Effect of chromophore exchange on the resonance Raman spectra of recombinant phytochromes

C. Kneip, D. Mozley, P. Hildebrandt*, W. Gärtner, S.E. Braslavsky, K. Schaffner

Max-Planck-Institut für Strahlenchemie, Postfach 101365, D-45413 Mülheim, Germany

Received 24 June 1997

Abstract The recombinant 65-kDa polypeptide of *phyA* oat phytochrome was expressed by yeast *Pichia pastoris* and assembled into two chromopeptides with the chromophores phytochromobilin (PΦB) and phycocyanobilin (PCB), respectively. The P_r and P_{fr} states of the two protein variants were characterized by resonance Raman (RR) spectroscopy and compared with native *phyA* oat phytochrome demonstrating that the deletion of the C-terminal half of *phyA* does not alter the structure of the chromophore site within the N-terminal half. Most of the RR spectral changes observed upon replacing PΦB by PCB can be attributed exclusively to altered vibrational mode compositions due to the different ring D substitutions (vinyl vs. ethyl), implying that the chromophore structures are largely the same for PΦB- and PCB-assembled phytochromes. Only in the P_r state may the RR spectral changes also reflect subtle differences of the PΦB and PCB conformations in the 65-kDa *phyA*, presumably brought about by the specific steric requirements of the vinyl and ethyl groups.

Key words: Phytochrome; Tetrapyrrole; Photoisomerization; Resonance Raman

1. Introduction

Phytochromes are a family of ubiquitous photoreceptors in plants controlling a variety of photomorphogenic processes [1,2]. Their prosthetic chromophore is the open-chain tetrapyrrole phytochromobilin (PΦB, Fig. 1A), which is covalently bound to the apoprotein. Light absorption of the parent state P_r (λ_{max}: 667 nm) leads to *E/Z* photoisomerization of one of the methine bridges [3,4], thereby initiating a reaction cascade which involves conformational changes of both the chromophore and protein [5]. The final product is the physiologically active state of phytochrome, P_{fr} (λ_{max}: 730 nm in *phyA* from etiolated plants), which does not only differ from P_r with regard to the chromophore configuration but also on the level of the tertiary structure of the protein. Determining the molecular changes during the photo-induced reaction pathway is a prerequiste for understanding the functioning of the photoreceptor. In particular, a detailed knowledge of the chromophoric structures in the various states of phytochrome is required. Such information would provide the key for elucidating the coupling of the structural changes of the chromophore and protein.

In previous studies, it has been shown that resonance Raman (RR) spectroscopy can provide the required information since this technique selectively probes the vibrational spectrum of the chromophore [6–14]. However, decoding the structural data from the spectra requires a safe vibrational assignment which is not yet available. Thus, concomitant to our studies on the vibrational analysis of tetrapyrroles [15,16], we have initiated measurements of the RR spectra of phytochromes varying in their protein composition and assembled with different chromphores. Such investigations may provide additional information about the character of the vibrational modes, thereby supporting the extraction of structural data from RR spectra. For such studies, it is thus essential to vary the protein sequence and to introduce chromophores with altered substitution pattern and selective isotope labels.

We report here results obtained with a recombinant phytochrome (*phyA*-type from oat), truncated at the C-terminal end [17]. This 65-kDa apoprotein variant (comprising amino acids 1–595) was expressed in the yeast *Pichia pastoris* and assembled with either the natural chromophore (PΦB) or phycocyanobilin (PCB, Fig. 1B). The latter differs from PΦB only by one substituent of ring D (C-18 ethyl instead of vinyl). These recombinant proteins have been studied previously by stationary and time-resolved UV-vis absorption spectroscopies [18,19]. Data demonstrated that deletion of the C-terminal half does not alter the spectral and kinetic properties of the chromophore. In the present work, the same recombinant proteins are studied in the P_r and P_{fr} forms by RR spectroscopy in order to gain more detailed structural information about the chromophores of these forms.

