128, 49 – 62; e) M. Martin, O. Gevert, H. Werner, *J. Chem. Soc. Dalton Trans.* **1996**, 2275 – 2283.

[5] M. Brookhart, B. Grant, A. F. Volpe, *Organometallics* **1992**, *11*, 3920 – 3922.

[6] Data for the X-ray structure analyses: **10**: Crystals from $CHCl_3$, $C_{59}H_{62}BClF_{24}O_2P_2Ru$ ($M_r = 1468.36$); crystal size $0.25 \times 0.20 \times 0.20$ mm^3; monoclinic, $P2_1/n$ (no. 14), $a = 20.287(4)$, $b = 15.739(2)$, $c = 20.271(5)$ Å, $\beta = 92.52(1)^\circ$, $Z = 4$, $V = 6466(2)$ Å^3, $\rho_{calcd} = 1.508$ g cm^{-3}; $T = 193(2)$ K; $2\theta = 50.06^\circ$; 11 694 reflections measured, 11 351 were unique ($R_{int} = 0.0325$), and 6414 observed ($I > 2\sigma(I)$); CAD4 (Enraf-Nonius), $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å), graphite-monochromated; LP and empirical absorption correction (Ψ scans, min. transm. 91.35 %). The structure was solved by the Patterson method (SHELXS-97); G. M. Sheldrick, *Acta Crystallogr. Sect. A* **1990**, *46*, 467) and refined with the full-matrix-least-squares method (SHELXL-97; G. M. Sheldrick, a program for crystal structure refinement, University of Göttingen, **1993**); $R_1 = 0.0678$, $wR_2 = 0.1293$ (for 6414 reflections with $I > 2\sigma(I)$), $R_1 = 0.1377$, $wR_2 = 0.1649$ (for all 11 351 data); data-to-parameter ratio 12.94; residual electron density $+0.585/-0.508$ e Å^{-3}. The hydrogen atoms H2A, H2B, and H9 were found in a differential Fourier synthesis and refined isotropically by setting the displacement parameter to 120 % of the equivalent isotropic U_{eq} value of C2 and C9. One CF_3 group of the anion was found rotation disordered. Two independent positions were found and refined anisotropically with the occupancy factors 80:20 with restraints to have the same U_{ij} components and the anisotropic displacement parameters in the direction of the bond. **13a**: Crystals from CH_2Cl_2, $C_{60}H_{61}BClF_{27}O_2P_2Ru$ ($M_r = 1536.36$); crystal size $0.21 \times 0.19 \times 0.15$ mm^3; monoclinic, $P2_1/n$ (no. 14), $a = 18.882(3)$, $b = 15.579(1)$, $c = 22.468(3)$ Å, $\beta = 91.40(2)^\circ$, $Z = 4$, $V = 6607(1)$ Å^3, $\rho_{calcd} = 1.544$ g cm^{-3}; $T = 173(2)$ K; $2\theta = 50.06^\circ$; 62 685 reflections measured, 11 647 were unique ($R_{int} = 0.0701$), and 7096 observed ($I > 2\sigma(I)$); IPDS (Stoe), $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å), graphite-monochromated. The structure was solved and refined as described for **10**; $R_1 = 0.0470$, $wR_2 = 0.1121$ (for 7096 reflections with $I > 2\sigma(I)$), $R_1 = 0.0825$, $wR_2 = 0.1270$ (for all 11 647 data); data-to-parameter ratio 11.92; residual electron density $+0.633/-1.108$ e Å^{-3}. The CF_3 group of the trifluoroacetato ligand was found disordered and refined anisotropically with restraints on U_{ij} and occupancy factors of 78:22. Also three of the CF_3 substituents on the $B(Ar_f)_4$ counterion were found rotation disordered and refined in the same way (occupancy factors: 66:36 (F4 – F6), 78:22 (F7 – F9) and 56:44 (F22 – F24). Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publications nos. CCDC-142920 (**10**) and CCDC-142921 (**13a**). Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

[7] C. Grünwald, O. Gevert, J. Wolf, P. González-Herrero, H. Werner, *Organometallics* **1996**, *15*, 1960 – 1962.

