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Resonance Raman spectroscopic study of the tryptic 39-kDa fragment of phytochrome

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Abstract The 39-kDa fragment of oat phytochrome *phyA*, obtained by tryptic digestion at the amino acids 65 and 425, was studied by resonance Raman spectroscopy. The parent state P_r reveals far-reaching similarities with that of the native phytochrome implying that the structures of the tetrapyrrole chromophore and its immediate protein environment are not affected by the proteolysis. However, the resonance Raman spectrum of the final product of the P_r phototransformation, denoted as P_{bl}, is more closely related to that of the P_{fr} precursor of the native phytochrome, i.e. meta-R_C, rather than to that of P_{fr} itself. The resonance Raman spectra indicate a high conformational flexibility of the chromophore in P_{bl} so that, unlike in P_{fr}, the tetrapyrrole rings C and D adopt a largely coplanar conformation. The protein interactions with ring D of the chromophore, which in the native phytochrome stabilize the specific chromophore structure of P_{fr}, cannot be established in the 39-kDa fragment due to the lack of the major C-terminal part of the protein. These findings, furthermore, support the view that the meta-$R_C \rightarrow P_{fr}$ transition is associated with a coupling of chromophore and protein structural changes that represent crucial events for the photoactivation of phytochrome.

Key words: Phytochrome; Tryptic fragment; Tetrapyrrole; Photoisomerization; Resonance Raman

1. Introduction

Phytochromes exert a variety of functions associated with photomorphogenic processes in plants [1,2]. These photoreceptors include an open-chain tetrapyrrole, phytochromobilin (PΦB), that serves as the chromophoric group (Fig. 1). PΦB is covalently bound via a thioether linkage to a cysteine (Cys-321) of the apoprotein. The biological function is linked to the photoinduced interconversion of the parent states P_r and P_{fr}. This transformation is initiated by a *E*,*Z* photoisomerisation of the methine bridge between rings C and D. Subsequent thermal reaction steps include relaxation processes of the chromophore and the protein which eventually lead to either of the parent states. As P_{fr} represents the physiologically active form of phytochrome, the protein structure of P_{fr} must exhibit specific structural properties that are recognised by signal transducing molecules. A prerequisite for understanding the functioning of phytochrome is, therefore, the elucidation of the coupling of chromophore and protein structural changes.

It is widely accepted that the functionally relevant changes of the protein structure in P_{fr} are associated with the C-terminal part of the protein [3]. Still, recombinant and tryptic phytochrome fragments, that range either from amino acid residues 1 to 595 (65 kDa) or 65 to 595 (59 kDa) only, i.e. lack the C-terminal part, display full photoreversibility and kinetics of the P_r/P_{fr} photointerconversion similar to the wild-type photoreceptor [4–6], in accord with the observation that the chromophore structure and the photoinduced structural changes of the recombinant 65-kDa phytochrome are essentially the same as well [7,8]. However, truncation at position 425 by tryptic digestion substantially affects the formation of P_{fr}, as indicated by the intensity reduction of the long-wavelength absorption band. While the first two intermediates in the $P_r \rightarrow P_{fr}$ phototransformation, lumi-R and meta-R_A (for the nomenclature of the photocycle intermediates, see [9]), can still be detected by low-temperature UV spectroscopy, the formation of the last intermediate meta-R_C as well as of P_{fr} is not observed any more [10]. Instead, a bleached species, P_{bl}, is formed. Thus, it was concluded that in this 39-kDa fragment spanning from amino acid residue 65 to 425, the chromophore–protein interactions during the $P_r \rightarrow P_{fr}$ phototransformation are significantly perturbed [10,11].

In this work, the 39-kDa fragment is studied by resonance Raman (RR) spectroscopy that selectively probes the vibrational spectrum of the chromophore [7,9,12–17]. Thus, this technique provides information about the molecular structure of the tetrapyrrole and its interactions with the protein environment. The present study is directed to determine similarities and differences between the active sites of the 39-kDa fragment and the native phytochrome, which eventually may contribute to a better understanding of the activation of phytochrome on a molecular level.

