyanin (PEC) (*32*, *33*). By contrast, the 15*E*-isomer of phytochrome is considerably red-shifted in Pfr. The molecular origin for this shift has been discussed controversially. Among others, a proton release upon conversion to Pfr or the formation of an iminoether at ring A have been proposed (*28*, *30*, *34*, *35*).

Light-induced Fourier transform infrared (FTIR) spectroscopy has been shown to be a powerful tool for investigating the molecular events occurring during the photoreactions of chromoproteins (*36*). In previous studies, we presented FTIR difference spectra of the intermediates of the photoreactions of PhyA and provided preliminary assignments to prominent spectral features (*37*). The progress in expression of recombinant phytochrome or fragments that can be reconstituted with different chromophores showing full photoreversibility provides access to selectively modified systems (*38*) and thereby a tool for the assignment and molecular interpretation of FTIR difference spectra. In this work, the C_1=O and the C_{19}=O stretching bands of the chromophore in PhyA65 in Pr, Pfr, and in the intermediates of both photoreactions have been assigned by a combination of ^{18}O labeling and chromophore modification. The comparison with spectra of model compounds, of the homologous protein from *Synechocystis*, and of the related phycoerythrocyanin, has allowed one to extract important conclusions with respect to protonation states, chromophore configurations, and chromophore−protein interactions. The conclusions apply also to the intact phytochrome, since its spectra and those of the fragment only differ in very small, albeit functionally potentially significant, details.

MATERIALS AND METHODS

Synthesis of the Selectively C_1=^{18}O-Labeled Phycocyanobilin Chromophore. Incorporation of the ^{18}O-label at the carbonyl group of ring A of the PCB-chromophore was achieved using PCB-dimethylester (PCBDME) as precursor. Shortly, it was converted to the 1-ethoxy-PCBDME (EtO−PCBDME) with Et_3OBF_4, and subsequently hydrolyzed with $^1H_2{}^{18}O$ (96 atom-%), resulting in PCBDME selectively ^{18}O-labeled at position 1 (Figure 1a). The ^{18}O-enrichment was 92% as verified by mass spectrometry. Deesterification of the propionic acid-methylester groups of the PCB pigment was performed as previously published (*39*).

Preparation of Recombinant 65 kDa Phytochrome. Recombinant N-terminal 65 kDa fragment of apophytochrome A of *Avena sativa* (phyA65) was obtained from heterologous expression of the encoding cDNA in the methylotrophic yeast *Hansenula polymorpha* essentially as described (*40*). The chromophores PCB and PEB were isolated following literature protocols (*41*, *42*), and PΦB was obtained from PEB by oxidation with DDQ (*41*). Pigments were purified by HPLC (reverse phase C18, acetonitrile, phosphate buffer, 7.5 mM, pH 7.6, 1 mL/min) and subsequently crystallized. Details of the apoprotein expression and of its assembly with PCB or PΦB can be found in earlier publications (*23*, *40*).

FTIR Measurements. The preparation of phytochrome samples for infrared spectroscopy and the experimental setup to obtain FTIR difference spectra at low temperature have been described (*37*). Spectral resolution was 4 cm^{-1}, and 256 scans were accumulated for each single beam spectrum before and after illumination. The band intensities of the spectra can be estimated from the strongest bands which extend from 0.1 milliabsorbance units (mOD) for the early photoproducts (lumi-R, meta-R_a) to 2 mOD for the Pfr spectrum (Figures 2 and 3). The different spectral intensities are due to the gradually reduced photoconversion at low temperatures. The conditions for obtaining the various phototransitions in phyA65 have been optimized by UV−vis spectroscopy.

