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Review

Transient radical pairs studied by time-resolved EPR

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Abstract

Photogenerated short-lived radical pairs (RP) are common in biological photoprocesses such as photosynthesis and enzymatic DNA repair. They can be favorably probed by time-resolved electron paramagnetic resonance (EPR) methods with adequate time resolution. Two EPR techniques have proven to be particularly useful to extract information on the working states of photoinduced biological processes that is only difficult or sometimes even impossible to obtain by other types of spectroscopy. Firstly, transient EPR yields crucial information on the chemical nature and the geometry of the individual RP halves in a doublet-spin pair generated by a short laser pulse. This time-resolved method is applicable in all magnetic field/microwave frequency regimes that are used for continuous-wave EPR, and is nowadays routinely utilized with a time resolution reaching about 10 ns. Secondly, a pulsed EPR method named out-of-phase electron spin echo envelope modulation (OOP-ESEEM) is increasingly becoming popular. By this pulsed technique, the mutual spin–spin interaction between the RP halves in a doublet-spin pair manifests itself as an echo modulation detected as a function of the microwave-pulse spacing of a two-pulse echo sequence subsequent to a laser pulse. From the dipolar coupling, the distance between the radicals is readily derived. Since the spin–spin interaction parameters are typically not observable by transient EPR, the two techniques complement each other favorably. Both EPR methods have recently been applied to a variety of light-induced RPs in photobiology. This review summarizes the results obtained from such studies in the fields of plant and bacterial photosynthesis and DNA repair mediated by the enzyme DNA photolyase.

Keywords: Transient EPR; Ouf-of-phase electron spin echo envelope modulation; Radical pair; Photosynthesis; Photolyase

1. Introduction

A large number of enzyme-catalyzed biochemical reactions are coupled to or directly involve electron transfer (ET) reactions. Usually, protein-bound inorganic or organic molecules participate as redox-active species in these processes. During the past decades, a growing number of proteins have furthermore been recognized in which aromatic amino acid side chains serve as protein-intrinsic redox partners. Thereby, these amino acids are functionally equivalent to cofactor molecules, and in some cases, mediate ET between organic and inorganic components of an ET chain. Since most of the organic cofactor molecules are diamagnetic in their ground state, one single ET event between two organic species results in a pair of paramagnetic, often ionic states. In the case of the involvement of an inorganic, mostly transition metal-based cofactor, one single ET step leads to a change of the metal's oxidation state, and thereby, to a transition between an integer to a half-integer spin state or vice versa of the respective cofactor coupled to a concomitant spin state change of the redox partner. Such changes in the magnetic and electronic properties of a pair of cofactor molecules can be favorably investigated by electron paramagnetic resonance (EPR) and optical spectroscopies. While optical spectroscopy has a clear advantage concerning the obtainable time resolution down to the femtosecond regime, the coupling of the reduction and the oxidation is generally only visible in parallel kinetics of the components in the absorbance-difference spectra belonging to the two different redox partners. Time-resolved EPR in contrast suffers from a comparatively low time resolution of presently about 10 ns but has the clear advantage of a direct observation of the coupling between the redox partners. This is due to the sensitivity of magnetic resonance methods to weak interactions that cannot be resolved by optical techniques on large molecules. The

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small magnetic interaction observable by EPR methods in pairs of paramagnetic molecules within proteins mainly is the dipolar electron spin–spin interaction.

This contribution is focused on pairs of paramagnetic states of organic cofactors and amino acid residues occurring as native transient radical pair (RP) states during ET processes in redox-active proteins. The examples discussed concern two classes of photoactive proteins, the photosystems of purple bacterial and oxygenic photosynthesis and the DNA repair enzyme photolyase.

The basic function of all photosynthetic reaction centers (RCs) is a light-induced vectorial transmembrane ET involving several consecutive RP states. The ET chain in type I RCs (Photosystem I (PS I) of oxygenic photosynthesis in cyanobacteria, green algae and plants, and the RCs of heliobacteria and green sulfur bacteria) comprises chlorophyll cofactors as the photoactive electron donors and [4Fe–4S] iron–sulfur clusters as terminal electron acceptors. For a collection of recent reviews regarding the structure and function of type I RCs, see e.g., the special issue of Biochimica et Biophysica Acta edited by Heathcote [1]. In type II RCs (Photosystem II (PS II) of oxygenic photosynthesis in cyanobacteria, green algae and plants, and the RCs of purple bacteria (pbRC)) the photoactive electron donors are again (bacterio)chlorophyll species, but the terminal acceptors are quinones. In PS II, a tyrosine amino acid residue couples the light-driven ET chain involving chlorophyll *a*, pheophytin *a*, and plastoquinone-9 molecules to the redox chemistry of a (presumably tetra-nuclear) manganese cluster, which constitutes the site of photosynthetic water oxidation and oxygen evolution. For recent reviews on PS II see e.g., Refs. [2,3].

