Radical Pairs in Proteins

DOI: 10.1002/anie.200803102

Direct Observation of a Photoinduced Radical Pair in a Cryptochrome Blue-Light Photoreceptor**

*Till Biskup, Erik Schleicher, Asako Okafuji, Gerhard Link, Kenichi Hitomi, Elizabeth D. Getzoff, and Stefan Weber**

Although proteins from the photolyase/cryptochrome family have a common three-dimensional fold, sequence homology, and redox-active flavin adenine dinucleotide (FAD) cofactor, they exhibit diverse activities.[1] In response to blue or UV-A light, they function physiologically in DNA repair, entrainment of the circadian clock, and other processes, such as stimulation of plant growth.[1–3] Members of the photolyase/cryptochrome family have been identified in various organisms ranging from bacteria to plants, animals, and humans.[1] Within this family of proteins, a phylogenetic cluster of genes originally identified from *Arabidopsis* and *Synechocystis* encode cryptochrome-like proteins, which are distinct from previously characterized "classic" plant (represented by *Arabidopsis* HY4) and animal (represented by *Drosophila* and *Homo sapiens*) cryptochromes, yet more closely resemble the latter.[4] Remarkably, the cryptochromes from this new cluster (Cry-DASH) have now been found throughout all kingdoms of life.[5] While multiple biological functions have been discussed, the availability of stable, recombinantly expressed, Cry-DASH proteins from diverse species provides the means of deciphering cryptochrome protein chemistry. Results from recent experiments indicate that Cry-DASH could work as a transcriptional regulator[4,5] as well as a DNA repair enzyme for single-stranded DNA.[6] Other experimental results suggest the participation of Cry-DASH in circadian input pathways.[7,8]

Redox reactions are proposed to play a key role in light-responsive activities of cryptochromes.[9,10] Both in vitro and in vivo experiments suggest that the FAD redox state is changed from fully oxidized (FAD^{ox}) to the radical form when it adopts the signaling state.[11,12] The results agree with the redox activity of photolyases.[13] In the latter, when starting from FAD^{ox}, photoinduced electron transfer (ET) produces a radical pair (RP), comprising an FAD and either a tyrosine or a tryptophan radical, which is directly observable by electron paramagnetic resonance (EPR) spectroscopy.[14–16]

ET-generated RPs are proposed to function as compasses for geomagnetic orientation in a large and taxonomically diverse group of organisms.[17–19] In organisms in which this process has been found to be light-dependent,[20] a sensor based on magnetic-field-sensitive RP chemistry could be in effect. However, also other mechanisms of magnetoreception based on, for example, iron-containing particles,[21] cannot be ruled out. Cryptochrome's potential for forming RPs upon blue-light excitation, by analogy with photolyases, and also its presence in the eyes of migratory birds,[22] make it a candidate photoreceptor-based magnetoreceptor.[18,23]

In principle, a compass based on RP photochemistry can be realized by 1) generation of a spin-correlated RP with coherent interconversion of its singlet and triplet states, 2) modulation of this interconversion by Zeeman magnetic interactions of the two electron spins with the geomagnetic field, and 3) sufficiently small interradical exchange and dipolar interactions such that they do not override the Zeeman interactions. Spin-correlated flavin-based RPs may be able to fulfill these criteria.[18,24,25] Hence, understanding the suitability and potential of cryptochromes for magnetoreception requires the identification of RP states and their origin, and the detailed characterization of magnetic interaction parameters and kinetics. Here, we use *Xenopus laevis* Cry-DASH proteins (referred to as *Xl*Cry-DASH) as a paradigm system to test the hypothesis that spin-correlated RPs can be induced in cryptochromes by blue light.