2. Materials and methods

2.1. Sample preparation

Isolation and purification of oat phytochrome *phyA* and sample preparation for Raman measurements are described in detail elsewhere [9,15]. The 39-kDa fragment was obtained by controlled digestion of the full-length protein with trypsin following the protocol published previously [10].

2.2. RR measurements

The RR spectra of P_r and P_{fr} were obtained with 1064-nm excitation by using a Bio-Rad Fourier transform Raman spectrometer equipped with a Nd-YAG laser (Spectra Physics, FC-106V, bandwidth $< 1\ cm^{-1}$). The phytochrome samples (ca. 0.1 mM) were photoconverted into the desired states and subsequently cooled down to −140°C as described previously [9]. The laser power at the sample was

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360 mW and the spectral resolution was 4 cm^{-1}. The raw spectra were corrected for the instrumental response as described in [18]. In some cases, the measured RR spectra include also contributions by Raman bands of the glass and quartz optics (at ca. 1070 and 760 cm^{-1}) as well as Raman bands of glycerol contaminations originating from the centricon cells used for concentrating the samples. The RR spectra of P_{fr} shown in this work were obtained after subtracting the residual contribution of P_r from the measured spectra using the characteristic RR bands of P_r as a reference [9]. Further details of the measurements and the experimental set-up are given elsewhere [7,9,15].

3. Results and discussion

The RR spectra of the P_r state of the native *phyA* of *Avena sativa* [P_r(*phyA*124)] and its 39-kDa fragment [P_r(*phyA*39)] reveal far-reaching similarities (Fig. 2). The positions of most of the bands are unchanged within the experimental accuracy of ± 1 cm^{-1}, and also the pattern of relative intensities is essentially the same for both species. Thus, it can be concluded that the chromophore structure of the P_r state, which for native *phyA*124 was ascribed to a protonated *ZZZasa* configuration [9], is preserved in *phyA*39. Small but clearly detectable spectral differences, however, are noted for the bands at 1569, 1519, and 1318 cm^{-1} of P_r(*phyA*39) in H_2O. They are downshifted by up to 3 cm^{-1} compared to P_r(*phyA*124). In our previous study, these bands have been assigned to modes of a protonated (cationic) chromophore that involve N–H in-plane (ip) deformation coordinates [9]. Whereas the mode ν_{95} at 1318 cm^{-1} includes the N–H ip deformation of ring D, the modes ν_{50} and ν_{51} at 1569 and 1517 cm^{-1}, respectively, originate from the NH groups of rings B and C. The corresponding mode of the deuterated tetrapyrrole (ν_{121}) is assigned to the band at 1073 cm^{-1} which is also downshifted by 2 cm^{-1} compared to P_r(*phyA*124).

Based on a comparison with the RR spectra of tetrapyrrole pigments with the same chromophore conformation and protonation state, i.e. C-phycocyanin and the P_r state of the phytochrome-like photoreceptor CPH1 from *Synechocystis* [19–21], it was concluded that particularly the frequencies of the N–H ip modes ν_{50} and ν_{95} (and ν_{121} in the deuterated pigments) sensitively reflect altered hydrogen-bonding interactions due to small structural differences at the chromophore binding sites. Therefore, the frequency shifts of these modes in P_r(*phyA*39) compared to P_r(*phyA*124) indicate subtle perturbations of the hydrogen-bonding network involving the tetrapyrrole. The same explanation may hold for the relatively small H/D downshift of the 1640-cm^{-1} band (ν_{46}) in P_r(*phyA*39) which is only -3 cm^{-1} compared to -6 cm^{-1} in P_r(*phyA*124). The overall similarity of the RR spectra of

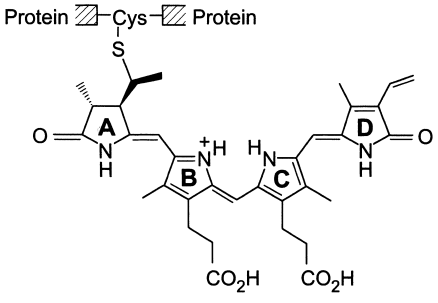

Fig. 1. Structural formula of PΦB.