RESULTS

The light-induced FTIR difference spectra of the photoreactions of recombinant phyA65 reconstituted with the native chromophore, phytochromobilin (phyA65-PΦB), are shown in Figure 2. The spectra were obtained from samples hydrated with ^1H_2O. Intermediates of the photoreaction were accumulated at low temperatures by irradiation of Pr and Pfr with red and far-red light, respectively, under the conditions given in the figure legend. Positive bands represent the photoproducts, while negative bands represent the parent states, viz. Pr in the spectra of the Pr → Pfr pathway (i.e., lumi-R, meta-R_a, meta-R_c, and Pfr), and Pfr

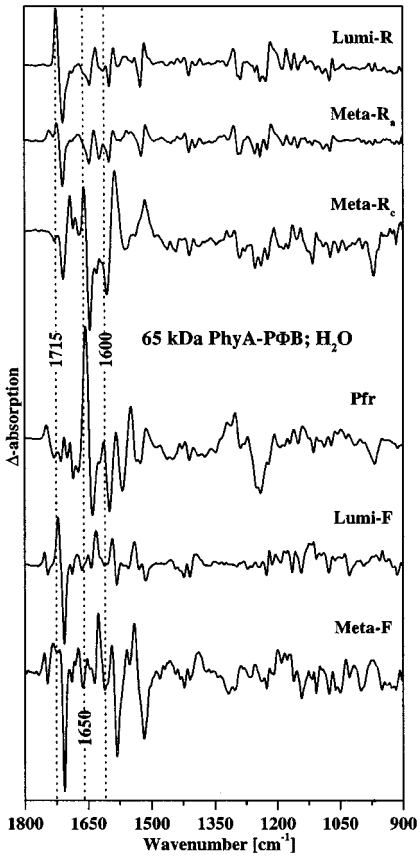

FIGURE 2: Light-induced FTIR difference spectra of the photoreactions of recombinant 65 kDa phytochrome assembled with phytochromobilin (phyA65-PΦB) recorded in ^1H_2O. From top to bottom: lumi-R ($T = -140$ °C); meta-R$_a$ ($T = -80$ °C); meta-R$_c$ ($T = -35$ °C); Pfr ($T = 0$ °C); lumi-F ($T = -140$ °C) and meta-F ($T = -70$ °C). Positive Bands represent the respective photoproduct, negative Pr (lumi-R, meta-R$_a$, meta-R$_c$, Pfr) and Pfr (lumi-F, meta-F).

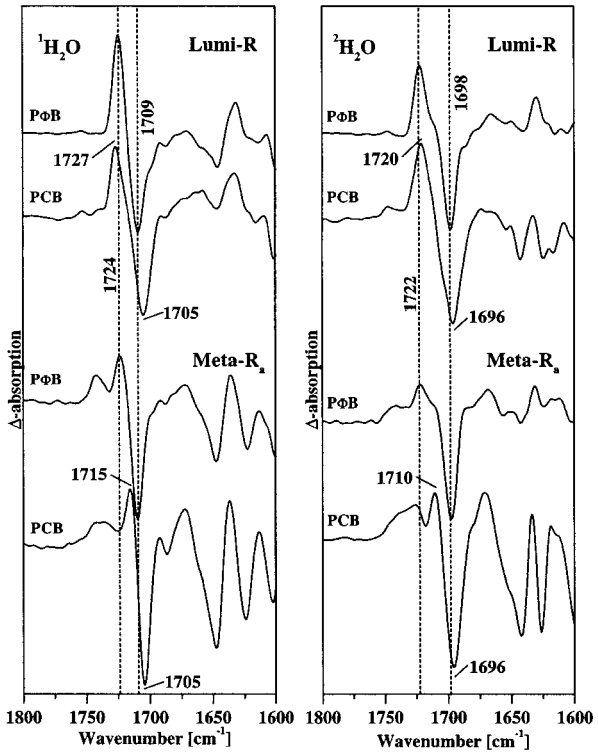

FIGURE 3: FTIR difference spectra of lumi-R and meta-R$_a$ recorded in ^1H_2O (left panel) and in ^2H_2O (right panel), respectively, in the region between 1800 and 1600 cm^{-1}. From top: lumi-R (phyA65-PΦB); lumi-R (phyA65-PCB); meta-R$_a$ (phyA65-PΦB); meta-R$_a$ (phyA65-PCB). The chromophores (PCB or PΦB) are indicated in each spectrum.

in the spectra of the reverse reaction (i.e., lumi-F and meta-F). The spectra are very similar to those of the full-length pigment isolated from oat seedlings (*37*). We therefore conclude that the recombinant N-terminal 65 kDa chromophore domain retains the basic spectroscopic features of the photoconversion while providing a considerably better S/N ratio.