A motif common to all structurally characterized members of the photolyase/cryptochrome family is a conserved chain of three tryptophan (Trp) residues for ET from the protein surface to the FAD cofactor.[26,27] Based on sequence alignment of *Xl*Cry-DASH with other family members of known structure,[4,26,28–30] we identified the putative Trp triad for ET (see Figure 1). In *Escherichia coli* DNA photolyase, a single photoinduced ET step from a nearby Trp reduces the FAD.[31] The resulting radical state on the Trp is subsequently transferred to the terminal Trp (W306).[13,32] Provided that the FAD is initially fully oxidized, ET generates a short-lived flavin-based RP species.[14–16] Subsequent proton release or uptake has been shown to eventually result in a RP state from the neutral radicals [$W306^{\bullet}\cdots FADH^{\bullet}$]. Because of the high

[*] Dr. E. Schleicher, A. Okafuji, Dr. G. Link, Prof. Dr. S. Weber
Institute of Physical Chemistry
Faculty of Chemistry, Pharmacy and Earth Sciences
Albert-Ludwigs-University
Albertstrasse 21, 79104 Freiburg (Germany)
Fax: (+49) 761-203-6222
E-mail: Stefan.Weber@physchem.uni-freiburg.de
T. Biskup
Department of Physics, Free University Berlin (Germany)
Dr. K. Hitomi, Prof. E. D. Getzoff
Department of Molecular Biology
and the Skaggs Institute for Chemical Biology
The Scripps Research Institute, La Jolla, CA (USA)

[**] This work was supported by the Deutsche Forschungsgemeinschaft (Sfb-498, project A2, and FOR-526), the U.S. National Institutes of Health (Grant R01 GM37684 to E.D.G.), and the Skaggs Institute of Chemical Biology (fellowship to K.H.). We thank J. R. Norris (University of Chicago) and R. Bittl (Free University Berlin) for helpful discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200803102.

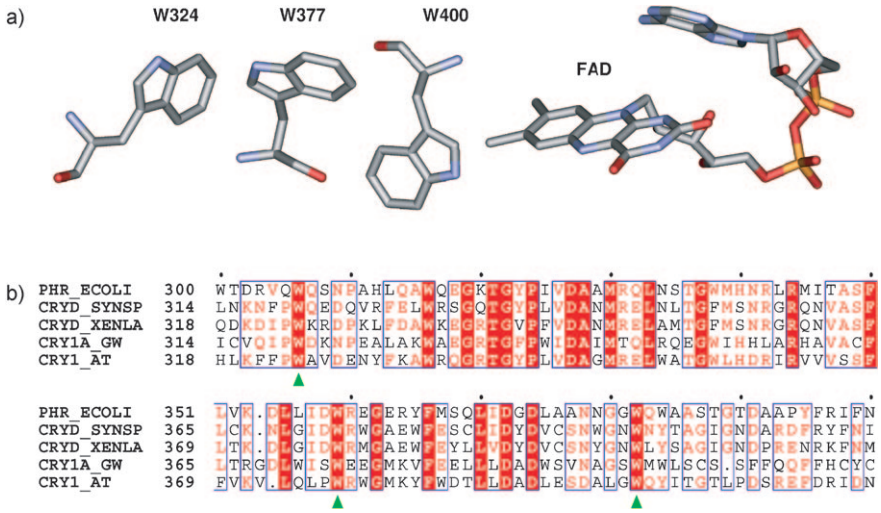

Figure 1. The conserved Trp triad of *Xl*Cry-DASH. a) Section from the three-dimensional protein structure homology model from the SWISS-MODEL repository (UniProt ID: CRYD_XENLA). b) Sequence alignment of five members of the photolyase/cryptochrome family. The conserved Trp residues of the putative ET chain in *E. coli* photolyase (PHR_ECOLI),[26] *Synechocystis* sp. Cry-DASH (CRYD_SYNSP),[4] *Xl*Cry-DASH (CRYD_XENLA), garden warbler (*Sylvia borin*) Cry-1a (CRY1a_GW), and *Arabidopsis thaliana* Cry-1 (CRY1_AT)[28] are marked with green triangles. Columns with an alignment score >0.7 are surrounded with a blue frame and the conserved amino acids colored red on white background. If the residues are strictly conserved, they are colored white on red background. The alignment was performed with MultAlin and further processed with ESPript 2.2.

structural conservation, we assume a similar ET mechanism for DASH cryptochromes.