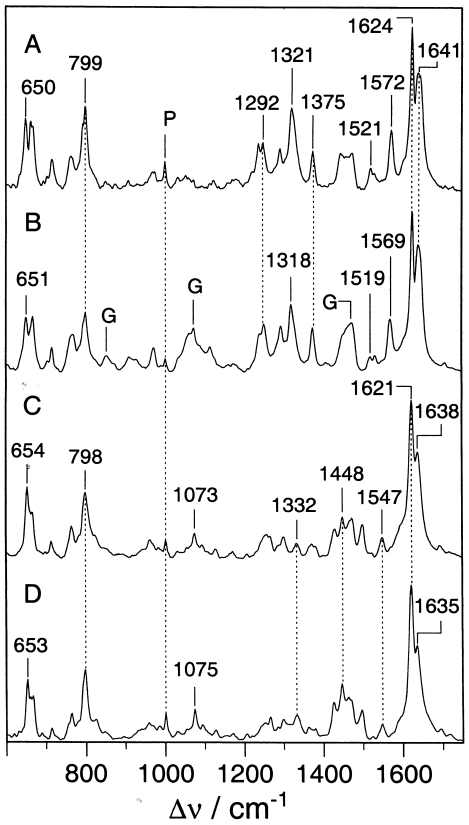

Fig. 2. RR spectra of the P_r state of (A) native *phyA*124 in H_2O, (B) the 39-kDa fragment in H_2O and (C) in D_2O, and (D) the native *phyA*124 in D_2O. All spectra were measured at -140°C with 1064-nm excitation. Raman bands of the protein and glycerol are denoted by 'P' and 'G', respectively.

P_r(*phyA*39) and P_r(*phyA*124), however, rules out major structural differences of the chromophore geometry and the immediate protein environment.

Upon irradiation with 660-nm light, the P_r state of the tryptic 39-kDa fragment is converted to the P_{bl} state. In our experiments, no intermediate states could be trapped in quantities that were sufficient for a satisfactory RR spectroscopic characterisation. Compared to the P_{fr} state of native *phyA*124, the first electronic transition of P_{bl} of the 39-kDa fragment is shifted to shorter wavelengths, from 725 to ca. 695 nm, and the oscillator strength is substantially lowered [10,22].

Fig. 3 compares the RR spectrum of the P_{bl} state of the 39-kDa fragment with those of the P_{fr} and meta-R_C states of native *phyA*124, all measured from samples in non-deuterated buffer. For both, meta-R_C(*phyA*124) and P_{bl}(*phyA*39), the resonance enhancement is comparatively weak as a result of relatively low oscillator strengths of the first electronic transition [10,23]. Consequently, vibrational bands of the apoprotein contribute more strongly to the measured spectra compared to P_{fr}(*phyA*124) [9]. These are, for instance, the bands at 1002 cm^{-1} (Phe), 1640 cm^{-1} (amide I), and the broad and structureless bands at ca. 1290 (amide III) and 1460 cm^{-1} (CH_2 vibrations of amino acid side chains) which, as well as bands originating from glycerol (vide supra), interfere with the RR bands of the chromophore. Nevertheless, for both P_{bl}(*phyA*39) and meta-R_C(*phyA*124) it is possible to analyse those spectral regions which include marker bands characteristic of the chromophore structure in P_{fr}(*phyA*124).