The spectra are generally characterized by large positive and negative bands above 1500 cm^{-1}, whereas numerous small bands are observed in the other spectral regions. A pronounced difference band around 1715 cm^{-1} (wavelengths of difference bands are given by the approximate zero crossing) shows up in the spectra of the early steps of the photoreactions (lumi-R, meta-R$_a$, and lumi-F), decreases in intensity during the progressing conversion (meta-R$_c$ and meta-F), and is absent in the spectrum of the transition to Pfr. In this spectral range, bands representing alterations of carbonyl and carboxyl groups are expected. The spectra of the late intermediates of both photoreactions, i.e., meta-R$_c$ and meta-F, as well the spectrum of the parent states show intensive bands in the regions of the amide-I (1680−1630 cm^{-1}) and amide-II modes (around 1550 cm^{-1}) indicating increasing conformational changes of the protein backbone during the respective transitions. Below 1500 cm^{-1}, all spectra of the Pr → Pfr pathway show a similar pattern of bands, with the exceptions of the negative bands around 970 and 1240 cm^{-1}. The former band only appears with the formation of meta-R$_c$ and Pfr, and the latter band mainly develops with the formation of Pfr, but a smaller broad negative absorbance is already superimposed onto the more narrow bands in meta-R$_c$. Reconstitution of the apoprotein with uniformly ^{13}C-labeled chromophores demonstrates that most of these bands are caused by the chromophore (data not shown). Since this so-called "fingerprint" region is expected to reflect the conformation of the chromophore, it can be deduced that in lumi-R some part of the prosthetic group has been already converted into the final Pfr-conformation, although, as will be shown below, rings A and D still undergo considerable further changes.

Reconstitution with phycocyanobilin (PCB) introduces an ethyl group instead of a vinyl group at position 18 of ring D (Figure 1a,b). In homology to recent spectroscopic investigations (*43*), we have found that in general the FTIR spectral patterns of the photoreactions are the same for phyA65-PΦB and phyA65-PCB. However, small but distinct alterations are observed in all spectra throughout the spectral range (data not shown). Here, we shall concentrate on the spectral range above 1680 cm^{-1}, where altered peak positions allow unequivocal assignments to distinct functional groups of the chromophore.

In Figure 3 we compare the lumi-R and meta-R$_a$ spectra of phyA65-PΦB and phyA65-PCB for measurements in ^1H_2O (left panel) and ^2H_2O (right panel). For normalization of the spectra, we used the negative band around 1411 cm^{-1} (Figure 2) which is hardly influenced by the ethyl for vinyl substitution at ring D [see the corresponding spectra of the full-length phytochrome (*37*)]. It is evident that this replace-

ment causes small but clearly resolvable shifts of the difference band around 1715 cm^{-1}. In phyA65-PΦB, the lumi-R difference band is located at 1724(+)/1709(−)cm^{-1}, and it shifts to 1727(+)/1705(−)cm^{-1} in phyA65-PCB (^1H_2O). In ^2H_2O, it is found at 1722(+)/1698(−)cm^{-1} for the phyA65-PΦB adduct and at 1720(+)/1696(−)cm^{-1} for phyA65-PCB. We have found previously that in the spectra of meta-R_a of the full-length pigment the maximum of this band is reduced in intensity, whereas the minimum stays unaltered (*37*). This is also observed for the phyA65-PΦB adduct where the band shows the same peak positions as in lumi-R, however, with considerably reduced intensity. In contrast, the maximum of this band in the spectra of the phyA65-PCB adduct is not only reduced but additionally shifted to lower wavenumbers and is found at 1715 cm^{-1} (^1H_2O) and 1710 cm^{-1} (^2H_2O), respectively. Furthermore, the band around 1740 cm^{-1} is obviously broadened in phyA65-PCB (Figure 3, lower spectra), suggesting the presence of additional small band(s) that are influenced by the chromophore variation. Additionally, the bands between 1670 and 1600 cm^{-1} show increased relative intensities in the phyA65-PCB spectra. Experiments with phyA65 reconstituted with uniformly ^{13}C-labeled chromophore clearly show that these bands are caused by the protein and are attributed to amide-I spectral changes. They reflect changes of the protein backbone (data not shown). Thus, as compared to phyA65-PΦB, the protein carrying the PCB chromophore has already undergone larger conformational changes toward meta-R_c, despite the low temperature.