2. Materials and methods

2.1. Sample preparation

The cDNA encoding amino acids 1–595 of oat *phyA* was extended at the 3′-end by an oligonucleotide encoding six histidine residues and a stop codon by PCR. This construct which carried *Eco*RI sites at each end was cloned into the *Eco*RI site of pHIL-D2 (Invitrogen) and used to transform *P. pastoris* cells according to manufacturer's instructions. After breaking the cells [18] the solution was cleared by ultracentrifugation, and the supernatant was incubated with either of the chromophores PΦB and PCB. The chromophores were isolated as described in [18]. The assembled chromoproteins were loaded onto a Ni-column, contaminating proteins were removed by washing with buffer, and the chromoprotein fraction was eluted from the column by treatment with an imidazole buffer. The resulting phytochrome-containing solution was then subjected to RR spectroscopy. Isolation and purification of oat *phyA* and sample preparation for Raman measurements are described elsewhere in detail [11,13].

2.2. RR measurements

The RR spectra of P_r and P_{fr} were obtained with 1064-nm excitation using a BioRad Fourier-transform Raman spectrometer equipped with a Nd-YAG laser (Spectra Physics, FC-106V, bandwidth < 1 cm^{-1}). The phytochrome samples were photoconverted into the desired states (P_r and P_{fr}) and subsequently cooled down to −140°C as described previously [13]. The concentration of the samples corresponded to optical densities of ≈ 1.0 (1-cm pathlength) for *phyA*

*Corresponding author. Fax: (49) 208-306-3951.
E-mail: hildebrandt@mpi-muelheim.mpg.de

PII S0014-5793(97)00969-1

and between 0.3 and 1.0 for the recombinant phytochromes, at the absorbance peak of the P_r form in each case. The laser power at the sample was 360 mW and the spectral resolution was 4 cm^{-1}. The raw spectra were corrected for the instrumental response as described in [20]. The measured RR spectra also include contributions by Raman bands of the glass and quartz optics (at $\approx$1070 and 760 cm^{-1}) as well as Raman bands of glycerol contaminations originating from the centricon cells used for concentrating the samples. These Raman bands, which are much more apparent in samples of low phytochrome concentrations, were subtracted to yield the spectra shown in this work. The RR spectra of P_{fr} shown in this work were obtained after subtracting the residual contribution of P_r from the measured spectra. Further details of the measurements and the experimental set-up are given elsewhere [13].

3. Results and discussion

The RR spectra of the P_r state of the native *phyA* (*Avena sativa*) and the recombinant protein assembled with PΦB (65-kDa *phyA*–PΦB; Fig. 2A,B) reveal far-reaching similarities (cf. [13]). The dominant bands, e.g. at 1639, 1624, 1572, and 1321 cm^{-1}, are at the same positions and exhibit the same RR intensities in both proteins. The only difference which might reflect a change in the RR spectrum beyond the experimental accuracy of ± 1 cm^{-1} concerns the doublet at 663 and 668 cm^{-1} (*phyA*) which is more clearly resolved in 65-kDa *phyA*–PΦB due to a subtle upshift of the latter component to 670 cm^{-1}. Thus, we can conclude that in the P_r state the RR spectrum of 65-kDa *phyA*–PΦB is essentially identical with that of native *phyA*, which implies that the configuration and conformation of the chromophore as well as its interactions with the protein environment are the same in both proteins. This conclusion also holds for the two P_{fr} states (Fig. 3A,B). Owing to the high sensitivity of RR spectroscopy towards subtle structural changes of the chromophore, the present results unambigously confirm the view that the deletion of the C-terminal half has no effect on the chromophoric site structure in the P_r and P_{fr} states. These findings also imply that dimer formation which does not occur in the 65-kDa protein does not influence the photoactive center.

