Chromophore structure of the physiologically active form (Pfr) of phytochrome

(bilipptides/NMR spectroscopy/cis–trans isomerization/tetrapyrroles)

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ABSTRACT Chromopeptides were prepared by proteolytic digestion of phytochrome (far-red absorbing form, Pfr) and of phytochrome. The phycoerythrobilin peptide, the chromophore of which is Z,Z,Z-configurated, was modified to the Z,Z,E isomeric chromophore. This peptide behaves similarly with regard to spectral and chromatographic properties and reactivity. We present evidence here, obtained by high-resolution 1H NMR spectroscopy, that both the modified phycoerythrobilin chromophore and the phytochrome chromophore obtained directly from Pfr are 15E-configurated.

Plant development is influenced by light in many ways. An important photoreceptor of higher plants is the chromoprotein phytochrome (1–3), which can mediate light-dependent irreversible differentiation (e.g., seed germination, flowering, and stem and leaf growth) and reversible modulations (e.g., leaflet or chloroplast movement, root tip adhesion, and transmembrane potentials).

A characteristic property of phytochrome in vivo (in the plant cell) and in vitro is its photoreversibility. The physiologically inactive (red-absorbing) Pfr form ($\lambda_{max} = 660$ nm) is transformed by red light to the physiologically active Pfr form ($\lambda_{max} = 730$ nm), which in turn is reconverted by far-red light to the Pfr form.

$$P_r \overset{660 \text{ nm}}{\rightleftharpoons} P_{fr}$$

The chemical structure of the Pfr chromophore (structure Ia), including its linkage to the protein, was elucidated by combination of oxidative degradation and UV/visible spectroscopy (4–6), by comparison of the cleaved chromophore with the product obtained by total synthesis (7, 8), and by high-resolution NMR spectroscopy of a chromopeptide (9). It is closely related to the structure of the phycoerythrin chromophore (structure Ib), which had been investigated with similar methods (3).

We recently have demonstrated (10) that chromopeptides can be obtained from the Pfr form of phytochrome that are different from Pfr chromopeptides with regard to the chromophore. The differences concern chromatographic properties, UV/visible spectra, and chemical reactivity (10). Pfr chromopeptides are readily transformed into Pfr chromopeptides by light. This transformation takes place slowly also in the dark. The relatively best stability of Pfr peptides was found at pH 2–4. Addition of bases in the dark apparently reverts the Pfr peptide immediately into the Pfr peptide, as monitored by the spectral properties and the stability towards irradiation. Reduction or oxidation of the Pfr chromophore also leads to partial transformation to the Pfr chromophore. The same properties as for the Pfr chromophore have been demonstrated for geometric isomers (E-configurated) prepared from all-Z-configurated Pfr chromopeptides and phycoerythrin chromopeptides (11). Similar properties also were reported for E-configurated bilinoides (12–17). It was concluded that the chromophore of the Pfr peptide should be either the 15E (structure Ila) or the 4E (structure IIIa) compound (11). The E-configurated phycoerythrin peptide should be the ethyl analogue IIb or IIib (11). We present evi-

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MATERIAL AND METHODS

The $^1$H NMR spectra were recorded either with a Bruker WM500FT NMR (quadrature detection) at 100°C or with a Bruker WP80FT NMR at 35°C. Both instruments were connected with the Aspect 2000 data system. The chemical shift values are in ppm from sodium trimethylsilylpropane sulfonate. Acetone was used as the internal standard (2.050 ppm). The solvent system was acetone-d$_6$/H$_2$O/CF$_3$COOH, 8:1:1 (vol/vol) (solvent system A), if not otherwise stated. The H$_2$O peak was reduced with the gated decoupling method.

Spectra were recorded at first of the Z,Z,E compounds Ia and Ib, respectively, and subsequently of the phototransformed products Ia and Ib, which were obtained by irradiation of the solutions directly in the NMR tube.

Chromopeptide from Phycocyanin. C-Phycocyanin was isolated from Spirulina maxima and digested with pepsin as described (11). Chromopeptides were purified by chromatography on Bio-Gel P-10 and on silica gel and by subsequent isoelectric focusing (11). The chromopeptide used in this study (pI = 5.03) had the amino acid composition Arg$_2$Lys$_2$Ala$_2$Gly$_2$Gly$_2$Asx$_2$Cys$_3$ (11). Photoisomerization was achieved after addition of 2 vol of mercaptethanol to 3 vol of 0.1 M Tris-HCl (pH 8.5) containing $2.4 \times 10^{-5}$ mol of chromopeptide as described (11). All subsequent steps were performed either in the dark or under dim-green safety light. The Z,Z,E-configurated chromopeptide IIb was isolated by chromatography on Bio-Gel P-10 and silica gel (11). The silica gel column that contained the adsorbed IIb was washed with water and then with acetone-d$_6$ to remove all nondeuterated solvents. The chromopeptide IIb then was eluted with solvent system A. The yield was $0.65 \times 10^{-7}$ mol, calculated from the UV/visible spectrum after photochemical reversion to the compound Ib, with $E_{665} = 35,500$ (19). The 500-MHz $^1$H NMR spectrum was recorded of this eluate (0.5 ml); the concentration of chromopeptide in the NMR tube was 0.13 mM.