^{18}O-labeling of the PCB chromophore at C_1 in the phyA65 causes clear band shifts above 1720 cm^{-1} in the spectra of Pfr, lumi-F, and meta-F. The respective spectra of the labeled (marked by an asterix) and unlabeled holoprotein are compared in Figure 4 in the region between 1800 and 1650 cm^{-1} for measurements in ^1H_2O (left panel) and ^2H_2O (right panel). In the Pfr spectrum (^1H_2O), a difference band at 1749(+)/1731(−)cm^{-1} nearly disappears upon ^{18}O labeling (upper two traces). This band is obviously shifted to lower frequencies and is now found at 1721(+)/1711(−)cm^{-1}. In the spectra of the intermediates of the reverse reaction (lumi-F and meta-F), the bands around 1750 cm^{-1} also nearly disappear upon isotopic labeling. While in lumi-F the shifted band is hidden under the large difference band around 1715 cm^{-1}, it can be discerned in the spectrum of meta-F around 1725 cm^{-1}.

The corresponding spectra recorded in ^2H_2O show very similar difference bands, which confirm these observations (Figure 4; right panel). The Pfr difference band at 1744(+)/1722(−)cm^{-1} is shifted by approximately 23 cm^{-1} to lower frequencies by the labeling and therefore almost vanishes in the spectrum of phyA65-PCB-C_1=^{18}O. Similarly, in the spectra of lumi-F and meta-F, the difference band at 1748(+)/1740(−)cm^{-1} is red-shifted. It is now hidden under the large band around 1710 cm^{-1} in lumi-F and shows up around 1719 cm^{-1} in meta-F.

In the other spectral regions of the spectra shown in Figure 4, as well in the spectra of lumi-R, no spectral changes caused by this isotopic labeling can be unequivocally identified. A corresponding band is seen in the spectrum of meta-R_a (data not shown). However, due to considerable overlap with other bands and due to its small size, it is difficult to be unequivocally identified. Thus, we can conclude that ring A

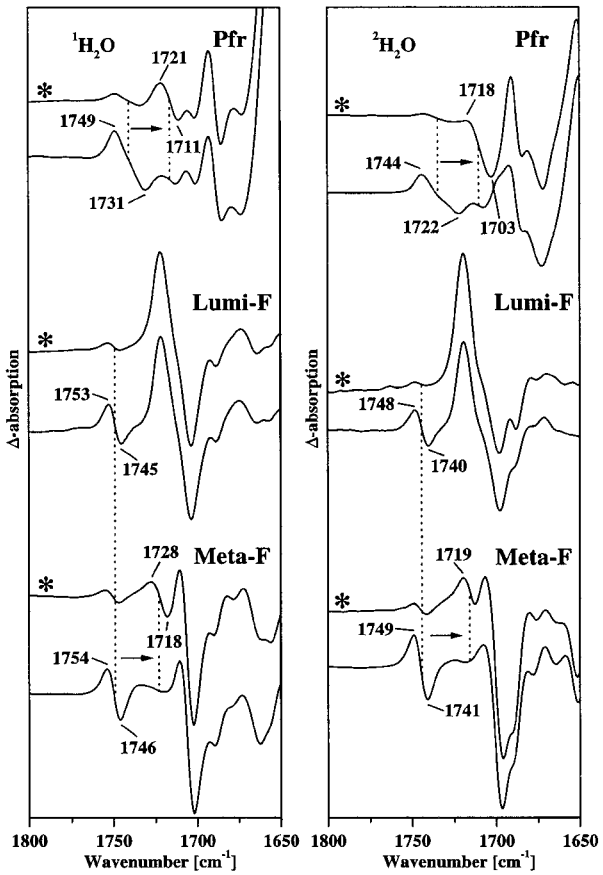

FIGURE 4: FTIR difference spectra of photopproducts of the phyA65-PCB adducts recorded in ^1H_2O (left panel) and in ^2H_2O (right panel), respectively, in the region between 1800 and 1650 cm^{-1}. The spectra of the pigment reconstituted with selectively isotopically labeled PCB are marked by an asterisk. From top to below: Pfr (phyA65-PCB-C_1=^{18}O); Pfr (phyA65-PCB); lumi-F (phyA65-PCB-C_1=^{18}O); lumi-F (phyA65-PCB); lumi-F (phyA65-PCB-C_1=^{18}O); meta-F (phyA65-PCB).

is only affected in the late steps of the Pr → Pfr phototransformation but in all steps of the reverse reaction. For a simple diatomic oscillator, the ^{18}O-induced isotope shift would amount to 41 cm^{-1}. Therefore, the shift of approximately 25 cm^{-1} observed for the C_1=O stretch is small, indicating considerable mixing with modes of ring A.