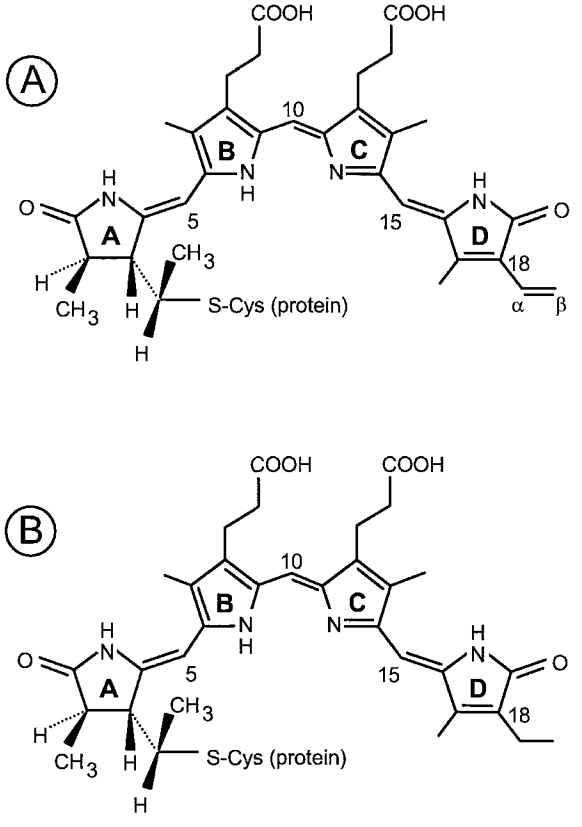

Fig. 1. Structural formulas of PΦB (A) und PCB (B).

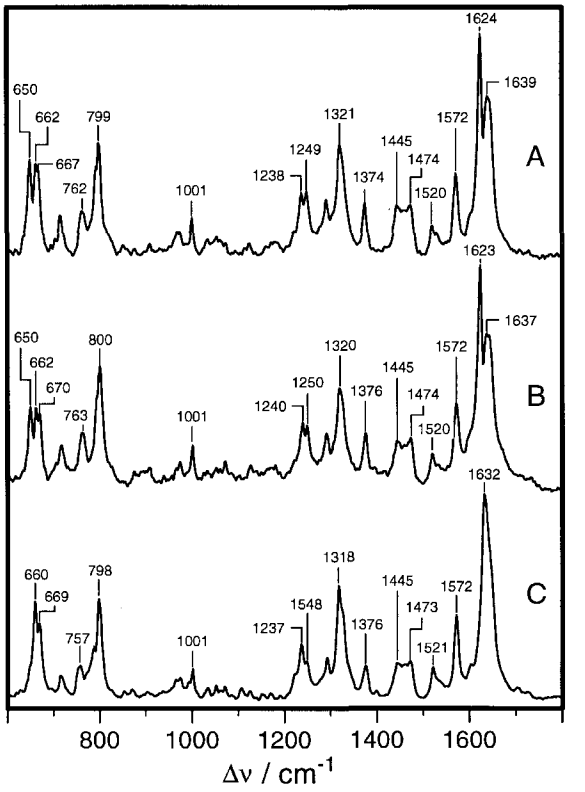

Fig. 2. RR spectra of the P_r state of (A) native *phyA*, (B) 65-kDa *phyA*–PΦB, and (C) 65-kDa *phyA*–PCB, excited at 1064 nm ($T = -140$°C).

Nevertheless, the recombinant protein assembled with PCB (65-kDa *phyA*–PCB) reveals a few distinct RR spectral differences in its P_r and P_{fr} states as compared to the 65-kDa *phyA*–PΦB variants (Figs. 2 and 3). The most pronounced difference is observed in the 1600-cm^{-1} region which, for P_r, is displayed on an expanded scale in Fig. 4. While all the bands between 1500 and 1600 cm^{-1} are at the same positions (and with similar relative RR intensities) in *phyA*, 65-kDa *phyA*–PΦB, and 65-kDa *phyA*–PCB, the latter protein reveals a distinct upshift of the 1624-cm^{-1} band to 1632 cm^{-1} so that it overlaps with the 1639-cm^{-1} band which appears to remain at the same position as in *phyA* and 65-kDa *phyA*–PΦB. In the RR spectrum of P_{fr}, we note a corresponding 5-cm^{-1} upshift of the 1598–cm^{-1} band, which is the counterpart of the 1624-cm^{-1} band of P_r, when PΦB is replaced by PCB. Hence, this particular band of P_r and P_{fr} most sensitively responds to the chemical modification of the ring D substituent, implying that the underlying mode involves substantial contributions from ring D vibrations. This finding and the invariance of all other bands in this region support the conclusion that the normal modes of linear tetrapyrroles are largely localized in individual pyrrole rings and adjacent methine bridges [15].