In photolyases (for a recent review, see e.g., Ref. [4]), RP states occur in the course of photoinitiated DNA repair and furthermore as intermediates during flavin adenine dinucleotide (FAD) cofactor photoreduction. The latter process is believed to take place to activate the enzyme in the case when the flavin is initially in a redox state different from the catalytically competent fully reduced $FADH^-$ form. Here, tryptophan and tyrosine residues act as protein-intrinsic electron donors that mediate ET between the flavin in the reactive center and exogenous reductants at the enzyme surface. As for photosystems, this review will only focus on the EPR detection of RP states in photolyases. For a general review on recent biophysical studies of this enzyme class, the reader is referred to the article by Weber in this issue.

2. Photosystems

2.1. Cofactor orientations

The first observation of spin-polarized EPR signals from a RP state in PS I of spinach chloroplasts has been reported by Blankenship et al. [5]. However, no appropriate theoretical model for the interpretation of the spectra was developed at that time. Thereafter, several studies on spin-polarized RPs have been performed, but also their interpretation lacked a sound theoretical description. Only in 1987 did two groups independently and almost simultaneously publish the same theoretical model of "spin-correlated radical pairs" (SCRP) [6,7]. This model provides the basis for the interpretation of all EPR-derived structural data on RPs so far.

The first application of the SCRP model to analyze the transient X- and K-band EPR spectra of the secondary RP state in PS I, $P_{700}^{\bullet+}\,A_1^{\bullet-}$, obtained by Stehlik et al. [8], yielded structural information on PS I, which was well before the successful crystallization and subsequent X-ray structure determination of this protein complex. Stehlik and coworkers found that the axis connecting the two carbonyl oxygens of phylloquinone (vitamin K_1, the secondary acceptor A_1 in PS I) is parallel to the axis connecting the donor P_{700} and the acceptor A_1 (see Fig. 1). In contrast, an angle of about 60° between the corresponding axes in pbRC was calculated from the X-ray structural model of the RC from (formerly) *Rhodopseudomonas viridis* [9,10]. The conclusion of Stehlik et al. was later tested spectroscopically by comparing the spin-polarized transient EPR spectra of the functionally equivalent states $P_{865}^{\bullet+}\,Q_A^{\bullet-}$ in pbRCs from

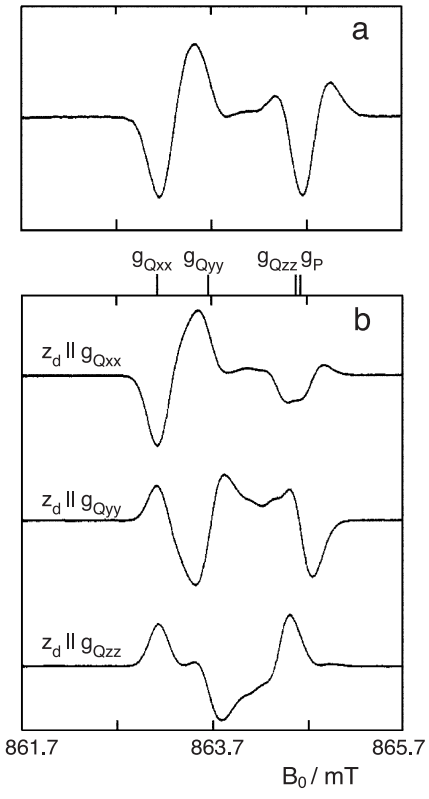

Fig. 1. (a) Room-temperature transient K-band EPR spectrum of PS I in freeze-dried perdeuterated *Synechococcus lividus* algae recorded at about 200 ns after pulsed laser excitation. (b) Simulations of the spectrum with the dipolar interaction vector z_d parallel to either one of the three principal axes of the *g*-tensor of $A_1^{\bullet-}$. Reprinted with permission from Ref. [8]. © 1989 American Chemical Society.

Rhodobacter sphaeroides and $P_{700}^{\bullet+}$ $A_1^{\bullet}$ in PS I [11]. These experiments confirmed the qualitative different alignment of the quinone acceptors in the two types of photosystems.