DISCUSSION

Because of its better expression and increased S/N, we have studied phyA65. However, with only few small differences, the obtained light-induced FTIR difference spectra (Figure 2) are in very good agreement with those of the full-length pigment (*37*). Consequently, the observed changes in infrared difference spectra of the full-length pigment mainly represent molecular changes of the N-terminal half of the pigment including the chromophore binding site, and phyA65 is an excellent model for the study of the molecular events during the photoreactions in this part of the protein. Since the expression of the 65 kDa apoprotein and of corresponding mutants as well as their assembly with native and artificial chromophores has become very effective (*23*, *24*, *40*, *44*), the different spectroscopic techniques are now able to demonstrate their full power. Although the primary photochemistry is the same, the full-length phytochrome needs to be studied to understand the signaling

mechanism. Therefore, the small but reproducible differences have to be further investigated.

Assignment of the C_{19}=O Stretching Vibration (Ring D). In a previous investigation of the intermediates of the photoreactions of native PhyA, we tentatively assigned the large difference band around 1715 cm^{-1} in the spectra of the early intermediates to the C_{19}=O group of ring D of the chromophore (*37*). Since the *Z,E* isomerization of the C_{15}=C_{16} double bond takes place during the formation of lumi-R (*45*), it can be expected that vibrational modes of ring D are particularly affected. The spectra of this investigation now provide direct evidence for this assignment: as demonstrated, in the lumi-R-spectrum the replacement of the vinyl by an ethyl group clearly influences the band at 1724(+)/1709(−)cm^{-1} (^1H_2O) (Figure 3, left panel, upper traces). Similar alterations are also observed in the spectra measured in ^2H_2O (Figure 3, right panel), as well as in the spectra of lumi-F and meta-F (data not shown). Since the modification is next to the 19-carbonyl group (Figure 1) of ring D, and since there is no direct interaction between rings A and D in the extended native conformation of the chromophore, this band must be assigned to the C_{19}=O stretching mode of ring D.

There is general agreement that the chromophore is protonated in Pr, but conflicting evidence is given for the protonation of Pfr and the intermediates in the Pr → Pfr photoreaction (*26*, *28*, *30*, *35*, *46*−*49*). From our infrared investigations of model compounds and using the tentative assignment of the C_{19}=O stretching mode in the lumi-R, meta-R_a, lumi-F, and Pfr difference spectra of native PhyA, we suggested that the chromophore is protonated throughout, viz. in Pr, in Pfr, and in the respective intermediates (*37*, *47*). This has now been confirmed by the unequivocal assignment of this mode in the difference spectra. It is important to note that for the measurements in ^1H_2O the frequency shifts induced by the replacement of the vinyl group for an ethyl group are different in sign for Pr and lumi-R. This shows that these shifts are not intrinsic properties of the modified chromophore but are probably caused by different chromophore−protein interactions, as will be shown detailed in the discussion of possible molecular causes for the upshift of the C_{19}=O stretching occurring in lumi-R and meta-R_a.