The replacement of PΦB by PCB also causes some spectral changes in the region below 1500 cm^{-1} both in P_r and P_{fr}. In

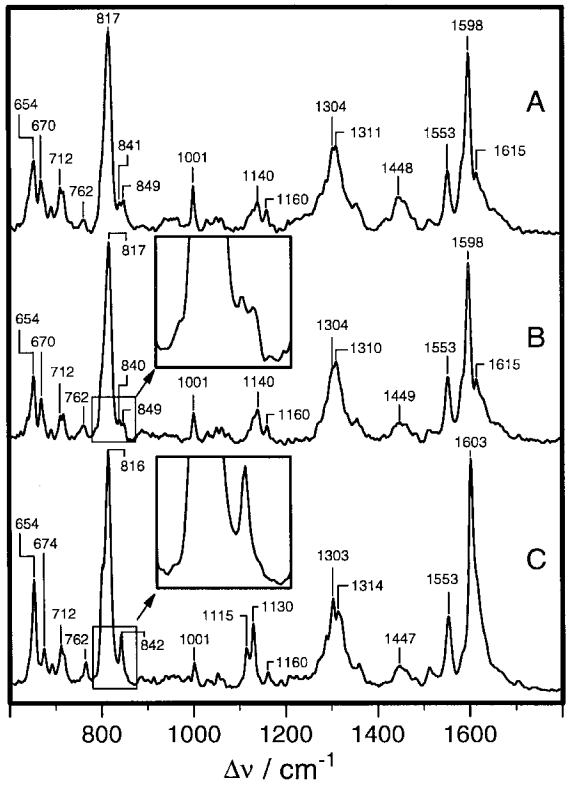

Fig. 3. RR spectra of the P_{fr} state of (A) native *phyA*, (B) 65-kDa *phyA*–PΦB, and (C) 65-kDa *phyA*–PCB, excited at 1064 nm ($T = -140°C$).

P_r, the maximum at 1320-cm^{-1} shifts down tolower wavenumbers in 65-kDa *phyA*–PCB, more clearly revealing the asymmetric bandshape with a shoulder at the high frequency side. In P_{fr}, the counterpart of this peak is found at ≈ 1310 cm^{-1} in 65-kDa *phyA*–PΦB and undergoes an even more pronounced splitting into two components at 1303 and 1314 cm^{-1} in 65-kDa *phyA*–PCB. The sensitivity of the latter major component towards PΦB → PCB substitution can readily be understood since modes involving the C_α–H rocking vibration of the vinyl substituents are expected between 1260 and 1330 cm^{-1} [15]. Evidently, the compositions of the normal modes in this region are scrambled upon substitution of the vinyl substituent by ethyl on ring D. A similar explanation may hold for the frequency shifts and intensity changes of the bands at 1240 and 1250 cm^{-1} of P_r, and at 1140 cm^{-1} of P_{fr}. The spectral changes of these bands may be related to the involvement of the vinyl C = C stretching and C_β–H_2 rocking coordinates in the underlying modes.