While in the studies of Stehlik et al. the structural parameters were determined by the analysis of the spin-polarized transient EPR spectra, a different approach to extract these parameters from time-resolved EPR data was established in the group of Kothe. The light-induced RP states in a magnetic field not only show a population of their eigenstates far from thermal (Boltzmann) equilibrium, but also carry additionally coherence between the eigenstates. This fact is an immediate consequence of the SCRP model and had been discussed early after the introduction of this model, e.g., by Hore [12]. The consequence of this coherence on the time evolution of the transient EPR signal was analyzed by Salikhov et al. [13]. Their analytical calculations of the EPR signal from a spin-polarized RP showed oscillatory time dependence. However, it was argued that the so-called quantum beat oscillations might be not detectable in experiments with real samples due to unfavorable interference effects of different oscillation frequencies [13]. In a parallel study using a numerical instead of an analytical approach to the problem, Bittl and Kothe [14] arrived at the same result of an oscillatory time dependence of the transient EPR signal. However, they concluded from their numerical calculations, which had been set up to model as closely as possible a realistic experimental situation, that the quantum beats should be observable in an EPR experiment with appropriate time resolution. Furthermore, it was concluded that the analysis of the oscillations provides an alternative way to extract geometrical parameters for the RP state from the time-resolved EPR data. The first successful experiment (see Fig. 2) proving the coherent nature of the RP state $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ in PS I [15] qualitatively corroborated the earlier result of Stehlik et al. of a rather parallel alignment of the carbonyl–oxygen axis of A_1 and the axis connecting P_{700} and A_1. Quantitatively, the analysis yielded a somewhat different geometry with a slight mismatch of 30° between these axes [16].

Meanwhile, both types of extraction of molecular-orientation parameters from transient EPR data have been refined and the discrepancy between them is now reduced. The data derived from the transient EPR spectra still give smaller values in the range between 0° and 10° [17] for the angle between the two axes compared to the 20° derived from the oscillatory time dependence of the transient EPR signal [18]. Irrespective of this minor discrepancy, it should be noted that the basic difference between the quinone–acceptor orientation in pbRC compared to PS I, initially concluded by Stehlik et al. in 1989 [8] from EPR data on PS I, is now well-corroborated by the X-ray crystallographic model of PS I [19], showing that in PS I, the axis connecting the two quinone oxygens points towards P_{700} [20].

2.2. Cofactor distances

The anisotropy of the dipolar electron spin–electron spin interaction within a RP is the source of the orientation information contained in the transient EPR spectra. The transient spectra are, however, rather insensitive on the magnitude of this dipolar interaction as long as it is smaller than the inhomogeneous broadening caused by unresolved hyperfine couplings [8]. In a theoretical study, Salikhov et al. [21] have shown that the magnitude of the dipolar interaction can be measured for a spin-polarized RP by a simple (Hahn-type) two-pulse spin echo experiment. The magnitude of the dipolar interaction is of interest because it depends as r^{-3} on the distance between the unpaired electrons in the RP and, therefore, contains distance information on the molecules forming the RP.

The spin echo for a spin-polarized RP is phase-shifted compared to the echo of the same spin system at thermal equilibrium and appears with the same signal phase as the applied microwave pulses. Therefore, the spin echo of a spin-polarized RP is called "out-of-phase echo". The amplitude of this out-of-phase echo is modulated due to the spin–spin interaction within the RP [21] as a function of the delay time τ between the two microwave pulses and termed

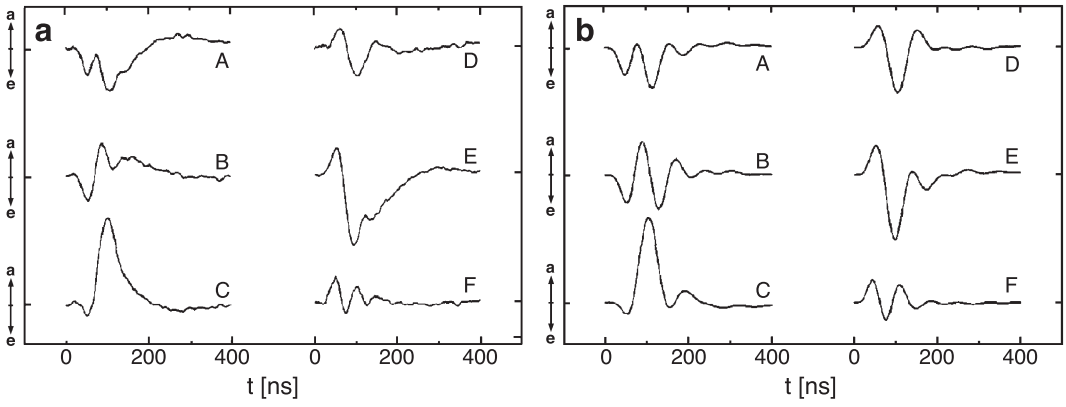

Fig. 2. Time evolution of the transverse magnetization of the light-induced RP state $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ in plant PS I of perdeuterated whole cells of cyanobacterium *S. lividus* at six different static magnetic fields B_0 of the transient EPR spectrum. (a) Experimental transients recorded at $T=295$ K. (b) Calculated transients from simulations using the SCRP model. Reprinted with permission from Ref. [15]. © 1991 Elsevier Science Publishers B.V.