Transient EPR (TREPR) spectroscopy, with a time resolution of up to 10 ns, allows real-time observation of short-lived RP states generated by pulsed laser excitation.[33] We compared the RP signals of the wild-type (WT) *Xl*Cry-DASH protein with that of a mutant (W324F) lacking the terminal Trp residue of the conserved putative ET chain (see Figure 1). In Cry-DASH proteins, the isoalloxazine moiety in FAD may assume three different redox states: fully reduced ($FADH^-$), one-electron oxidized ($FAD^{\bullet-}$ or $FADH^{\bullet}$), and two-electron oxidized (FAD^{ox}), which can be identified by their characteristic optical absorptions (Figure 2). The pronounced absorbance near 380 nm is due to the second chromophore, methenyl tetrahydrofolyl polyglutamate.[5] *Xl*Cry-DASH with homogeneous FAD^{ox} can be prepared from a mixture of the three FAD redox states by treatment with potassium ferricyanide.[14] FAD^{ox} has been chosen as the initial state because 1) it was found to be the physiologically relevant dark state in plant and animal cryptochromes,[34–36] and 2) potential spin-polarized RP intermediates can be generated from FAD^{ox} by photoinduced ET.[14–16]

In Figure 3, the TREPR signal of WT *Xl*Cry-DASH recorded at a physiologically relevant temperature (274 K) is depicted in three dimensions as a function of the magnetic field B_0 and the time t after pulsed laser excitation at 460 nm. In contrast to conventional continuous-wave EPR spectroscopy, which requires magnetic-field modulation to improve the signal-to-noise ratio, TREPR is recorded in a direct detection mode, so as not to constrain the time resolution of the experiment. Consequently, positive and negative signals indicate the enhanced absorptive (A) and emissive (E) electron-spin polarization of the EPR transitions, respectively.[37,38]

Upon photoexcitation, WT *Xl*Cry-DASH readily forms a spin-polarized paramagnetic species, which we assigned to a RP based on the spectral shape and narrow width of the signal. (A spin-polarized flavin triplet state detected under comparable experimental conditions would span more than 150 mT as a result of the large spin–spin interactions between the two unpaired electrons.[39]) The time evolution of the TREPR signal reveals that the RP state lives for at least 6 μs; a more precise determination is not possible because the exponential signal decay is predominantly determined by relaxation of the spin polarization to the Boltzmann equilibrium populations. The spectrum of *Xl*Cry-DASH recorded after 500 ns (Figure 4) resembles those obtained recently from light-induced transient RP species (comprising flavin and amino acid radicals) resulting from FAD photoreduction of photolyases.[15,16] In the *E. coli* enzyme, the TREPR-observed RP state was assigned to [W306$^{\bullet+}$···FADH$^{\bullet}$], W306 being the terminal Trp residue of the Trp triad. To unravel the origin of the RP signal in *Xl*Cry-DASH, we examined the W324F mutant, which lacks the equivalent terminal Trp (W324; see Figure 1). Under identical experimental conditions, the W324F protein does not exhibit any TREPR signal (see Figure 4). We therefore conclude that W324 is either the ultimate electron donor in ET to the flavin or constitutes an

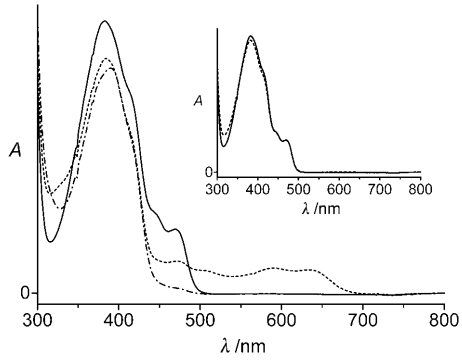

Figure 2. Optical absorption spectra of *Xl*Cry-DASH recorded at 273 K show the FAD cofactor in different oxidation states: FAD^{ox} (——), $FADH^{\bullet}$ (-----), and $FADH^-$ (–•–•). The inset shows *Xl*Cry-DASH with the FAD^{ox} cofactor before illumination (——) and *Xl*Cry-DASH reoxidized by aerial oxygen after 12 h of blue-light illumination (-----). This is to demonstrate that the protein remains intact in terms of its cofactor contents even upon intensive light-illumination conditions.