Spectral changes in the C–H out-of-plane bending modes (HOOP modes) are of particular interest inasmuch as they are regarded as sensitive indicators for conformational changes (torsions) of the methine bridges of linear tetrapyrroles [8,13]. In P_{fr}, there is a redistribution of the RR intensities between 840 and 850 cm^{-1} in 65-kDa *phyA*–PCB as compared to 65-kDa *phyA*–PΦB suggesting that the underlying modes include the C_α-H HOOP coordinate. In fact, modes of this character and with frequencies in this range were calculated for biliverdin dimethyl ester by the semi-empirical AM1 method [15]. The corresponding bands of P_r cannot be identified unambiguously. They possibly overlap with the 800-cm^{-1} band since the intensity of its high-frequency shoulder is reduced in 65-kDa *phyA*–PCB when compared to 65-kDa *phyA*–PΦB.

The RR spectra of P_r reveal distinct spectral changes of the 763- and 650-cm^{-1} bands which remain largely unchanged in P_{fr}. While the band at 763-cm^{-1} shifts down by 6 cm^{-1}, the 650-cm^{-1} band disappears in 65-kDa *phyA*–PCB.

Most of the RR spectral differences between 65-kDa *phyA*–PΦB and 65-kDa *phyA*–PCB can be attributed to a redistribution of the normal mode composition resulting from the C-18 vinyl/ethyl substitution since analogous spectral differences have been observed for the recombinant phytochromes from the cyanobacterium *Synechocystis* assembled with either PΦB and PCB (*synech*–PΦB; *synech*–PCB) [21]. This is particularly true for the frequency shifts and intensity changes of the bands at 1624, 1320, and 1240 cm^{-1} of P_r, and the 1598- and 1310-cm^{-1} bands of P_{fr}. It can be ruled out, therefore, that these alterations of the RR spectra reflect conformational differences of the chromophore brought about by different protein–tetrapyrrole interactions involving the C-18 substituents of ring D. Hence, these bands can be regarded as marker bands for the ring D constitution. This may also be true for the 650-cm^{-1} band of *phyA* which remains at this frequency in *synech*–PΦB and 65-kDa *phyA*–PΦB. In the PCB-assembled phytochromes, however, this band disappears. Instead, the intensities increase at 656 and 660 cm^{-1} in *synech*–PCB and 65-kDa *phyA*–PCB, respectively. Since a predominant contribution of the vinyl torsional coordinate is expected for a mode at this frequency according to quantum chemical force field calculations [22], the change in the 650-cm^{-1} region upon

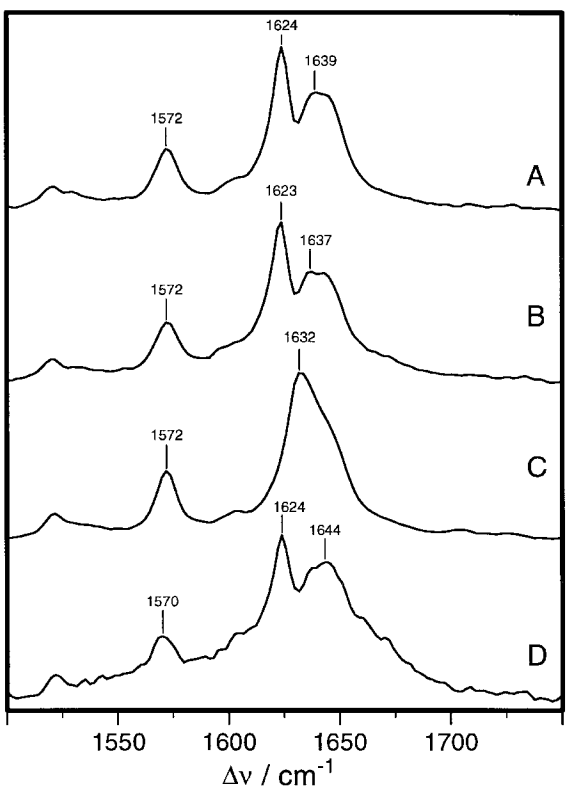

Fig. 4. Expanded view (1500–1750 cm^{-1}) of the RR spectra of the P_r state of (A) native *phyA*, (B) 65-kDa *phyA*–PΦB, (C) 65-kDa *phyA*–PCB, and (D) of phytochrome formed from the endogeneous chromophore of *P. pastoris* during expression, excited at 1064 nm ($T = -140°C$).