out-of-phase electron spin echo envelope modulation (OOP-ESEEM) (see Fig. 3). The first experiment showing the theoretically predicted OOP-ESEEM [21,22] has been performed on the state $P_{865}^{\bullet+}\,Q_A^{\bullet-}$ in pbRC [23]. The observed dipolar interaction corresponds to a distance which agrees well with the one measured between P_{865} and Q_A in the X-ray structural model for the pbRC of *R. sphaeroides* (see Ref. [24] for a recent new structure and references therein).

Pulsed EPR experiments on the corresponding states $P_{700}^{\bullet+}\,A_1^{\bullet-}$ [25–27] and $P_{680}^{\bullet+}\,Q_A^{\bullet-}$ [28,29] in the two photosystems of oxygenic photosynthesis, PS I and PS II, respectively (see Fig. 3), yielded distances between the primary donors and the quinone acceptors before such information was available from X-ray crystallography. In both cases, the data from independently conducted experiments by two groups were in very good agreement with each other. For $P_{700}^{\bullet+}\,A_1^{\bullet-}$ in PS I, a distance of 25.4 Å, and for $P_{680}^{\bullet+}\,Q_A^{\bullet-}$ a distance of 27.4 Å have been deduced from the dipolar spin–spin coupling. For PS I with known assignment of the state $P_{700}^{\bullet+}$ and $A_1^{\bullet-}$ to a molecular species in the structural model, the EPR distance has meanwhile been corroborated by X-ray crystallographic data [19]. For $P_{680}^{\bullet+}\,Q_A^{\bullet-}$, the distance of 27.4 Å corresponds to an assignment of $P_{680}^{\bullet+}$ to P_{D1} or P_{D2} in the X-ray crystallographic model of PS II [30]. The EPR result obtained on wild-type PS II is in good agreement with an optical study on genetically modified PS II [31] that also yielded an assignment of $P_{680}^{\bullet+}$ to mainly P_{D1} and a variable contribution of P_{D2}.

