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Photoreceptors

Chromophore Heterogeneity and Photoconversion in Phytochrome Crystals and Solution Studied by Resonance Raman Spectroscopy**

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Phytochromes constitute a family of sensory photoreceptors ubiquitous in plants, bacteria, and fungi.[1] The light-sensing cofactor, a methine-bridged tetrapyrrole, is covalently bound to the apoprotein through a thioether linkage (Figure 1). Upon light absorption in the parent state Pr, the chromophore undergoes a $Z \rightarrow E$ double-bond isomerization of the methine bridge between the rings C and D, followed by thermal relaxation steps, thereby coupling structural changes in the chromophore and protein. The final step is the formation of the Pfr state, which is the physiologically active form of the protein in plant phytochromes. The molecular mechanism of the Pr→Pfr photoconversion is not yet understood, but a major breakthrough has been recently achieved with the determination of the three-dimensional structures of the Pr states of bacterial biliverdin(BV)-binding phytochromes, considered to be representative models for this photoreceptor family.[2] These structures, however, refer to the chromophore-binding domain (CBD) and lack the PHY domain, which is assumed to be essential for the Pr→Pfr photoconversion. In addition, radiation-damage-induced release of the chromophore as well as limited diffraction resolution leave open the possibility that the chromophore geometry, particularly in the region of the thioether bond and ring A, may require further structural refinement.[2c] In fact, spectroscopic studies on phytochromes in solution have led to conflicting conclusions with respect to conformation and the structural homogeneity of the chromophore.[3,4] For these reasons, it is highly desirable to analyze the chromophore structure of phytochromes in solution and in crystals using the same method.

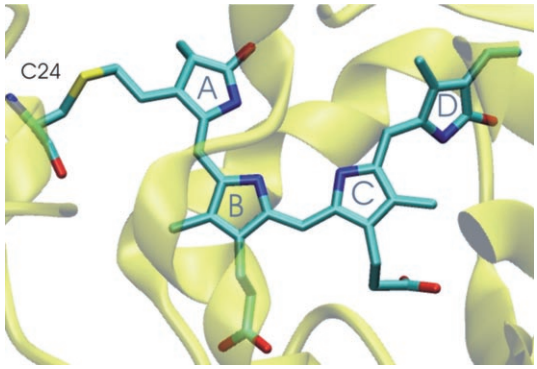

Figure 1. *ZZZssa* geometry of biliverdin (BV) in the Pr state of the chromophore-binding domain of DrBphP as derived from the crystal structure data.[2b]

In this work we have employed resonance Raman (RR) spectroscopy, which exclusively probes the vibrational bands of the cofactor and thus provides information about the tetrapyrrole structure.[3] RR spectra of photoreceptor crystals were first reported for bacteriorhodopsin,[5] but owing to the rigorous resonance conditions only photostationary mixtures could be probed. Here we have used 1064 nm excitation to avoid initiation of photochemical processes of phytochrome by the Raman probe beam.[3] Thus, for the first time, RR spectra of the pure parent state and an intermediate in a photoreceptor crystal have been obtained.

Crystals of the CBD of the wild-type (WT) and the Y307S variant phytochrome from *Deinococcus radiodurans* (DrBphP) as well as a construct from *Agrobacterium tumefaciens* phytochrome Agp1, which contains both the CBD and the PHY domains (Agp1-M15), were obtained as previously described.[2a,b,6] For RR experiments, protein

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crystals were kept in buffer and, for large crystals, typically one crystal was in the focus of the laser beam. Since different crystals afforded identical RR spectra, anisotropy effects could be ruled out. Increasing the laser power from 15 to 700 mW did not cause spectral changes, indicating that the near-infrared laser line does not damage the crystals. Photoconversion products of the solution samples and the crystals were generated by red-light irradiation as described previously.[3] All spectra were measured at −140 °C. Spectra of the protein crystals include large contributions from the Raman bands of the surrounding buffer which, however, do not obscure the RR bands that are indicative of the methine bridge geometry of tetrapyrroles.[3]

The RR spectra of the parent Pr states of the WT full-length DrBphP and the WT CBD in frozen solutions are identical, indicating that the structure of the chromophore and its interactions with the protein environment are preserved in the CBD fragment despite the lack of the PHY and histidine kinase domains (see Figure S1 in the Supporting Information). In both cases, the chromophore is protonated as shown by the N–H in-plane bending mode (ip) of the N–H groups in rings B and C at 1576 cm^{-1} that disappears in D_2O. A very similar spectrum is obtained for the CBD fragment of the Y307S mutant in solution although the marker bands are downshifted by 1–2 cm^{-1} relative to the WT CBD (Figure 2).