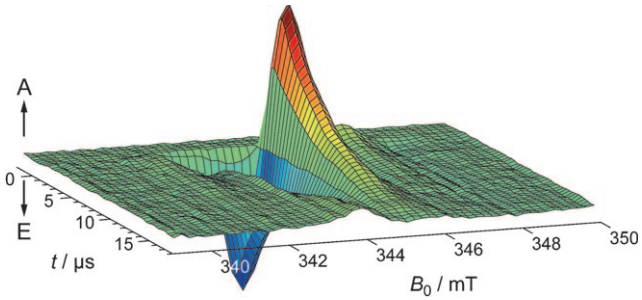

Figure 3. Complete TREPR data set of *Xl*Cry-DASH measured at 274 K. To monitor potential shape changes in the TREPR signal caused by gradual sample degradation, spectra were recorded from low to high magnetic field followed by detection in the opposite magnetic-field direction. Each time profile is the average of 120 acquisitions recorded with a laser pulse repetition rate of 1.25 Hz, a microwave frequency of 9.68 GHz, and a power of 2 mW at a detection bandwidth of 100 MHz. A: enhanced absorption; E: emission.

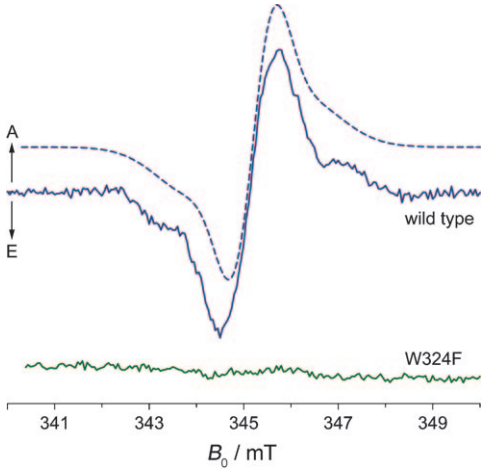

Figure 4. TREPR spectrum of WT (solid blue curve) and W324F (solid green curve) *Xl*Cry-DASH recorded 500 ns after pulsed laser excitation. Experimental parameters were as those used for Figure 3. The dashed curve shows a spectral simulation of the WT protein EPR data using the following parameters: $\mathbf{g}_{FAD} = (2.00431, 2.00360, 2.00217)$, $\mathbf{g}_{Trp} = (2.00370, 2.00285, 2.00246)$, $\mathbf{\Omega}(\mathbf{g}_{FAD}\cdots\mathbf{g}_{Trp}) = (126.5°, 76.5°, 246.5°)$, $D = -0.36$ mT, $E = 0$, $\mathbf{\Omega}(\mathbf{g}_{FAD}\cdots\mathbf{D}) = (0°, 109.9°, 110.5°)$, $J = +0.24$ mT.

integral part of the Cry-DASH ET pathway leading from the protein surface to the FAD.