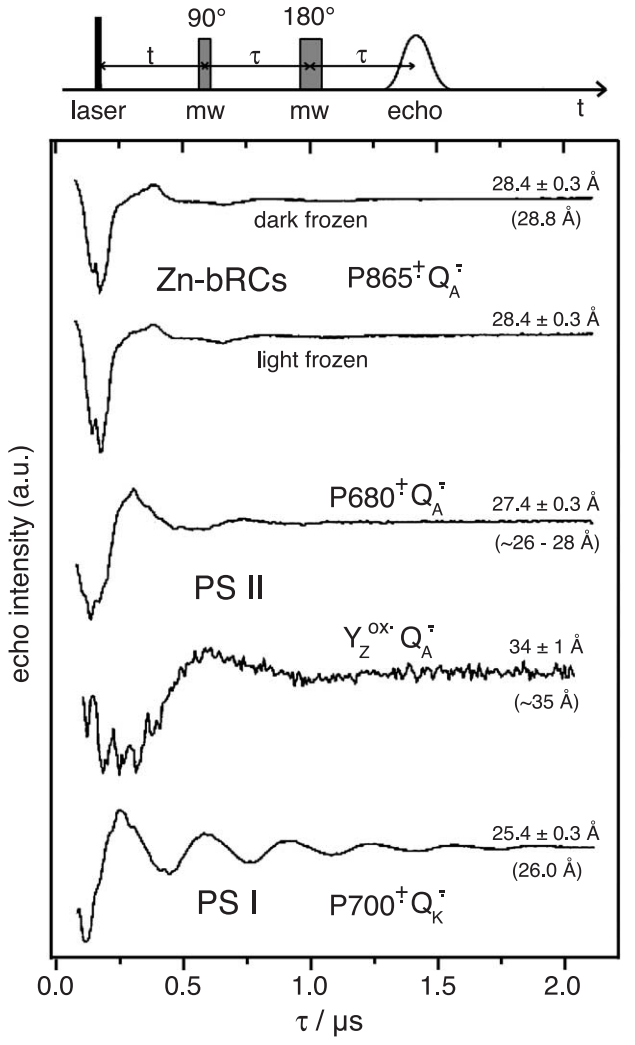

Fig. 3. Electron spin echo modulation (OOP-ESEEM) due to the electron spin–spin interaction for various RP states in the photosystems: $P_{865}^{\bullet+}\,Q_A^{\bullet-}$ in Zn-substituted pbRCs of *R. sphaeroides*, $P_{680}^{\bullet+}\,Q_A^{\bullet-}$ and $Y_Z^{\bullet ox}Q_A^{\bullet-}$ in PS II from spinach, and $P_{700}^{\bullet+}\,A_1^{\bullet-}$ in PS I from *Synechococcus elongatus*. Distances evaluated from the modulation frequencies are compared with those from X-ray crystallography (in brackets). Reprinted with permission from Ref. [73]. © 2002 American Chemical Society.

In PS II it has been possible to observe pulsed EPR data on a spin-polarized RP involving a radical state of an amino acid residue. Such a system with one partner in the spin-polarized RP being a paramagnetic state of an organic cofactor and the other an amino acid radical has so far only been observed by transient EPR on the DNA-repair enzyme photolyase (see below). The state studied in Ref. [32] is the spin-polarized RP state $Y_Z^{ox\bullet}Q_A^{\bullet-}$ that is generated by ET from the redox-active amino acid Y_Z to the oxidized species $P_{680}^{\bullet+}$.

The observation of the $Y_Z^{ox\bullet}Q_A^{\bullet-}$ RP in PS II was possible by working with liquid-solution preparations of the RC. Thereby, the time constant for the Y_Z to $P_{680}^{\bullet+}$ ET has been on the same time scale as the delay times between the microwave pulses used to observe the OOP-ESEEM. A variation of the delay time *t* between the laser flash inducing the ET process and the microwave pulses can then be used to discriminate the consecutive RP states. The dipolar frequencies of the two RP states contribute to the time evolution of the OOP-ESEEM signal as a function of the delay time *t*. The relative intensities of the dipolar frequencies of the two RP states is given by their relative population at a specific delay time *t* determined by the time constant for the ET between the two RP states. Using a very short delay time *t* results in an OOP-ESEEM time trace that almost exclusively contains the dipolar frequencies of the early RP state $P_{680}^{\bullet+}\,Q_A^{\bullet-}$. At rather long delay times *t*, the time evolution of the OOP-ESEEM is dominated by the dipolar frequencies of the later RP state $Y_Z^{ox\bullet}Q_A^{\bullet-}$ (see Fig. 1 from Ref. [32]).

The distance between $Y_Z^{ox\bullet}$ and $Q_A^{\bullet-}$ deduced from the OOP-ESEEM at long delay times *t* is 34 Å. In the X-ray crystallographic model of PS II at a 3.8-Å resolution only two amino acid side chains, the two symmetrically placed redox-active tyrosines Y_Z and Y_D, have been assigned [30]. The EPR-derived distance between $Y_Z^{ox\bullet}$ and $Q_A^{\bullet-}$ coincides with the distance between Y_Z and Q_A in the structure model. This coincidence may be used to justify the assignment of an amino acid side chain in a structure model at 3.8 Å resolution.

The confidence in the possibility to use EPR-derived structural data to justify assignments in low or medium

resolution X-ray crystallographic models is based on the results obtained on the RP state $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ in PS I. As already discussed above, the relative orientation of the carbonyl bond direction of A_1 with respect to P_{700} as well as the distance between the two molecules had been determined by EPR before such information became available from X-ray crystallography. Localization of the phylloquinone molecule A_1 in PS I has proven a very difficult task for X-ray crystallography from the initial structural model at 6 Å resolution [33] to the first model at 4 Å resolution [34] due to the small size of the head group of this molecule. In the corresponding structure models, different putative binding sites for A_1 had been proposed. Pulsed EPR experiments on single crystals of PS I allowed to determine the angle between the axis connecting $P_{700}^{\bullet+}$ and $A_1^{\bullet-}$ and crystallographic axes [35]. Together with the distance between the two molecules, the information on the connecting vector between these moieties in the crystallographic unit cell allowed to identify the binding site of A_1 by EPR spectroscopy on the RP state $P_{700}^{\bullet+}$ $A_1^{\bullet-}$. The EPR-derived information on the A_1-binding site has been used to assign an electron density in a refined structural model of PS I at the 4-Å resolution level [36]. This assignment has later been corroborated by the PS I structure at 2.5 Å resolution [19].