In the C=C stretching region, the RR spectrum of P_{fr}(*phyA*124) in H_2O displays a prominent band at 1598 cm^{-1} attributable to the mode ν_{47} which predominantly includes the C=C stretching of the C–D methine bridge (Figs. 3, 4) [9]. This band is substantially downshifted compared to P_r(*phyA*124) and, hence, is regarded as a characteristic indicator of the specific *ZZE* configuration of the chromophore in P_{fr}(*phyA*124). As this band is at a similar position in P_{fr}(*phyA*124), P_{bl}(*phyA*39) and meta-R_C(*phyA*124), it is very likely that the gross configuration of the tetrapyrrole chromophore is not altered in all three forms. Furthermore, the 1553-cm^{-1} band of P_{fr}(*phyA*124), attributable to the N–H ip deformation of the rings B and C (ν_{50}), is found at the same position also in P_{bl}(*phyA*39) and meta-R_C(*phyA*124), albeit with a markedly lower intensity. The substantial 20-cm^{-1} downshift compared to P_r(*phyA*124) has been interpreted in terms of alterations of the hydrogen-bonding interactions of rings B and C following the photoisomerisation of the chromophore [9]. Thus, these structural rearrangements associated with the inner pyrrole rings appear to occur in the 39-kDa fragment as well. However, the corresponding N–H ip mode of ring D (ν_{95}), which in P_{fr}(*phyA*124) is assigned to the band at 1311 cm^{-1}, is upshifted by 6 and 5 cm^{-1} in P_{bl}(*phyA*39) and meta-R_C(*phyA*124), respectively. These findings suggest that both meta-R_C(*phyA*124) and P_{bl}(*phyA*39) differ from P_{fr}(*phyA*124) in details of the chromophore–protein interactions involving ring D which, in turn, may be related to slightly different conformations of the C–D methine bridge.

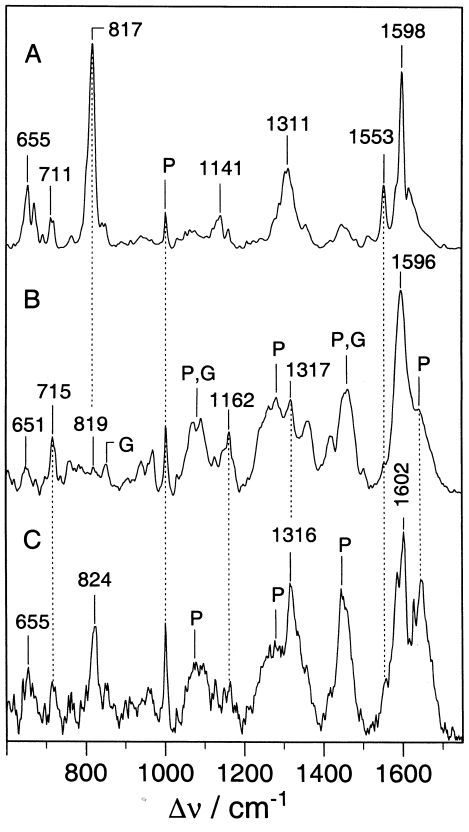

Fig. 3. RR spectra of (A) the P_{fr} state of native *phyA*124 in H_2O, (B) the P_{bl} state of the 39-kDa fragment in H_2O, and (C) the meta-R_C state of native *phyA*124 in H_2O. All spectra were measured at −140°C with 1064-nm excitation. Raman bands of the protein and glycerol are denoted by 'P' and 'G', respectively.