Chromopeptide from Phytochrome. Phytochrome was extracted from etiolated oat seedlings (Avena sativa L., var. Pirol, Baywa München, Federal Republic of Germany) and purified to the step of brushite chromatography as described by Song et al. (20); 130 mg of the small phytochrome (60 kilodaltons) was
collected and applied to a DEAE-Sepharose column (Pharmacia; 5 × 20 cm), which was equilibrated with 10 mM Tris-HCl buffer, pH 7.4/10 mM KCl/1 mM EDTA/1 mM NaN₃. The adsorbed phytochrome was washed with the same buffer and then eluted with the same buffer containing 250 mM KCl. The eluate was concentrated by precipitation with ammonium sulfate at 50% saturation. The precipitate was dissolved in the equilibration buffer and dialyzed against the same buffer. The resulting solution (172 ml), which contained 100 mg of phytochrome (purity index A₆₆₅ = 1:20), was then irradiated at 660 nm to saturation. All subsequent steps were carried out either in the dark or under dim-green safety light. The resulting P₆ solution (=75% P₆/25% P₅) was acidified with HCl to pH 1.5, and ascobic acid was added to a final concentration of 50 mM. The mixture was incubated with 100 mg of pepsin for 1 hr at 37°C under argon gas. The resulting peptides were purified by chromatography on Bio-Gel P-10 and on silica gel as described (11, 18). The silica gel column, which contained the adsorbed P₆ peptide IIa, was washed with water to remove colorless peptides and then with acetone-d₄ to completely remove nondeuterated solvents. The P₆ peptide IIa was then desorbed with a mixture of acetone-d₄/H₂O/CF₃COOH, 85:10:5 (vol/vol) (solvent system B), and stored in this solution (0.5 ml) under argon gas at ~18°C until use. The concentration of IIa was 94 μM calculated from the UV/visible spectrum after phototransformation to Ia, with E₆₀₀ = 32,000 (21). The amino acid composition was Arg₁, Gly₂, His₂, Asp₂, Thr₁, Leu₂, Ile₂, Va₁, Leu₁, Ala₁, Gly₂, Glx₁, Ser₁, Thr₀, Asx₀, Cys₀, and Proline was not determined.

RESULTS AND DISCUSSION

The most prominent feature in the ¹H NMR spectrum of E-configurated bilindiones is the downfield shift of the methine proton (encircled in structure IV) at the E-configurated double bond in comparison with the respective Z isomer (13-17). The methine proton of the opposite, Z-configurated methine bridge shows, if any, only a smaller upfield shift. This has been reported for compounds V-IX, which can be considered as model compounds for chromopeptides from phytochrome and phyocyanin. The difference in the chemical shift for E- minus Z-configurated compounds is apparently independent of the solvent used for ¹H NMR measurement (see Table 2 and discussion below). Therefore, it was important for our problem to record all methine signals of the chromophore (protons at C-5, C-10, and C-15, see structures I-III). Unfortunately, the methine proton next to a hydrogenated ring (5-H in I, II, III, V, and VIII) is easily exchanged in a H⁺ (or D⁺)-catalyzed reaction against ¹H in H₂O (9, 20), CH₃OH (22), and CF₃COOH (23). This could possibly lead to loss of the desired information. The ¹H NMR spectrum of the P₆ chromophore had been recorded in deuteropyridine (9) and that of model chromophores in deuterochloroform. The latter solvent was unsuitable for the chromopeptide because of lack of solubility. As outlined above, the P₆ chromophore is transformed into the P₅ chromopeptide by bases like pyridine. Therefore, it was necessary to find a solvent that meets the requirements for solubility and stability of the P₆ chromophore and for lack of deuteration exchange at 5-H. The last purification step leaves the P₆ chromoprotein adsorbed at a silica gel column. Elution in a sharp zone would yield a highly concentrated solution of the chromopeptide and eventually allow the transfer into a deuterated solvent without lyophilization. Therefore, the solvent system also had to be designed for good elution of P₆ chromoprotein from the adsorbed state. After a number of experiments with various solvents, the solvent system B proved to be most suitable. The silica gel column is washed at first with deuterated acetone to remove all protonated solvents. This treatment does not elute the chromopeptide. The chromopeptide is then eluted with the indicated solvent mixture in a sharp zone. The solvent mixture allows ¹H NMR measurements that yield sharp, easily interpretable resonances even at low temperatures. At 10°C, the stability of the P₆ chromophore is sufficient, and the methine signals are undisturbed by the temperature-dependent H₂O signal. 5-H is kept in its protonated form by the nondeuterated solvents (H₂O and CF₃COOH). This proton exchange does not lead to line broadening because the exchange is slow in terms of the NMR time scale.