2.3. Functional aspects

An aspect of PS I that had been rather controversially discussed for a long time was the identity and the functional role of the secondary electron acceptor A_1. Several reports questioned either the mandatory involvement of a secondary electron carrier between the primary acceptor A_0 and the iron–sulfur clusters in PS I or questioned the assignment of A_1 to a phylloquinone molecule (for reviews, see e.g., Refs. [37,38]). Both of these questions have been resolved mainly based on time-resolved EPR data. EPR experiments using quinone depletion and reconstitution with either the native phylloquinone, deuterated phylloquinone or non-native quinones [39–41] provided clear evidence for the assignment of A_1 to the phylloquinone in PS I. Kinetic data for the ET from A_1 to the iron–sulfur cluster F_X has also been obtained by pulsed EPR [42] as well as by direct-detection EPR [43].

Kinetic studies on the ET from A_1 to F_X are presently again a controversial subject. The X-ray crystallographic model of PS I shows the same approximate C_2 symmetry of two branches of cofactors as observed in the pbRC and in PS II. In pbRC and PS II, the structural symmetry is clearly broken on the functional level with ET between the two symmetry-related quinones and one of the quinones (Q_A) being tightly bound while the second quinone (Q_B) moves in and out of the protein matrix during the RC function. In PS I, however, there is no obvious reason for a drastic (qualitative) functional difference of the two symmetry-related cofactor branches. In an optical study on PS I with genetically modified amino acid surroundings of the two phylloquinones [44], two different kinetic phases have been assigned to ET from the A_1 molecule bound to the A- and the B-subunit of PS I to F_X, respectively. This would correspond to a bi-directional ET in PS I involving both branches of cofactors. Using time-resolved EPR, no direct evidence for an involvement of both cofactor branches in ET has been found so far. In contrast, for the low temperature conditions where most of the structural EPR studies mentioned above have been performed, involvement of exclusively the phylloquinone bound to the A-subunit of PS I has been shown by experiments on the RP state $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ [45] on the same set of PS I mutants from the green algae *Chlamydomonas reinhardtii* as used in the optical study at physiological conditions [44].

The exclusive involvement of the A_1 bound to the A-subunit of PS I in low temperature ET has recently also been corroborated for cyanobacterial PS I from *Synechocystis* sp. PCC 6803 by time-resolved ENDOR experiments on $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ in PS I with modified amino acids in the binding sites of both phylloquinone molecules [46]. In a companion study, optical and transient EPR spectroscopy at physiological conditions have been applied to the same set of cyanobacterial PS I mutants [47]. By transient EPR, no evidence for a fast ET component from A_1 to F_X could be detected. Furthermore, the problem in the assignment of kinetic components in the optical data to ET along the different branches in PS I has been discussed [47]. Indirect arguments in favor of a bi-directional ET in PS I have been derived from pulsed EPR experiments measuring the decay time of the spin-polarized signal of $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ at low temperatures after pre-reduction [48]. However, again the assignment of different kinetic components to ET in both branches of PS I is not unique.

While the question about uni- or bi-directional electron transfer in PS I still seems to be an open question, investigation of the RP state $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ contributed to an important result regarding the functional properties of the quinone acceptor in PS I. Using a genetic approach, mutants of *Synechocystis* sp. PCC 6803 have been prepared which lack the ability to synthesize phylloquinone [49]. A severe impairment of the function of PS I and thereby of the photosynthetic apparatus would be expected by the lack of this cofactor. Nevertheless, these mutants are able to grow photo-autotrophically [49]. The analysis of the RP state $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ in these mutants by time-resolved EPR and ENDOR [50] provided strong evidence that instead of the native phylloquinone, a plastoquinone-9 molecule occupies the A_1 site of PS I and that it is competent in participating in ET from the primary acceptor A_0 to the iron–sulfur cluster F_X. This is surprising since plastoquinone-9 is part of the ET chain in PS II and works there as Q_A/Q_B at drastically different mid-point potentials of about −100 mV compared to the about −800 mV of A_1 in PS I. This result shows that the functional properties of the specific cofactor are less governed by the chemical identity of the quinone than by the protein matrix.