[8] P. Schwab, R. H. Grubbs, J. W. Ziller, *J. Am. Chem. Soc.* **1996**, *118*, 100 – 110; b) S. T. Nguyen, R. H. Grubbs, J. W. Ziller, *J. Am. Chem. Soc.* **1993**, *115*, 9858 – 9859.

[9] Cambridge Structural Database, October 1999 Release; F. H. Allen, O. Kennard, *Chem. Des. Autom. News* **1993**, *8*, 31 – 37.

[10] P. González-Herrero, unpublished results.

[11] F. Friebolin, *Basic One- and Two-Dimensional NMR Spectroscopy*, 2nd ed. VCH, Weinheim, **1993**.

[12] a) W. R. Roper, *J. Organomet. Chem.* **1986**, *300*, 167 – 190; b) H. P. Kim, R. J. Angelici, *Adv. Organomet. Chem.* **1987**, *27*, 51 – 111; c) H. Fischer, P. Hofmann, F. R. Kreissl, R. R. Schrock, U. Schubert, K. Weiss, *Carbyne Complexes*, VCH, Weinheim, **1988**; d) A. Mayr, H. Hoffmeister, *Adv. Organomet. Chem.* **1991**, *32*, 227 – 324; e) L. J. Baker, G. R. Clark, C. E. F. Rickard, W. R. Roper, S. D. Woodgate, L. J. Wright, *J. Organomet. Chem.* **1998**, *551*, 247 – 259, and references therein.

[13] A. Dobson, D. S. Moore, S. D. Robinson, M. B. Hursthouse, L. New, *Polyhedron* **1985**, *4*, 1119 – 1130.

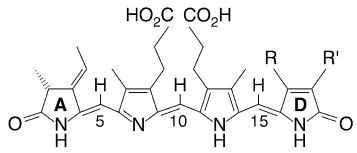
[14] K. H. Dötz, H. Fischer, P. Hofmann, F. R. Kreissl, U. Schubert, K. Weiss, *Transition Metal Carbene Complexes,* Verlag Chemie, Weinheim, **1983**.

Model Studies of Phytochrome Photochromism: Protein-Mediated Photoisomerization of a Linear Tetrapyrrole in the Absence of Covalent Bonding**

Ingo Lindner, Silvia E. Braslavsky, Kurt Schaffner, and Wolfgang Gärtner*

Dedicated to Professor Günther Wilke on the occasion of his 75th birthday

Open-chain tetrapyrroles, such as phytochromobilin (**1**) and phycocyanobilin (**2**), serve as chromophores in a number of chromoproteins. Their photoreactivity is largely regulated by the protein environment. Whereas **2** functions as an



1 R = CH_3, R' = $CH{=}CH_2$
2 R = CH_3, R' = CH_2CH_3
3 R = $CH{=}CH_2$, R' = CH_3

antenna chromophore in the light-harvesting pigments of cyanobacteria,[1] **1** is the chromophore of the plant photoreceptor phytochrome,[2] where it reversibly photoisomerizes around the $C_{15}-C_{16}$ double bond thereby converting the physiologically dormant P_r form into the active P_{fr} state.[2, 3] This photochromic control, triggered by a double-bond isomerization, is not restricted to **1**. For example, **2** in the phytochrome of the alga *Mesotaenium caldariorum* functions as a photochemical trigger analogous to **1**.[4] Furthermore, **2** can also assume this role in recombinant phytochromes of higher plants.[5] In all these photoreceptors, the chromophore is invariably bound by a covalent thioether bond to a cysteine residue of the protein.

We have now explored whether covalent chromophore bonding to the apoprotein is a prerequisite to the phototrigger function. We find, for the first time, that such is not the case. Rather, the chromophore binding pocket of the apoprotein can accommodate a chromophore sufficiently well to control the host – guest interactions driving the $P_r \rightleftarrows P_{fr}$ photocycle, without the need for covalent bonding.