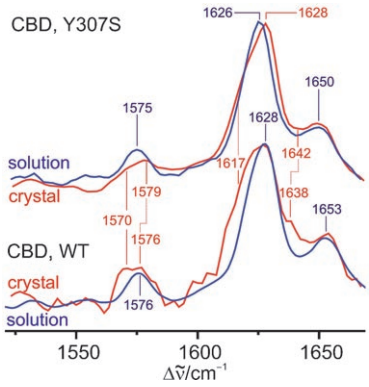

Figure 2. RR spectra in the marker band region of the Pr state of the WT CBD(DrBphP) (bottom) and the Y307S CBD(DrBphP) (top) in solution (blue) and in the crystalline state (red).

In the RR spectra of the crystalline CBDs the characteristic features of the Pr state are observed, indicating that the chromophore structure in solution and in the crystal is the same. However, in contrast to Y307S CBD, the bands of WT CBD crystals are considerably broadened and more asymmetric than those of the protein in frozen solution (Figure 2), suggesting an increased heterogeneity in the crystal. Specifically, the width of the prominent 1628 cm^{-1} peak (C=C stretching of the C–D methine bridge[3]) has increased from 16 to ca. 26 cm^{-1} as a result of additional bands on the low- and high-frequency sides. The extra bands at ca. 1617 and ca. 1638 cm^{-1} are attributable to the C=C stretchings of the C–D and A–B methine bridges, respectively. Also the N–H ip bending mode appears as a doublet with band components at ca. 1576 and 1570 cm^{-1}.

The spectra of crystals of Agp1-M15 show the opposite behavior to the spectra of the protein in solution (Figure 3). Here the solution spectrum displays a signature characteristic

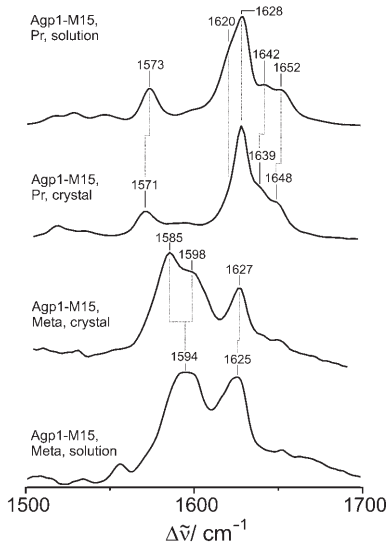

Figure 3. RR spectra in the marker band region of the Pr state of Agp1-M15 in solution and in crystals, compared with the Meta-Rc-like states of Agp1-M15 in the crystal and in solution obtained after red-light illumination at 20 °C and −30 °C, respectively, and subsequent subtraction of contributions from the nonphotolyzed Pr state.

of two chromophore conformations as reflected by the doublets at 1652 and 1642 cm^{-1} (A–B stretching) and at 1628 and 1620 cm^{-1} (C–D stretching). The RR spectra of Agp1-M15 crystals, however, display a sharpening of the 1628 cm^{-1} band that can readily be attributed to the intensity decrease of the 1620 cm^{-1} shoulder. Furthermore, the intensity distribution of the high-frequency doublet is altered at the expense of the 1652 cm^{-1} component. These findings indicate that crystallization of Agp1-M15 leads to a more, albeit not fully, uniform chromophore conformation.