Specific Chromophore−Protein Interactions in Meta-R_a. From the appearance of additional band(s) around 1730 cm^{-1} and the increased bands around 1640 cm^{-1} attributed to amide-I bands in the meta-R_a spectra of phyA65-PCB, it can be deduced that the photoreaction of phyA65-PCB has already proceeded further than in phyA65-PΦB. The ethyl group in the PCB chromophore is more flexible than the vinyl group, relieving some steric interaction with the protein. It appears plausible that this allows the protein to undergo larger conformational changes at this low temperature. The meta-R_a spectra of phyA65-PΦB show the C_{19}=O stretch at positions similar to lumi-R; however, the intensity of the maximum of this band is considerably reduced (Figure 2, upper traces). In the corresponding spectrum of phyA65-PCB, the positive part of the C=O-band being now located at 1715 cm^{-1} (^1H_2O) and 1710 cm^{-1} (^2H_2O) exhibits a considerable downshift, whereas the minimum, representing Pr, is unaltered (Figure 3, lower traces). From the downshift it can be suggested that in comparison to the native chromophore, ring D of the PCB-chromophore shows a considerably different behavior in meta-R_a. Changes in hydrogen bonding of the carbonyl or NH group with the protein, which are influenced by the C_{18} substituent, can explain these spectral features. In addition, as will be suggested below, a difference in the torsion of the methine bridge connecting rings C and D may cause this alteration. The modified steric interaction would be the cause for both molecular explanations.

Assignment of the C_1=O Stretching Vibration (Ring A). The assembly of the isotopically labeled chromophore PCB−C_1=^{18}O (Figure 1a) to the phytochrome apoprotein allows the identification of the C=O stretch of the carbonyl group of ring A in the infrared spectra of the photoreactions. In the spectra of Pfr, lumi-F, and meta-F, significant shifts are observed for the relatively weak difference bands above 1730 cm^{-1} (^1H_2O) and 1720 cm^{-1} (^2H_2O). They are nearly absent in the spectra of the isotopically labeled compound (Figure 4). Therefore, these difference bands definitely represent changes of the carbonyl group of ring A during the photoconversions. The observed very small residual bands in this spectral region are obviously due to the portion of unlabeled PCB which amounts to 10%.

Upon formation of Pfr from Pr, the frequency of the C=O stretching vibration of ring A is shifted from 1731 to 1749 cm^{-1} (Figure 4, left panel, second trace). The assigned infrared spectra of model compounds exclude that a deprotonation takes place in this transition, since then a downshift of the C_1=O stretching mode should have been observed also for this mode, although the influence of protonation on the C=O stretch of ring A is smaller than in the case of ring D (10 vs 15 cm^{-1}) (*50*). The influence of protonation on the C=O stretch of ring A can be explained by the resonance structure of the O=C−N=C− part of ring A, which via the nitrogen lone-pair provides some coupling with the conjugated system. The structural data of PCB in the α-subunit of C-phycocyanin indicate that this part is almost planar (*25*) promoting the resonance structure. Since this planarity is an intrinsic property of the chromophore, this applies also to phytochrome.

Molecular Interpretation of the Shifts of the C=O Frequencies of Rings D and A. Possible molecular mechanisms causing a change in the carbonyl frequency of ring D have been discussed previously (*37*), and since we now have assigned the carbonyl mode of ring D, they are shortly summarized here. An obvious influence is hydrogen bonding to the C=O group, lowering the frequency. Furthermore, since the downshifts caused by $^1H/^2H$-exchange indicate coupling of the C=O modes with the in-plane NH bending modes of the same ring, changes in the NH frequency can also induce shifts of the C=O stretch. Such alterations could be caused by different hydrogen bonding of the NH group to protein residues, a stronger bond increasing the frequency, thus increasing the coupling, and thereby causing an upshift of the C=O stretch. Thus, the shifts induced by $^1H/^2H$-exchange provide information on this hydrogen bond: if the shifts differ in the parent state and photoproduct, a change in hydrogen bonding can be assumed. For the PΦB pigment, a considerable change of this hydrogen bond occurs during the Pr → lumi-R transition, the isotope shift amounting to 2 (lumi-R) and 11 cm^{-1} (Pr) (Figure 3, upper traces). Thus, the NH group appears considerably less hydrogen bonded

to the environment in lumi-R. The reduced shift in lumi-R, caused by a reduced coupling, precludes this change in hydrogen bonding of the NH group as a mechanism for the upshift of the C═O mode in lumi-R (*37*). However, our infrared studies of model compounds have identified an additional factor influencing the C═O stretching frequency: the degree of conjugation. The higher the conjugation, the lower is the frequency. If the ring is saturated (such as ring A), the mode is at a considerably higher frequency (30 to 40 cm^{-1}) (*47*). In addition, the frequency of ring D is upshifted by 15 cm^{-1} upon protonation of the chromophore, which can be only explained by the extensive conjugation (*50*). Thus, it appears plausible that twists around the methine bridge connecting ring C and D, which reduce the conjugation, will cause an upshift.