Further information on the RP state [W324$^{\bullet}$···FADH$^{\bullet}$] in *Xl*Cry-DASH was obtained from spectral simulations performed on the basis of the correlated-coupled RP mechanism (CCRP model),[37,38] outlined in more detail in the Supporting Information. The calculations were performed using published **g**-tensor parameters for FAD[40] and Trp[41] neutral radicals. The relative orientations of the principal axes of both **g**-tensors and the dipole–dipole coupling tensor were taken from the homology model (see Figure 1) and kept fixed. The strength of the dipolar coupling ($D = -0.36$ mT, $E = 0$) between FADH$^{\bullet}$ and W324$^{\bullet}$ was estimated based on the point-dipole approximation, $D(r)/\text{mT} = -2.78/(r/\text{nm})^3$, assuming an interradical distance r of 2.0 nm between the points of highest unpaired electron-spin density, C(4a) and C(3), in FADH$^{\bullet}$ and W324$^{\bullet}$, respectively. Different overall inhomogeneous spectral line widths of Gaussian shape were considered in the calculations for both radicals. We restricted our simulations to TREPR spectra observed at very early time points to avoid spectral alterations arising from anisotropic spin relaxation. The good agreement between calculated and experimental TREPR spectra (Figure 4) supports our hypothesis that W324 is the terminal electron donor to FAD. However, satisfactory simulation of the overall E/E/A/A polarization pattern of the WT spectrum required two simultaneous assumptions: 1) a pure electronic singlet state as RP precursor, which is consistent with findings from optical spectroscopy on the equivalent RP state in photolyase,[42] and 2) a positive value for the exchange interaction J; in other words, the triplet configuration of the RP is energetically favored by $2J$ over the singlet RP configuration. J is assumed to fall off exponentially with the interradical distance r, $J(r) = J_0 \exp(-\beta r)$,[43] where a β value of (14 ± 2) nm^{-1} was proposed for ET in proteins.[44] In our simulations, we obtained the best fit at $J = +0.24$ mT, which is larger than the value reported for the primary RP in bacterial photosynthesis ($|J| = 0.9$ mT at $r = 1.8$ nm[45]) when scaled to the same interradical distance. Given that the bridging aromatic residues W377 and W400 in *Xl*Cry-DASH might be conducive to an efficient J coupling between FADH$^{\bullet}$ and W324$^{\bullet}$, our value appears reasonable, but it could well be in error by a factor of 2 to 4 because of uncertainties in model geometry and possible reorientations of FAD with respect to W324 in the RP relative to the ground state. The large radical–radical interactions in the *Xl*Cry-DASH RP seemed, at first, to preclude a sufficiently strong response to the earth's weak magnetic field, yet the exchange and dipolar interactions might substantially cancel each other, as recently suggested by Efimova and Hore.[25] In this case, the geomagnetic field could affect product yields in cryptochrome, allowing it to function as the magnetoreceptor in the avian compass.

In conclusion, we have demonstrated that Cry-DASH readily forms a RP species upon blue-light excitation. We proved spin correlation in the RP by directly observing electron-spin-polarized EPR transitions in real time. Our observations support the conservation of this photo-induced reaction and its biological relevance among cryptochrome/photolyase proteins. We furthermore present the first spectral simulations for a flavin-based spin-correlated RP which allowed us to extract the exchange interaction parameter that is difficult to estimate based solely on the three-dimensional protein structure. Our simulations suggest 1) RP formation from a singlet-state precursor, and 2) exchange interactions of significant magnitude, such that they may not be neglected when RPs of the type of [W324$^{\bullet}$···FADH$^{\bullet}$] are considered as candidate spin states in a RP mechanism of geomagnetoreception. Thus, the studies presented here show that the RPs of cryptochromes have fundamental properties appropriate for a magnetic compass.

Experimental Section

*Xl*Cry-DASH was expressed and purified in the dark, as described previously.[5] For TREPR studies, protein samples stored in buffer (0.3 M NaCl, 0.1 M Tris·HCl, pH 8.0, 30–50% (v/v) glycerol) were supplemented with 5 mM potassium ferricyanide, $K_3[Fe(CN)_6]$, and incubated overnight to ensure homogeneity of the FAD oxidation state. After removal of excess $K_3[Fe(CN)_6]$ by ultrafiltration, samples were supplemented with 5 mM $K_3[Fe(CN)_6]$ and 35% (v/v) glycerol and used for TREPR, or supplemented with 10 mM EDTA, and subsequently illuminated at 273 K with blue light (Halolux 100HL, Streppel, Wermelskirchen-Tente, Germany) selected with a 430–470 nm band filter to generate reduced states of the FAD. Concentrations of the individual FAD redox states were estimated based on their published absorbance coefficients (M. S. Jorns, B. Wang, S. P. Jordan, L. P. Chanderkar, *Biochemistry* **1990**, *29*, 552) using a Shimadzu UV-1601PC spectrophotometer.