PΦB → PCB substitution is readily understood. The frequency difference in *synech*–PCB and 65-kDa *phyA*–PCB, 656 vs. 660 cm^{-1}, can be regarded as an indication for slightly different chromophore conformations in these two proteins.

The P_r band at around 763 cm^{-1} may serve as a similar conformation indicator for the *synech* and *phyA* phytochromes. The band shifts down by ca. 6 cm^{-1} in both *synech*–PΦB and *synech*–PCB whereas in the assembled *phyA* phytochromes such a downshift is only found for 65-kDa *phyA*–PCB and no effect is observed for 65-kDa *phyA*–PΦB. It appears, therefore, that this band reflects subtle conformational changes of the chromophores resulting from differences in the chromophore binding pocket (*phyA* vs. *synech*) and the tetrapyrrole (PCB vs. PΦB). Such differences may reflect the properties of the C-18 substituents, vinyl being somewhat smaller and more polar(izable) than ethyl. Although the nature of the underlying mode of the 763-cm^{-1} band is not yet clear, it is likely that ring deformation vibrations are involved which should be particularly sensitive towards changes of the chromophore conformation.

The fact that the kinetic behavior of the recombinants 124-kDa *phyA*–PΦB and 124-kDa *phyA*–PCB is different while the behavior of recombinant 124-kDa *phyA*–PΦB is very similar to that of native oat *phyA*, also reflects a different specific interaction of the C-18 substituents (vinyl in PΦB and ethyl in PCB) with the protein environment and is in line with the present results [18].

It has been noted previously [23] that *P. pastoris* is capable to produce a tetrapyrrole which can be incorporated into the phytochrome peptide during expression. This endogeneous tetrapyrrole biosynthesis is relatively efficient when the cells grow in the dark but is down-regulated by light. The phytochromes, the RR spectra of which are discussed above, have been assembled in vitro from apoproteins expressed in cells grown under illumination. By contrast, the RR spectrum in Fig. 4D reflects the P_r state of a phytochrome assembled entirely in vivo in cells which had been kept in the dark permanently. No additional chromophore has been added to the protein after isolation so that exclusively the endogeneously produced tetrapyrrole of *P. pastoris* is incorporated.

Based on the marker bands which are specific for the ring D constitution it is now possible to establish the nature of the endogeneous tetrapyrrole of *P. pastoris*. The spectrum displays a distinct band at 1624 cm^{-1} which is characteristic for 65-kDa *phyA*–PΦB. It shifts to 1632 cm^{-1} in 65-kDa *phyA*–PCB and overlaps with the adjacent 1642-cm^{-1} band. Hence, the endogeneous chromophore is unequivocally identified as PΦB, confirming an earlier suggestion based on UV-vis absorption spectroscopy [23].

4. Conclusion

The present study has shown that the 65-kDa recombinant phytochrome is a convenient model system to investigate the chromophoric site by spectroscopic techniques. It will be possible to extend such studies to other tetrapyrroles including isotopomers incorporated in these protein variants [24]. There is no evidence of an effect of the C-terminus on the chromophore structures in the P_r and P_{fr} states. The comparison of the RR spectra of PΦB- and PCB-containing phytochromes has revealed a number of bands originating from modes localized in ring D. Hence, these bands hold promise to be sensitive markers for structural changes in this part of the chromophore domain during the photo-induced reaction cycle.

Acknowledgements: We thank W. Schlamann for the purification and preparation of the *phyA* samples, and T. Huestege and G. Koc for the preparation and chromatographic purification of the chromophores. P.H. gratefully acknowledges a Heisenberg Fellowship by the Deutsche Forschungsgemeinschaft. D.M. was supported in part by the HCM EC Grant ERBCHBGCT 930313.