Because of the many influences on the C═O stretch of ring D discussed above, it would be desirable to have some reference data, in which the geometry and/or the environment are defined. Replacing the hydrophobic solvent CCl_4 by the hydrogen bond donor C^2HCl_3 causes a downshift of the C═O stretch of the unprotonated chromophore from 1696 to 1685 cm^{-1} (*47*, *50*). However, in solution, the chromophore exhibits a different (cyclic) geometry and has, due to its pronounced flexibility, also a less defined conformation (*51*, *52*). The chromophore geometries are known, however, in another biliprotein, viz. phycoerythrocyanin. In the α-subunit, the PVB chromophore undergoes a *Z*,*E* isomerization in the native state. The crystal structure has been determined at 2.7 Å resolution in the form of the heterotrimer ($(\alpha\beta)_3$) (*53*). Since the *Z*,*E* isomerization also involves the methine bridge connecting rings C and D (*33*), FTIR difference spectroscopy could identify the C═O stretching of ring D (*54*). As we have argued previously, Asp87 serves as the counterion for the protonation of the chromophore. Although it belongs to the α-subunit, it is close to the neighboring *β*-subunit: the C═O group of ring D is in a hydrophobic environment, contacting the ring of Phe74 and the main chain of His74 both located on the *β*-subunit. Our infrared studies showed that in the α-monomer, as compared to the hetereotrimer, the corresponding C═O frequency of the initial state is located at 1710 cm^{-1}, (*54*). The structural data indicate that rings C and D are somewhat twisted around the methine bridge (*53*). However, it is not sufficient to interrupt conjugation. Therefore, protonation still shifts up the C═O stretch of ring D.

Using these reference data, the position of the C═O stretch in Pr at 1709 cm^{-1} indicates that this group is also in a hydrophobic environment. In addition, since studies of model compounds have shown that this mode of the protonated chromophore in the hydrogen bonding solvent C^2HCl_3 is found around 1702 cm^{-1} (*50*), a hydrogen bonding environment of this group in the Pr state can be clearly excluded. Therefore, the large upshift to 1724 cm^{-1} observed in the transition to lumi-R (Figure 3) cannot be explained by an environmental change of the C═O group and, as explained above, of the neighboring NH group. Therefore, we suggest the following mechanism.

Since the C═O frequency is influenced by the degree of conjugation, it appears plausible that the upshift in lumi-R can be explained by twists of the methine bridges, especially that between rings C and D, since this would reduce the conjugation. This mechanism is supported by the observation that for all photoreactions of bilin pigments so far studied by us, some of which differ greatly in their amino acid composition, such an upshift is a common feature of the first photointermediate: lumi-R and lumi-F (this work and (*37*)) of plant phytochrome; in *Synechocystis* cyanochrome, the photoproduct of Pr obtained below −40 °C and the photoproduct of Pfr obtained below −100 °C (*55*), and the low-temperature photoproduct of phycoerythrocyanin (*50*). Because of the different protein and chromophore systems, a twist of the chromophore around the isomerizing methine bridge appears the most plausible explanation for this common frequency upshift. This is further supported by the downshift of a band in the transition to lumi-R from 1599 to 1589 cm^{-1} (Figure 1), which is also explained by a reduction in conjugation: replacement of the vinyl group by an ethyl group also induces a downshift on both the Pfr and lumi-R bands by approximately 6 cm^{-1} (data not shown) indicating that it mainly represents the C═C stretch of ring D.