The boundary conditions for the influence of the protein matrix on the absorption and photochemical properties of the phytochrome chromophore have not yet been studied thoroughly. The regioselective double-bond photoisomerization at

[*] Prof. Dr. W. Gärtner, Dipl.-Chem. I. Lindner, Prof. Dr. S. E. Braslavsky, Prof. Dr. K. Schaffner
Max-Planck-Institut für Strahlenchemie
Postfach 101365, 45413 Mülheim an der Ruhr (Germany)
Fax: (+49) 208-306-3951
E-mail: gaertner@mpi-muelheim.mpg.de

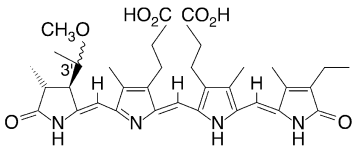
[**] This work is part of the PhD thesis of I. Lindner, Max-Planck-Institut für Strahlenchemie, Mülheim an der Ruhr, and Gerhard-Mercator-Universität, Duisburg, **2000**. We thank Tanja Berndsen, Gül Koç, and Helene Steffen for technical assistance.

C-15 in the protein-bound form of open-chain tetrapyrroles, such as **1** and **2**, is attributed to a steering effect of the surrounding protein matrix. In homogeneous organic solutions they are presumed to preferentially photoisomerize around the C-10 double bond.[6] Moreover, the 10*E* isomer of **2** is thermally unstable and reverts to the *Z* form within nanoseconds at ambient temperature.[6, 7]

It has also been found that the phytochrome A (phyA) photochromism is influenced by only a few amino acid residues,[8] and that both ligation of **2** to the apoprotein and photochromism depend critically on the two propionate side chains of **2**, which appear to position the chromophore prior to binding and to maintain its conformation.[8a] Furthermore, we have shown[9] that variation of ring D substitution in the phytochromobilin isomer **3** is tolerated in the P_r/P_{fr} photocycle, while the absorption maximum of the P_{fr} form is selectively blue shifted (that is, shifted to shorter wavelengths).

Finally, the significance of this study using a phytochrome-like protein with a covalently nonbonding chromophore is accentuated by the fact that a *bacterio*phytochrome has recently been found[10] which possesses a histidine rather than the usual cysteine at the site for covalent bonding and, thus, broadens the native structural variability of the phytochrome family.

When recombinant oat apophyA 65[11] was incubated with **4**, a methanol adduct to **2**,[12] the chromophore was embedded in the apoprotein. The resulting complex, apophyA 65/**4**,



4

proved capable of undergoing a photoreversible transformation reminiscent of the phytochrome photocycle ($\lambda_{max} = 643$ and 705 nm, somewhat blue shifted but analogous to the P_r and P_{fr} forms of recombinant phyA 65-**2**, respectively; Figure 1 A), even though the methanol addition blocks the attachment site (C-3′) on the chromophore and prevents covalent bonding of **4** to the apoprotein (Scheme 1[14]).[15] Repetitional cycling at appropriate irradiation wavelengths ($\lambda = 614$ and 714 nm for excitation of the P_r- and P_{fr}-like forms, respectively) did not cause any loss of the 643 and 705 nm absorbances.

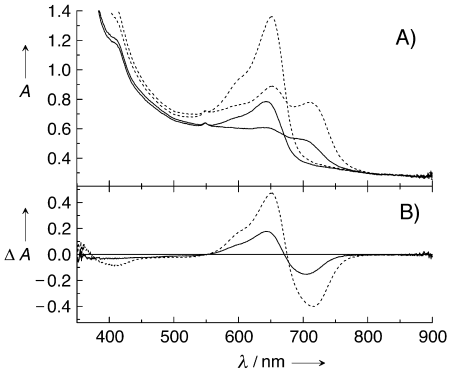
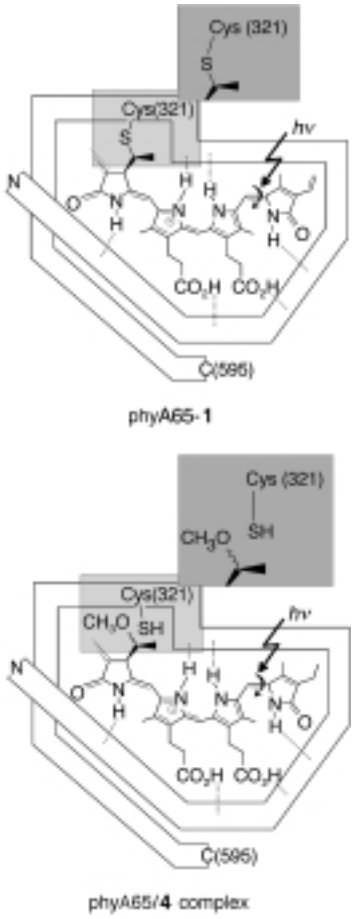