The first aim was to unequivocally localize the methine signals of the chromophore in this acid solvent system. For this purpose, the influence of acid was investigated with the model compound VIIIa. This compound exhibits a relatively simple ¹H NMR spectrum (17). The identification of 5-H is facilitated not only by the ¹H⁻¹H exchange in acids (see above) but also by the splitting of this signal into a doublet. It can be seen (Table 1) that addition of increasing amounts of trifluoroacetic acid to a solution of VIIIa in acetone leads to a pronounced downfield shift of the signal of 10-H, whereas the effect is less dramatic for 5-H and 15-H. Interestingly, the distance between the signals for 5-H and 15-H remains constant throughout the acidification experiment. Addition of water to VIIIa in acetone does not significantly change the positions of the methine signals (not shown).

The next step was the identification of the methine resonances in the ¹H NMR spectrum of the phytochrome peptide (IIb). These resonances were easily identified in pyridine-d₅/H₂O (Table 2) because the position is intermediate between those reported for pyridine and for H₂O (24). In solvent system A, a small upfield shift of the signals for 5-H and 15-H and a larger downfield shift of the signals for 10-H was observed (Table 2). The chemical shifts of these methine resonances are close to those of the model compounds VIIIa (compare with values in Table 1). The assignment of methine resonances of the P₆ chromophore Ia was achieved by comparison with published data for Ia (9) and with data for the phyocyanin chromophore Ib and the model compound VIIIb (Tables 1 and 2).

The crucial ¹H NMR experiment with the phytochrome peptide (IIa and Ia) is shown in Fig. 1; that with the phyocyanin peptide (Ib and Ib) is shown in Fig. 2. Because some dark reversion (IIa → Ia and IIb → Ib, respectively) is inevitable when handling the peptides as discussed earlier (10), two sets of signals are observed due to IIa and Ia (Fig. 1) or IIb and Ib (Fig. 2). The signals of IIa and Ib are identified by the light sensitivity of these peptides. Irradiation of the sample in the NMR tube with white light causes disappearance of the signals of IIa

<table>
<thead>
<tr>
<th>Trifluoroacetic acid, mM</th>
<th>Chemical shift*, ppm</th>
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<tbody>
<tr>
<td>5-H</td>
<td>15-H</td>
</tr>
<tr>
<td>0</td>
<td>5.57</td>
</tr>
<tr>
<td>0.03</td>
<td>5.58</td>
</tr>
<tr>
<td>0.3</td>
<td>5.58</td>
</tr>
<tr>
<td>3.0</td>
<td>5.62</td>
</tr>
<tr>
<td>30.0</td>
<td>5.93</td>
</tr>
</tbody>
</table>

* Taken from 80 MHz ¹H NMR spectra. The chemical shift values are in ppm from acetone-d₅ (2.05 ppm).

Doublet, J = 1.2 Hz.
and IIb and an increase of the corresponding signals of Ia and Ib, respectively.

The \(^1H\) NMR spectrum of the E-configured phycocyanin peptide (Fig. 2) reveals that it has the 15E configuration (IIb) and not the 4E configuration (IIIb). This can be concluded from an inspection of \(^1H\) NMR data of E-configured model compounds (Table 3). The 15-H signal is shifted downfield by 0.16–0.27 ppm by the \(Z \rightarrow E\) isomerization at the 15,16 double bond. The other methine signals (i.e., of 5-H and 10-H for \(Z \rightarrow E\) isomerization at C-15) exhibit, if any, only small shifts during the isomerization, whereas the isomerization at the 4,5 double bond leads to a strong shift of the 5-H signal and, in one case, of the 10-H signal (Table 3). These shifts seem to be (more or less) independent of the solvent used for NMR measurements and of the \(\beta\)-substituents of the compounds.