The position of the C═O stretch of ring A in Pr is characteristic of a moderate hydrogen bonding environment (for protonated PCB in C^2HCl_3 this mode is located at 1735 cm^{-1}) (*50*). Since ring A is saturated, the degree of conjugation of the C═O group is considerably reduced as compared to ring D. Therefore, twists of the methine bridge connecting rings A and B will have a reduced effect on the C═O stretch. The isotope shift induced by $^1H/^2H$-exchange is much larger in Pr than in Pfr (9 vs 5 cm^{-1}). Again, this shows that the environment of the NH group of ring A is altered, the hydrogen bond being weakened in Pfr. However, similarly as has been discussed for lumi-R with respect to the C═O stretch of ring D, this change in hydrogen bonding cannot explain the upshift of the C═O stretch in Pfr, since this would rather cause a downshift. With the formation of Pfr larger conformational changes of the protein take place. Therefore, a change in hydrogen bonding of the C═O group of ring A with the protein environment is plausible. Our studies of model compounds showed that the C═O stretch of the unprotonated 2,3-dihydrobiliverdin chromophore shifts from 1724 (C^2HCl_3) to 1734 cm^{-1} (CCl_4) (*47*, *50*). Thus, the upshift observed in Pfr (1749 cm^{-1}) vs Pr (1731 cm^{-1}), which does not occur in the earlier intermediates, is well explained by a transition to a more hydrophobic environment, although a larger twist of ring A vs ring B may still contribute. However, the additional smaller upshifts observed in lumi-F (apparent band position 1753 cm^{-1}) and meta-F (apparent band position 1754 cm^{-1}) vs Pfr cannot be explained by a further increase in hydrophobicity since the band position of Pfr already is characteristic of a very hydrophobic environment. Therefore, we ascribe it to a stronger twist of the methine bridge. Such a stronger twist appears plausible for the intermediates of the backreaction. This twist is released with the formation of Pr, which involves larger structural changes of the protein.

It is interesting to note that in the forward reaction the carbonyl mode of ring A only appears with the formation of Pfr, whereas in the backreaction, it is already modified with the formation of the first intermediate lumi-F. From resonance Raman experiments, it has been suggested that the forward reaction involves a two-step isomerization, first around the double bond (producing the C_{15}-*E*, *syn* geometry), and subsequently around the single bond (producing the final

C_{15}-*E*, *anti* geometry), whereas in the backreaction a simultaneous isomerization takes place (*29*). This would fit into our observations: in lumi-R, the distortion of the chromophore is mainly constrained to twists between rings C and D, causing the upshift of the carbonyl mode of ring D. When the photoreaction proceeds further by rotation around the single bond and by invoking larger structural changes of the protein, the CO of ring A is influenced (as mentioned above, perhaps already altered in meta-R_a). With the formation of Pfr, the strain of the chromophore between rings C and D is largely relaxed, and because of the C_{15}-*E*, *anti* geometry, the environment of the carbonyl of ring D is not drastically altered. This results in a C_{19}═O stretching frequency very similar to that found in Pr. In the backreaction, because of the simultaneous bond rotation, twists between rings A and B are already produced in lumi-F, in addition to the twists between rings C and D discussed above. Because of the larger conformational changes of the protein, they are released in Pr. However, it should be mentioned that this analysis relies on the C_{15}-*Z*, *syn* geometry in Pr, for which no unequivocal proof is available. In any event, our data clearly demonstrate that the backreaction is not just the reversal of the forward reaction. In the latter, ring A is only influenced at the later stages of the reaction, whereas in the former it is already altered in the first step. Such different pathways in the isomerization agrees with the observations that for the forward reaction a barrier is present in the electronic excited state, leading to a relatively long formation time of lumi-R, whereas the formation of lumi-F in the back reaction is much faster (*20*, *56*, *57*).

Recently, a new mechanism of the Pr → Pfr photoreaction was proposed from spectral homologies between phytochrome and model compounds (*35*). The authors suggest that in Pfr the chromophore is not isomerized, but a serylimino-ether is formed (i.e., a serine residue is bound via an etherbridge to ring A of the chromophore). However, this would imply the disappearance of the carbonyl group of ring A in Pfr. The presence of this carbonyl in Pfr (Figure 4, second traces), as is presented in our work, definitely rules out this mechanism.

ACKNOWLEDGMENT

We are very thankful to Prof. A. Gossauer and Dr. N. Engel (University of Fribourg, Switzerland) for giving initial impulses for the synthesis of ^{18}O-labeled chromophore. We thank W. Schyja (Freiburg, Germany) for practical help in handling the chromophores. We also acknowledge the use of the mass spectroscopy facility of the Department of Organic Chemistry and Biochemistry, University of Freiburg.

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BI0156916