The band doublets at ca. 1650 and 1640 cm^{-1}, 1628 and 1617 cm^{-1}, and 1576 and 1570 cm^{-1} that are clearly visible in the RR spectra of Agp1-M15 in solution and crystals as well as in WT CBD(DrBphP) crystals indicate the coexistence of at least two, potentially very similar, conformers. Most likely, the underlying structural differences refer to different torsional angles of the A–B and C–D methine bridges, which have a great effect on the frequencies of the A–B and C–D stretches,[3] and to the hydrogen-bond interactions of the pyrrole rings B and C. Crystallization affects this heterogeneity for Agp1-M15 and WT CBD(DrBphP) but not for Y307S CBD(DrBphP) for which the highest resolution structure has been obtained.[2b] The strikingly different behavior in Agp1-M15 is evidently related to the interactions between the PHY and the CBD domains which stabilize a more homogeneous chromophore structure in the crystal than in solution. Note that chromophore structural heterogeneity has also been concluded from spectroscopic studies on plant and cyanobacterial phytochromes.[4,7]

Irradiation of Y307S CBD(DrBphP) crystals at 20 °C did not lead to spectral changes, indicating that the chromophore is incapable of photoisomerization. Again, these CBD fragments in solution behave in a different manner, since they can be photoconverted to a Meta-Rc like state (see Figure S2 in the Supporting Information), suggesting that the failure of CBD(DrBphP) crystals to undergo photoreactions at all is a result of crystal packing effects. In contrast, the photocycle of crystalline Agp1-M15 including the essential PHY domain proceeds to the Meta-Rc state leading to a spectrum that is similar albeit not identical to that of the Meta-Rc state of Agp1-M15 in solution (Figure 3). The final relaxation process to Pfr is blocked and observed only for Agp1-M15 in solution.[8] The complete phototransformation to Pfr in the crystalline state most likely requires the rupture of the crystal packing consistent with the large-scale protein structural changes upon Meta-Rc→Pfr conversion.[8a]

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[1] a) N. C. Rockwell, Y. S. Su, J. C. Lagarias, *Annu. Rev. Plant Biol.* **2006**, *57*, 837; b) P. H. Quail, *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 85.
[2] a) J. R. Wagner, J. S. Brunzelle, K. T. Forest, R. D. Vierstra, *Nature* **2005**, *438*, 325; b) J. R. Wagner, J. Zhang, J. S. Brunzelle, R. D. Vierstra, K. T. Forest, *J. Biol. Chem.* **2007**, *282*, 12298; c) X. Yang, E. A. Stojkovic, J. Kuk, K. Moffat, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12571.
[3] a) M. A. Mroginski, D. H. Murgida, D. von Stetten, C. Kneip, F. Mark, P. Hildebrandt, *J. Am. Chem. Soc.* **2004**, *126*, 16734; b) D. H. Murgida, D. von Stetten, P. Hildebrandt, P. Schwinté, F. Siebert, S. Sharda, W. Gärtner, M. A. Mroginski, *Biophys. J.* **2007**, *93*, 2410.
[4] J. J. van Thor, M. Mackeen, I. Kuprov, R. A. Dwek, M. R. Wormald, *Biophys. J.* **2006**, *91*, 1811.
[5] L. S. Sanii, A. W. Schill, C. E. Moran, M. A. El-Sayed, *Biophys. J.* **2005**, *89*, 444.
[6] P. Scheerer, N. Michael, J. H. Park, S. Noack, C. Förster, M. A. S. Hammam, K. Inomata, H. W. Choe, T. Lamparter, N. Krauß, *J. Struct. Biol.* **2006**, *153*, 97.
[7] P. Schmidt, T. Gensch, A. Remberg, W. Gärtner, S. E. Braslavsky, K. Schaffner, *Photochem. Photobiol.* **1998**, *68*, 754.
[8] a) D. von Stetten, S. Seibeck, N. Michael, P. Scheerer, M. A. Mroginski, D. H. Murgida, N. Krauss, M. P. Heyn, P. Hildebrandt, B. Borucki, T. Lamparter, *J. Biol. Chem.* **2007**, *282*, 2116; b) K. Inomata, M. A. S. Hammam, H. Kinoshita, Y. Murata, H. Khawn, S. Noack, N. Michael, T. Lamparter, *J. Biol. Chem.* **2005**, *280*, 24491.