The \(^1H\) NMR spectrum of the \(P\_c\) chromoprotein (Fig. 1) reveals that it also has the 15E configuration (IIa) rather than the 4E configuration (IIIa). The signal-to-noise ratio is smaller than for the phycocyanin peptide because of a lower concentration of the \(P\_c\) peptide available for the NMR investigation. Nevertheless, the signals for 5-H (and 10-H) can well be assigned. The 15-H signal is less easily detected in the spectrum of the \(P\_c\) peptide because a spike interferes with it. However, a small signal disappears by irradiation exactly at the position expected for 15-H for the 15E compounds.

Comparison of the methine resonances before (\(P\_c\) peptide) and after irradiation (\(P\_c\) peptide) correspond well with methine resonances of the 15E- and 15Z-model chromophores (Table 3) but not with those of 4E (VIc and IXc) and 4Z (VIa and IXa)

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Chromophore & 5-H & 15-H & 10-H & Solvent & Ref. \\
\hline
Phycocyanin Ib & 5.87 & 6.08 & 7.29 & Pyridine-\(\text{d}_5\) & 24 \\
 & 5.94 & 6.32 & 7.41 & \(\text{d}_5\)/Pyridine-\(\text{d}_5\) & 24 \\
 & 5.95 & 6.36 & 7.32 & 10\% pyridine-\(\text{d}_5\)/Pyridine-\(\text{d}_5\) & This paper* \\
E-Configurated & & & & & \\
phycocyanin IIb & & & & & \\
& 5.999 & 6.239 & 7.601 & System A & This paper* \\
\(P\_c\) Ia & 5.90 & 6.16 & 7.26 & Pyridine-\(\text{d}_5\) & 9 \\
& 5.995 & 6.338 & 7.618 & System B & This paper* \\
\(P\_c\) Ia & 5.938 & 6.533 & 7.630 & System B & This paper* \\
\hline
\end{tabular}
\caption{Methine signals of the \(^1H\)-NMR spectrum of bilindiones in various solvents}
\end{table}

\textsuperscript{*} \(^1H\)-NMR (80 MHz) assignments for the methine signals of phycocyanin chromopeptide. The spectra were taken on a Bruker WP80 FTNMR with Aspect 2000 data system at 35\(^\circ\)C. The chemical shifts in pyridine-\(\text{d}_5\)/Pyridine-\(\text{d}_5\) are reported in ppm from tetramethylsilane (internal standard, 0.0 ppm). The chemical shifts in solvent systems A and B were reported in ppm from acetone-\(\text{d}_6\) (2.05 ppm).

\textsuperscript{†} \(^1H\)-NMR (500 MHz) assignments for the methine signals of phytochrome chromopeptide. The spectra were taken on a Bruker WM500 FTNMR with Aspect 2000 data system at 10\(^\circ\)C at Bruker, Analytische Messtechnik (Karlsruhe, Federal Republic of Germany). The chemical shifts are reported in ppm from acetone-\(\text{d}_6\) (2.05 ppm).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Partial 500 MHz \(^1H\) NMR spectrum of phytochrome chromopeptides (94 \(\mu\)M) in solvent system B at 10\(^\circ\)C (see footnote † of Table 2). (Lower) \(P\_c\) chromopeptide (Ia) containing some \(P\_c\) chromopeptide (Ia) due to dark reversion. (Upper) \(P\_c\) chromopeptide (Ia) obtained from Lower by irradiation with white light.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Partial 500 MHz \(^1H\) NMR spectrum of phycocyanin chromopeptide (130 \(\mu\)M) in solvent system A at 10\(^\circ\)C. (Lower) (15E)-Phycocyanin chromopeptide (IIb) containing some (15Z)-phycocyanin chromopeptide (Ib) due to dark reversion. (Upper) (15Z)-Phycocyanin chromopeptide (Ib) obtained from Lower by irradiation with white light.}
\end{figure}
compounds. Furthermore, it has been demonstrated that 2,3-dihydrobilindiones with bulky substituents at C-3 do not form stable 4E isomers (25).

Geometric isomerization of the phytochrome chromophore during the photoconversion of native phytochrome (P₇ → P₉) had been suggested early in phytochrome research (26-28). The present paper strongly supports this proposal and, furthermore, localizes the Z → E isomerization within the chromophore. The suggested chromophore isomerization points to an interesting connection between the plant photoreceptor phytochrome and the animal visual pigment rhodopsin in which Z → E isomerization of the chromophore has been considered as the primary photoprocess (29). Further experiments are needed to reveal whether this possibility exists also in phytochrome. In particular, it has to be checked whether Z → E isomerization is compatible with experimental results, which point to an intramolecular proton transfer as the primary photoreaction in phytochrome (30-32).

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