Time-resolved detection of EPR following pulsed laser excitation was performed using a laboratory-built spectrometer.[15] Pulsed optical excitation of the samples was provided by a Nd:YAG laser (Spectra Physics GCR-11) pumping an optical parametric oscillator (Opta BBO-355-vis/IR, Opta GmbH, Bensheim, Germany) tuned to a wavelength of 460 nm (pulse width 6 ns; pulse energy 4 mJ).

Received: June 27, 2008
Revised: August 13, 2008
Published online: December 4, 2008

Keywords: electron transfer · EPR spectroscopy · flavins · radical pairs · singlet–triplet transition

[1] C. Lin, T. Todo, *Genome Biol.* **2005**, *6*, 220.
[2] A. R. Cashmore, *Cell* **2003**, *114*, 537.
[3] A. Losi, *Photochem. Photobiol.* **2007**, *83*, 1283.
[4] R. Brudler, K. Hitomi, H. Daiyasu, H. Toh, K.-i. Kucho, M. Ishiura, M. Kanehisa, V. A. Roberts, T. Todo, J. A. Tainer, E. D. Getzoff, *Mol. Cell* **2003**, *11*, 59.
[5] H. Daiyasu, T. Ishikawa, K.-i. Kuma, S. Iwai, T. Todo, H. Toh, *Genes Cells* **2004**, *9*, 479.
[6] C. P. Selby, A. Sancar, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17696.
[7] P. Facella, L. Lopez, A. Chiappetta, M. B. Bitonti, G. Giuliano, G. Perrotta, *FEBS Lett.* **2006**, *580*, 4618.
[8] S. A. Brunelle, E. S. Hazard, E. E. Sotka, F. M. Van Dolah, *J. Phycol.* **2007**, *43*, 509.
[9] O. Froy, D. C. Chang, S. M. Reppert, *Curr. Biol.* **2002**, *12*, 147.
[10] B. Giovani, M. Byrdin, M. Ahmad, K. Brettel, *Nat. Struct. Biol.* **2003**, *10*, 489.
[11] M. Merrow, T. Roenneberg, *Cell* **2001**, *106*, 141.
[12] R. Banerjee, E. Schleicher, S. Meier, R. Muñoz Viana, R. Pokorny, M. Ahmad, R. Bittl, A. Batschauer, *J. Biol. Chem.* **2007**, *282*, 14916.
[13] C. Aubert, M. H. Vos, P. Mathis, A. P. M. Eker, K. Brettel, *Nature* **2000**, *405*, 586.
[14] Y. M. Gindt, E. Vollenbroek, K. Westphal, H. Sackett, A. Sancar, G. T. Babcock, *Biochemistry* **1999**, *38*, 3857.
[15] S. Weber, C. W. M. Kay, H. Mögling, K. Möbius, K. Hitomi, T. Todo, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1319.
[16] S. Weber, *Biochim. Biophys. Acta Bioenerg.* **2005**, *1707*, 1.
[17] K. Schulten, *Festkörperprobleme*, *Vol. 22* (Ed.: J. Treusch), Vieweg, Braunschweig, **1982**, p. 61.
[18] T. Ritz, S. Adem, K. Schulten, *Biophys. J.* **2000**, *78*, 707.
[19] T. Ritz, P. Thalau, J. B. Phillips, R. Wiltschko, W. Wiltschko, *Nature* **2004**, *429*, 177.
[20] W. Wiltschko, R. Wiltschko, *J. Comp. Physiol. A* **2005**, *191*, 675.
[21] J. L. Kirschvink, J. L. Gould, *Biosystems* **1981**, *13*, 181.
[22] M. Liedvogel, K. Maeda, K. Henbest, E. Schleicher, T. Simon, C. R. Timmel, P. J. Hore, H. Mouritsen, *PLoS ONE* **2007**, *2*, e1106.