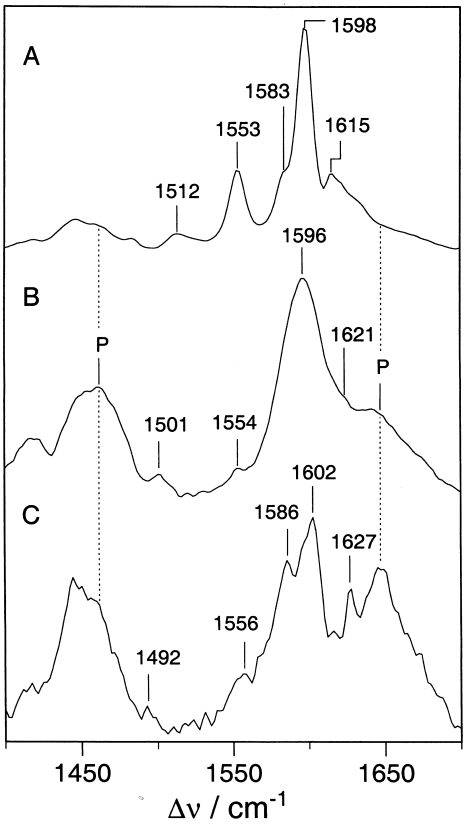

Fig. 4. Expanded view of the RR spectra in the C=C stretching region of (A) the P_{fr} state of native *phyA*124 in H_2O, (B) the P_{bl} state of the 39-kDa fragment in H_2O, and (C) the meta-R_C state of native *phyA*124 in H_2O. All spectra were measured at −140°C with 1064-nm excitation. Raman bands of the protein are denoted by 'P'.

Such an explanation may also account for the striking intensity difference at around 820 cm^{-1}. Whereas in P_{fr}(*phyA*124) the 817-cm^{-1} band is the most intense RR band in the spectrum, a substantially lower intensity is observed for the corresponding bands of meta-R_C(*phyA*124) at 824 cm^{-1} and, particularly, of P_{bl}(*phyA*39) at 819 cm^{-1}. These bands are attributed to a C–H out-of-plane mode of a methine bridge, presumably of the C–D bridge [9,12,15–17]. Furthermore, it has been suggested that a high RR intensity of this mode is indicative for a torsion of the methine bridge single bond. For P_{fr}(*phyA*124), such an idea implies a non-coplanar conformation of the rings C and D whereas in meta-R_C(*phyA*124) and, particularly, in P_{bl}(*phyA*39) the deviation from coplanarity should be much smaller. This interpretation is consistent with the conclusion that the interactions of ring D with the immediate protein environment are qualitatively different in meta-R_C(*phyA*124) and P_{bl}(*phyA*39) compared to P_{fr}(*phyA*124). It is likely that this conformational difference compared to P_{fr}(*phyA*124) is the origin of the reduction of oscillator strength of the first electronic transition in meta-R_C(*phyA*124) and P_{bl}(*phyA*39).

Stabilisation of the distorted tetrapyrrole conformation in P_{fr}(*phyA*124) requires either a sterically particularly constrained chromophore pocket or other protein–chromophore interactions (e.g. hydrogen bonding) or both, which are not provided in the 39-kDa fragment obtained from native *phyA*124. Instead, the loosening of the tertiary protein struc-

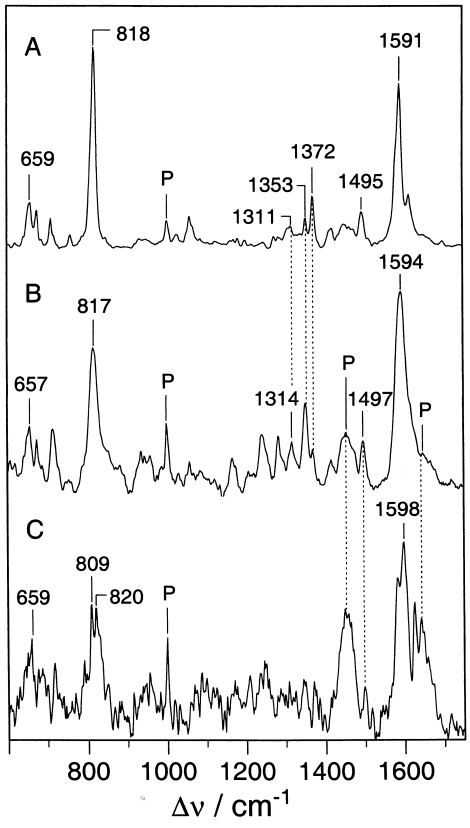

Fig. 5. RR spectra of (A) the P_{fr} state of native *phyA*124 in D_2O, (B) the P_{bl} state of the 39-kDa fragment in D_2O, and (C) the meta-R_C state of native *phyA*124 in D_2O. All spectra were measured at −140°C with 1064-nm excitation. Raman bands of the protein are denoted by 'P'.