References

[1] P.H. Quail, M.T. Boylan, K. Dehesh, J. Nieto-Solelo, B.M. Parks, J.M. Teppermann, D.E. Somers, and D. Wagner, in: G. Coruzzi, and P. Puigdomenech (Eds.), Plant Molecular Biology, Springer, Heidelberg, 1994, pp. 391–400.
[2] Whitelam, G.C. and Harberd, N.P. (1994) Plant Cell Environ. 17, 615–625.
[3] Rüdiger, W. and Thümmler, F. (1991) Angew Chem. Int. Ed. Engl. 30, 1216–1228.
[4] Lagarias, J.C. and Rapoport, H. (1980) J. Am. Chem. Soc. 102, 4821–4828.
[5] S.E. Braslavsky, W. Gärtner, and K. Schaffner, Plant Cell Environ., 1997, in press.
[6] Fodor, S.P.A., Lagarias, J.C. and Mathies, R.A. (1988) Photochem. Photobiol. 48, 129–136.
[7] Farrens, D.L., Holt, R.E., Rospendowski, B.N., Song, P.-S. and Cotton, T.M. (1989) J. Am. Chem. Soc. 111, 9162–9169.
[8] Fodor, S.P.A., Lagarias, J.C. and Mathies, R.A. (1990) Biochemistry 29, 11141–11146.
[9] Tokutomi, S., Mizutani, Y., Anni, H. and Kitagawa, T. (1990) FEBS Lett. 269, 341–344.
[10] Mizutani, Y., Tokutomi, S., Aoyagi, K., Horitsu, K. and Kitagawa, T. (1991) Biochemistry 30, 10693–10700.
[11] Hildebrandt, P., Hoffmann, A., Lindemann, P., Heibel, G., Braslavsky, S.E., Schaffner, K. and Schrader, B. (1992) Biochemistry 31, 7957–7962.
[12] Mizutani, Y., Tokutomi, S. and Kitagawa, T. (1994) Biochemistry 33, 153–158.
[13] Matysik, J., Hildebrandt, P., Schlamann, W., Braslavsky, S.E. and Schaffner, K. (1995) Biochemistry 34, 10497–10507.
[14] Andel III, F., Lagarias, J.C. and Mathies, R.A. (1996) Biochemistry 35, 15997–16008.
[15] Smit, K., Matysik, J., Hildebrandt, P. and Mark, F. (1993) J. Phys. Chem. 97, 11187–11900.
[16] Matysik, J., Hildebrandt, P., Smit, K., Korkin, A., Mark, F., Gärtner, W., Braslavsky, S.E., Schaffner, K. and Schrader, B. (1995) J. Mol. Struct. 348, 225–228.
[17] Gärtner, W., Hill, C., Worm, K., Braslavsky, S.E. and Schaffner, K. (1996) Eur. J. Biochem. 236, 978–983.
[18] Schmidt, P., Westphal, U.H., Worm, K., Braslavsky, S.E., Gärtner, W. and Schaffner, K. (1996) J. Photochem. Photobiol. B: Biology 34, 73–77.
[19] A. Remberg, Ph.D. Thesis, University of Essen, MPI für Strahlenchemie, Mülheim an der Ruhr, 1997.
[20] P. Hendra, C. Jones, and G. Warnes, Fourier Transform Raman Spectroscopy, Ellis Horwood, New York, 1991.
[21] A. Remberg, I. Lindner, T. Lamparter, J. Hughes, C. Kneip, P. Hildebrandt, S.E. Braslavsky, W. Gärtner, and K. Schaffner, K., submitted.
[22] K. Nemeth, F. Mark, C. Kneip, and P. Hildebrandt, unpublished results.
[23] Wu, S.-H. and Lagarias, J.C. (1996) Proc. Natl. Acad. Sci. USA 88, 10387–10391.
[24] Knipp, B., Kneip, C., Matysik, J., Gärtner, W., Hildebrandt, P., Braslavsky, S.E. and Schaffner, K. (1997) Chem. Eur. J. 3, 363–367.