Figure 1. P_r and P_{fr} absorption (A) and P_r-P_{fr} difference absorption spectra (B) of recombinant oat phyA 65-**2** (dashed lines) and apophyA 65/**4** complex (complete lines), after saturating red and far-red irradiations. The spectra of phyA 65-**2**, as shown here, were recorded in the competition experiment with apophyA 65/**4** and **2**.



Scheme 1. Schematic representations of the chromophore pockets of P_r phytochrome[14] (top) and the P_r-like apophyA 65/**4** complex (bottom). The arrows indicate the $Z \rightarrow E$ double-bond photoisomerization that leads to the P_{fr} forms.

The noncovalent incorporation of the methanol adduct in the apophyA 65/**4** complex was demonstrated conclusively by a competition experiment. Upon addition of **2** to the P_r-like form of the complex under conditions known[11b,c] to bring about spontaneous covalent bonding to the apoprotein, the methanol adduct **4** was promptly replaced in the binding pocket. The familiar chromoprotein, the P_r form of phyA 65-**2**,[11] was formed instead (Figure 1) and was capable of undergoing the characteristic P_r/P_{fr} photocycle (recombinant phyA 65-**2**: $\lambda_{max} = 653$ nm for P_r, and 718 nm for P_{fr}[11c]).

The absorption intensity of the P_{fr}-like form of apophyA 65/**4** is somewhat lower, relative to P_r, than is customary for the P_{fr} forms with covalently bound chromophores (**1** or **2**), and the spectral shape suggests the presence of more than just two absorbing components (Figure 1 A). Nevertheless, in the difference absorption spectrum of apophyA 65/**4** (Figure 1 B), the intensities of the P_r- and P_{fr}-like features are comparable

  1433-7851/00/3918-3270 $ 17.50+.50/0 *Angew. Chem. Int. Ed.* **2000**, *39*, No. 18

to those of wild-type phytochromes. The greater breadth of the absorption bands, relative to those of P_r and P_{fr}, possibly reflects a wider conformational population of chromophores in the binding pocket than is encountered in the phytochromes. It transpires that the covalent bonding to Cys 321 (the binding position in the oat phytochrome) is essential for a conformationally uniform incorporation of the chromophore into the protein. Undoubtedly, this arrangement is further supported by hydrogen bridges and dipolar interactions. In the absence of the covalent bonding, these nonbonding forces are insufficient to selectively provide the optimum conformation. Nevertheless they are good enough to ensure an arrangement which is reasonably stable—albeit one which allows exchange of the methanol adduct **4** with the unsaturated phycocyanobilin (**2**)—and is capable of performing a phototrigger function that mimics the phytochrome photochromism.