3. Photolyases

3.1. DNA repair

Limited information on the RP states occurring during photorepair of UV-induced DNA damages by the enzyme photolyase is available from EPR. This is due to a number of properties of this enzymatic reaction that precludes direct observation of RPs under ambient conditions: first, the splitting of the cyclobutane pyrimidine dimer (CPD) lesion into the two thymine bases by reductive photoinduced ET from the flavin cofactor is extremely fast. At room temperature, the reaction cycle is completed within less than 2 ns, slowing down to a few nanoseconds at lower temperatures [51]. Even with the fastest instrumentation available to date. This is at least one order of magnitude too fast to be directly probed by time-resolved continuous-wave EPR (transient EPR) and pulsed EPR techniques. Second, the reaction is not cyclic and repair results in products that are chemically different from the reactants. Separation of the repaired DNA segment upon release from the photolyase enzyme followed by docking to further damaged DNA requires time and thus precludes fast repetitive accumulation of EPR signals to improve signal-to-noise ratio. To overcome this problem, the EPR experiment would have to be performed while continuously renewing the sample (photolyase bound to damaged DNA) using flow-cell technology. While such instrumentation is available up to W-band frequencies, the amount of enzyme bound to homogeneously damaged DNA is limited. Third, photoexcitation has to be performed using blue to near-ultraviolet light at wavelengths below 440 nm. Using such high-energetic radiation, the risk of photodamaging the enzyme under formation of paramagnetic side products is high and thus complicates a quantitative analysis of the resulting mixture of EPR spectra. Finally, the photolyase enzyme used for spectroscopic studies usually lacks an antenna molecule that is supposed to widen the absorption cross-section to longer wavelengths and to increase the absorbance at longer wavelengths. While photolyase deficient of this light-harvesting cofactor is still capable of repairing DNA, its quantum yield for repair is low. Consequently, the expected EPR signal strength is weak.

Despite these problems, several attempts to probe ET from the flavin to the DNA lesion by time-resolved EPR have been made. Kim et al. [52] observed short-lived EPR transients using continuous-wave EPR with a modulation of the magnetic field. The kinetic traces detected after irradiation of a photolyase/substrate mixture by flashes of a xenon lamp rose and decayed rapidly with the 35 μs instrument time constant. Because the repair reaction is much faster (see above), the observed EPR transients cannot reflect the true time course of transient RP formation and decay. The transients reported were gradually reduced and eventually were not detectable after prolonged optical excitation of the sample. The decrease in signal was believed to result from DNA repair and not due to enzyme degradation. Nevertheless, the EPR signal strength was too low to succeed in recording a RP EPR spectrum. Therefore, no information on spin polarization, signal splittings and other information on magnetic interactions were obtained.

A few years later, Rustandi and Jorns [53] examined light-induced ET from the FAD to the DNA lesion using continuous-wave EPR with a modulation of the excitation-light source (modulation frequency, 500 Hz). The experiment was performed at cryogenic temperatures ($T = 4$ K). Under such conditions, DNA repair does not occur and the photogenerated RP could only undergo backward ET reactions. Thus, the system ends in the initial state that is then ready for a further photoexcitation/probe cycle. Upon signal integration, a spin-polarized EPR spectrum with an emission/enhanced-absorption polarization pattern was detected, which provides clear evidence for a non-Boltzmann electron spin distribution of a RP. In the SCRP model, however, an emission (E)/enhanced-absorption (A) pattern is predicted for each of the two radicals in a singlet-born RP provided the exchange coupling J is positive. Therefore, an E/A/E/A pattern would be expected. However, when the difference in the g-factors of the individual RP halves is very small, the central peaks may overlap and cancel each other, resulting in an apparent E/A signal. The rather weak E/A-polarized signals observed in Rustandi and Jorns' studies may be partly due to cancellation between overlapping $FADH^{\bullet}$ and $T<>T^{\bullet -}$ polarizations. Because the experiments were performed using the light-modulation technique, the modulation frequency (500 Hz) precluded detection of RP intermediates in real time. Therefore, neither the lifetime of the species nor the rates for ET were obtained.

Clearly, the enzymatic DNA-repair reaction deserves re-examination with EPR methods with high time resolution. From the polarization pattern, the relative orientation of the RP halves, and from a pulsed echo-experiment, the distance between the redox-partners could be determined. The latter information would be particularly useful, as to date no crystal-structure of photolyase bound to photodamaged DNA is available, and thus the precise distance between the flavin cofactor and the CPD during enzymatic activity is unknown.

3.2. Flavin cofactor photoreduction

By far, most flavoproteins stabilize their flavin cofactor in the fully oxidized redox form. This is also the state in which photolyase is prepared when isolated under aerobic conditions. Like many other proteins with flavin cofactors, photolyase may be reduced in a step-wise fashion until the FAD reaches the catalytically active, fully two-electron-reduced redox state, $FADH^{-}$, when illuminated in the presence of exogenous electron donors, such as ethylene diamine tetraacetic acid or dithiothreitol. This general procedure was first described by Massey and Palmer [54] who opened up a novel method for preparing semi-reduced and reduced flavoproteins from their fully oxidized forms.