[23] H. Mouritsen, U. Janssen-Bienhold, M. Liedvogel, G. Feenders, J. Stalleicken, P. Dirks, R. Weiler, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14294.
[24] I. A. Solov'yov, D. E. Chandler, K. Schulten, *Biophys. J.* **2007**, *92*, 2711.
[25] O. Efimova, P. J. Hore, *Biophys. J.* **2008**, *94*, 1565.
[26] H.-W. Park, S.-T. Kim, A. Sancar, J. Deisenhofer, *Science* **1995**, *268*, 1866.
[27] M. Byrdin, V. Sartor, A. P. M. Eker, M. H. Vos, C. Aubert, K. Brettel, P. Mathis, *Biochim. Biophys. Acta Bioenerg.* **2004**, *1655*, 64.
[28] C. A. Brautigam, B. S. Smith, Z. Ma, M. Palnitkar, D. R. Tomchick, M. Machius, J. Deisenhofer, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 12142.
[29] Y. Huang, R. Baxter, B. S. Smith, C. L. Partch, C. L. Colbert, J. Deisenhofer, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17701.
[30] T. Klar, R. Pokorny, J. Moldt, A. Batschauer, L.-O. Essen, *J. Mol. Biol.* **2007**, *366*, 954.
[31] M. Byrdin, A. P. M. Eker, M. H. Vos, K. Brettel, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 8676.
[32] Y. F. Li, P. F. Heelis, A. Sancar, *Biochemistry* **1991**, *30*, 6322.
[33] R. Bittl, S. Weber, *Biochim. Biophys. Acta Bioenerg.* **2005**, *1707*, 117.
[34] M. Ahmad, N. Grancher, M. Heil, R. C. Black, B. Giovani, P. Galland, D. Lardemer, *Plant Physiol.* **2002**, *129*, 774.
[35] C. Lin, D. E. Robertson, M. Ahmad, A. A. Raibekas, M. S. Jorns, P. L. Dutton, A. R. Cashmore, *Science* **1995**, *269*, 968.
[36] A. Berndt, T. Kottke, H. Breitkreuz, R. Dvorsky, S. Hennig, M. Alexander, E. Wolf, *J. Biol. Chem.* **2007**, *282*, 13011.
[37] G. L. Closs, M. D. E. Forbes, J. R. Norris, *J. Phys. Chem.* **1987**, *91*, 3592.
[38] P. J. Hore, D. A. Hunter, C. D. McKie, A. J. Hoff, *Chem. Phys. Lett.* **1987**, *137*, 495.
[39] R. M. Kowalczyk, E. Schleicher, R. Bittl, S. Weber, *J. Am. Chem. Soc.* **2004**, *126*, 11393.
[40] C. W. M. Kay, R. Bittl, A. Bacher, G. Richter, S. Weber, *J. Am. Chem. Soc.* **2005**, *127*, 10780.
[41] R. Pogni, M. C. Baratto, C. Teutloff, S. Giansanti, F. J. Ruiz-Dueñas, T. Choinowski, K. Piontek, A. T. Martínez, F. Lendzian, R. Basosi, *J. Biol. Chem.* **2006**, *281*, 9517.
[42] a) K. B. Henbest, K. Maeda, P. J. Hore, M. Joshi, A. Bacher, R. Bittl, S. Weber, C. R. Timmel, E. Schleicher, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 14395; b) A. W. MacFarlane IV, R. J. Stanley, *Biochemistry* **2001**, *40*, 15203.
[43] F. J. J. De Kanter, R. Kaptein, R. A. Van Santen, *Chem. Phys. Lett.* **1977**, *45*, 575.
[44] C. C. Moser, J. M. Keske, K. Warncke, R. S. Farid, L. Dutton, *Nature* **1992**, *355*, 796.
[45] R. J. Hulsebosch, I. V. Borovykh, S. V. Paschenko, P. Gast, A. J. Hoff, *J. Phys. Chem. B* **1999**, *103*, 6815.