Experimental Section

Compound **4**: **2** and **4** were obtained from *Spirulina platensis* upon methanolysis in the routine preparation of **2**.[16] After treating the lyophilized cyanobacteria (500 g) for 16 hr with refluxing MeOH under argon in the dark, the bilin extract was purified by reverse-phase column chromatography. Preparative HPLC (Chromasil RP-18, 1 mL min^{-1}, MeCN:phosphate buffer(7:18, 7.5 mM, pH 7.6)) separated **2** and **4** (12:1). Acetonitrile was removed from the latter fraction in vacuo and the residue was extracted with CH_3Cl:MeOH (19:1). Compound **4** was crystallized from CH_2Cl_2:*n*-hexane at $-70\,^{\circ}C$. UV/Vis (100 mM Tris, 200 mM NaCl, 10% glycerol, 0.2% DMSO, pH 8.0): $\lambda_{max} = 348$, 593 nm. ^{1}H NMR (500 MHz, C_5D_5N, ^{1}H/^{1}H-COSY): $\delta = 1.24$ (t, ^3J(H,H) = 7.52 Hz, 3H; H_3C-18^2), 1.31 (d, ^3J(H,H) = 6.30 Hz, 3H; H_3C-3^2), 1.43 (d, ^3J(H,H) = 7.35 Hz, 3H; H_3C-2^1), 1.97, 2.07, 2.12, 3.30 (4s, 3H each; H_3C-7^1, -17^1, -13^1, and H_3CO-3^1, respectively), 2.49 (m, 2H; H_2C-18^1), 2.78, 3.06 (2m, 1H each; HC-2 and -3, respectively), 2.85 (m, 4H,; H_2C-8^2 and -12^2), 3.10, 3.18 (2t, ^3J(H,H) = 7.42, 7.23 Hz, 2H each; H_2C-8^1 and -12^1, respectively), 3.75 (m, 1H; HC-3^1), 5.68, 6.06, 7.30 (3s, 1H each; HC-5, -15, and -10, respectively); the signals were assigned by decoupling and NOESY experiments. HR-MS (ESI positive ion mode): $C_{34}H_{42}N_4O_7Na$ [M^+Na]: calcd 641.2951, found 641.2999.

The structure of **4**[12] was confirmed by MS and comparison of the ^{1}H NMR spectra with in-house recorded data for **2**, and literature data for the dimethylesters of **2**,[17] **4**,[13] and adducts of **2** with short Cys-containing peptides.[18] The sample of **4** that was used for the assembly with apophyA 65 was homogeneous by HPLC, MS, and ^{1}H NMR spectroscopy. In particular, it was not contaminated with other tetrapyrrole components, such as **2**.

Assembly of the apophyA 65/**4** complex and irradiation: Oat apophyA 65[11] (crude lysate of *Hansenula polymorpha* yeast cells;[11a] 4.5 nmol in 600 μL phosphate buffer, pH 7.6) was incubated with **4** (5 nmol in 1 μL DMSO) at ambient temperature. The apoprotein concentration was determined prior to incubation, by assembly of an aliquot of the apoprotein solution with excess **2**.

The solution of apophyA 65/**4** complex was irradiated with light from a slide projector bulb (250 W; distance between sample cell and bulb was approximately 20 cm; excitation wavelengths of $\lambda = 614 \pm 7$ and 714 ± 7 nm were determined for $P_r \rightarrow P_{fr}$ and $P_{fr} \rightarrow P_r$, respectively, with interference filters). Complete conversions (maximum absorption values) required irradiation periods of about 10 min. Absorption spectra were recorded with a Shimadzu spectrophotometer UV2102PC.

Competition experiment with apophyA 65/**4** complex and **2**: Compound **2** (1.7 nmol in 1 μL DMSO) was added to a sample of the P_r-like form of the apophyA 65/**4** complex (600 μL). The mixture was kept in the dark for several min prior to consecutive irradiations at $\lambda = 614 \pm 7$ (interference filter; $P_r \rightarrow P_{fr}$) and approximately 720 nm (cut-off filter; $P_{fr} \rightarrow P_r$). Absorption spectroscopic analyses were performed prior to the first and after each irradiation.

Received: April 11, 2000 [Z 14972]

[1] W. M. Schluchter, A. N. Glazer in *The Phototrophic Prokaryotes* (Eds.: G. A. Peschek, W. Löffelhardt, G. Schmetterer), Kluwer, New York, **1999**, pp. 83–95.

[2] S. E. Braslavsky, W. Gärtner, K. Schaffner, *Plant Cell Environ.* **1997**, *20*, 700–706.

[3] W. Rüdiger, F. Thümmler, E. Cmiel, S. Schneider, *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 6244–6248, and references therein.

[4] S.-H. Wu, M. T. McDowell, J. C. Lagarias, *J. Biol. Chem.* **1997**, *272*, 25700–25705.