ture upon tryptic fragmentation causes a higher conformational flexibility of the tetrapyrrole in P_{bl}(*phyA*39) which leads to a substantial broadening of most of the RR bands. This broadening, which is particularly pronounced for the 1596-cm^{-1} band (Fig. 4), is attributed to a conformational heterogeneity of the chromophore, i.e. a distribution of *ZZE* isomers with different C–D methine bridge conformations. It appears to be that already subtle details of the protein folding can affect this distribution such that a P_{fr}-like chromophore structure is favoured as it is found in the deuterated *phyA*39: the RR spectrum of the deuterated P_{bl}(*phyA*39) reveals a substantial intensity increase of the 817-cm^{-1} band as compared to the non-deuterated P_{bl}(*phyA*39) (Fig. 5). This intensity increase suggests a conformational change towards the chromophore structure in P_{fr}(*phyA*124) which may result from slight changes of the hydrogen-bonding network in the chromophore binding pocket due to the deuteration of the amide backbone. This structural rearrangement is not reversed by H/D re-exchange as witnessed by the RR spectrum measured of such a sample, in which the relatively high intensity of the 817-cm^{-1} band is preserved (data not shown).

In contrast to *phyA*39, the P_{fr} chromophore structures of the tryptic 59-kDa and the recombinant 65-kDa fragments of *phyA*, both of which still possess the peptide segment 426–595 are essentially the same as in *phyA*124, as indicated by the RR spectra [7,24]. Thus, it may be that a site for the specific protein–chromophore interactions, which enable the formation and stabilisation of the P_{fr} state, has been eliminated proteolytically with the removal of the peptide segment 426–595. Nevertheless, a 39-kDa fragment expressed by the yeast *Pichia pastoris* is able to adopt a P_{fr}-like form exhibiting a 700-nm absorption band of similar strength as P_{fr}(*phyA*124) [22]. For this recombinant fragment, yeast-specific chaperonines or post-translational modifications, not available to the proteolytic fragment of *phyA*, may support a protein folding which stabilises a chromophore structure more similar to P_{fr}(*phyA*124) even in the absence of the peptide segment 426–595.

4. Conclusion

The loss of the C-terminus – in particular, the segment of amino acid residues 426–595 – in the 39-kDa fragment adversely affects the interactions of the apoprotein with the tetrapyrrole chromophore. This conclusion, as derived from the present RR spectroscopic study, confirms previous suggestions [10] that protein–chromophore interactions are weakened compared to native *phyA*124. In the P_r state structural perturbations are relatively small so that the 39-kDa fragment represents a good model for phytochrome, particularly, in the context of current attempts to determine the three-dimensional structure of this photoreceptor by X-ray crystallography. On the other hand, the lack of the major C-terminal part of the protein significantly perturbs the $P_r \rightarrow P_{fr}$ phototransformation such that the last step, the transition from meta-R_C(*phyA*124) to P_{fr}(*phyA*124), is essentially blocked in the 39-kDa fragment. This transition, which most likely involves a partial rotation around the C–D methine bridge single bond associated with changes of the interactions of ring D with the protein environment, evidently requires a specific folding of the apoprotein in the vicinity of the chromophore binding site. Conversely, this conclusion is consistent with the view that the transition from meta-R_C(*phyA*124) to P_{fr} is associated with protein structural changes which, in turn, may be relevant for the functioning of the photoreceptor.

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