[5] a) J. C. Lagarias, D. M. Lagarias, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 5778–5780; b) T. D. Elich, J. C. Lagarias, *J. Biol. Chem.* **1989**, *264*, 12902–12908; c) T. Kunkel, K.-J. Tomizawa, R. Kern, M. Furuya, N.-H. Chua, E. Schäfer, *Eur. J. Biochem.* **1993**, *215*, 587–594; d) C. Hill, W. Gärtner, P. Towner, S. E. Braslavsky, K. Schaffner, *Eur. J. Biochem.* **1994**, *223*, 69–77; e) A. Ruddat, P. Schmidt, C. Gatz, S. E. Braslavsky, W. Gärtner, K. Schaffner, *Biochemistry* **1997**, *36*, 103–111.

[6] S. E. Braslavsky, D. Schneider, K. Heihoff, S. Nonell, P. F. Aramendía, K. Schaffner, *J. Am. Chem. Soc.* **1991**, *113*, 7322–7334.

[7] $Z \rightarrow E$ Isomerization around either of the lateral double bonds (C-5 and C-15) is effected by reversible addition of nucleophiles to the central C-10 methine prior to photoreaction, see: H. Falk, *The Chemistry of Linear Oligopyrroles and Bile Pigments*, Springer, New York, **1989**, pp. 462–463.

[8] a) S. H. Bhoo, T. Hirano, H.-Y. Jeong, J.-G. Lee, M. Furuya, P.-S. Song, *J. Am. Chem. Soc.* **1997**, *119*, 11717–11718; b) A. Remberg, P. Schmidt, S. E. Braslavsky, W. Gärtner, K. Schaffner, *Eur. J. Biochem.* **1999**, *266*, 201–208.

[9] I. Lindner, B. Knipp, S. E. Braslavsky, W. Gärtner, K. Schaffner, *Angew. Chem.* **1998**, *110*, 1943–1946; *Angew. Chem. Int. Ed.* **1998**, *37*, 1843–1846.

[10] S. J. Davis, A. V. Vener, R. D. Vierstra, *Science* **1999**, *286*, 2517–2520.

[11] a) D. Mozley, A. Remberg, W. Gärtner, *Photochem. Photobiol.* **1997**, *66*, 710–715; b) W. Gärtner, C. Hill, K. Worm, S. E. Braslavsky, K. Schaffner, *Eur. J. Biochem.* **1996**, *236*, 978–983; c) A. Remberg, A. Ruddat, S. E. Braslavsky, W. Gärtner, K. Schaffner, *Biochemistry* **1998**, *37*, 9983–9990.

[12] Methanol addition to **2** is expected to afford the two C-3′ epimers of **4**.[13] However, neither the ^{1}H NMR spectrum nor the HPLC results indicate any heterogeneity attributable to a mixture of epimers in the sample of crystalline **4** used in this work. An assignment of its C-3′ configuration on the basis of the available analytical data was not possible.

[13] A. Gossauer, R.-P. Hinze, R. Kutschan, *Chem. Ber.* **1981**, *114*, 132–146.

[14] For a discussion of the 5*Z*,10*Z*,15*Z* configuration of the chromophore in P_r phytochrome, see: C. Kneip, P. Hildebrandt, W. Schlamann, S. E. Braslavsky, F. Mark, K. Schaffner, *Biochemistry* **1999**, *38*, 15185–15192.

[15] For the induction of P_r- and P_{fr}-like in-vitro spectral shifts in organic solutions, see: a) M. Stanek, K. Grubmayr, *Chem. Eur. J.* **1998**, 1653–1659; b) M. Stanek, K. Grubmayr, *Chem. Eur. J.* **1998**, 1660–1666.

[16] W. Kufer, H. Scheer, *Hoppe–Seyler's Z. Physiol. Chem.* **1979**, *360*, 935–956.

[17] W. J. Cole, D. J. Chapman, H. W. Siegelman, *Biochemistry* **1968**, *7*, 2929–2935.

[18] J. E. Bishop, H. Rapoport, A. V. Klotz, C. F. Chan, A. N. Glazer, P. Füglistaller, H. Zuber, *J. Am. Chem. Soc.* **1987**, *109*, 875–881.

  1433-7851/00/3918-3271 $ 17.50+.50/0