In photolyase, the FAD cofactor lies deeply buried in the enzyme, with limited access to the surface through a narrow opening, which can host a CPD when flipped out of the DNA double helix. FAD photoreduction in CPD photolyase of *Escherichia coli* is aided by a redox-active tryptophan amino acid residue at the enzyme surface. The specific amino acid was first identified by a comprehensive point-mutational study in which each individual tryptophan was replaced by phenylalanine [55]. Only Trp-306 → Phe mutation abolished photoreduction of $FADH^{\bullet}$ without affecting other excited-state properties of the flavin cofactor. Trp-306 is situated at a distance of approximately 20 Å apart from FAD in a small loop region at the enzyme surface and is believed to mediate ET to exogenous electron donors. In their absence, backward ET from the reduced flavin to the oxidized Trp-306 takes place. To bridge the distance between these two redox partners, a chain of tryptophan residues, Trp-359 and Trp-382, was postulated to provide an efficient ET pathway between these moieties. This notion is based on the X-ray crystal structure of the enzyme that was published in 1995 by Park et al. [56]. The chain of tryptophan amino acids is well-conserved throughout the photolyase enzyme family [57,58] and even through the more recently discovered cryptochromes that do not repair DNA but participate in blue-light reception and regulation of circadian rhythms [59].

By time-resolved EPR, a spin-polarized radical signal following xenon flash-lamp excitation of photolyase was first discovered by Essenmacher et al. [60] and Kim et al. [61]. Erroneously, the signal was assigned to a single radical species generated as a consequence of single-electron reduction of the FAD cofactor in the neutral semiquinone form, $FADH^{\bullet}$, to the fully-reduced $FADH^{-}$. Isotope labeling demonstrated that tryptophan is the origin and suggested, based on its characteristic hyperfine pattern, that it occurs in its cation-radical form. The spin polarization was believed to arise from photoexcitation of the doublet $FADH^{\bullet}$ into the excited doublet state of the flavin radical, which then decays by intersystem crossing to yield the quartet state. The long-lived quartet state of $FADH^{\bullet}$ was then believed to be quenched either by a H-atom transfer, generating the tryptophan neutral radical and $FADH_2$, or by an ET to generate the tryptophan cation radical and $FADH^{-}$.

In a later study by Gindt et al. [62] this mechanism was dismissed. From the correlation of the stationary EPR signal arising from $FADH^{\bullet}$ and the transient response following enzyme photoexcitation, it was concluded that the observed spin-polarized EPR signal actually originates from a RP that is generated from the fully oxidized form of the FAD cofactor. Since the spin-polarized signal amplitude decreased upon addition of a triplet quencher, it was proposed that the triplet of the fully oxidized FAD cofactor is the primary electron acceptor that abstracts an electron from tryptophan to form a RP, $FAD^{\bullet -}Trp^{\bullet +}$. The characteristic splitting of the signal was assigned to the hyperfine interaction of the strongly coupled β-protons at the link of the indole ring of tryptophan to the protein backbone. The flavin part of the RP signal has not been disentangled due to the lack of information on the flavin's *g*-tensor and spectral width. Such information has only recently become available from high-frequency/high-field EPR experiments performed at 360 GHz/12.8 T [63]. With this information at hand, however, it should now be feasible to quantitatively understand the RP line shape and from this to obtain information on the geometry of the RP.

The first direct-detection EPR study of the photoreduction reaction has been performed with (6–4) photolyase, an enzyme that specifically repairs the (6–4) photodamage in DNA [64]. Despite the conservation of the tryptophan ET chain, a different spin-polarized EPR signal compared to that in CPD photolyase has been observed (see Fig. 4). The RP transient-EPR spectrum recorded at 1.4 μs after pulsed laser excitation is composed of broad spectral wings at 343.0 and 348.6 mT and a narrow emission/enhanced absorption polarized signal. The broad features are centered at $g = 2.0034$, as is typical for a neutral flavin radical [63], and hence have been assigned to the flavin part of the RP. Its characteristic (derivative-like) line shape arises from extensive destructive averaging of the broad and overlapping absorptively and emissively polarized signal contributions within the line pair belonging to $FADH^{\bullet}$. The narrow feature with emission/enhanced absorption polarization is centered at $g = 2.0048$. Such a high *g*-value is characteristic for a

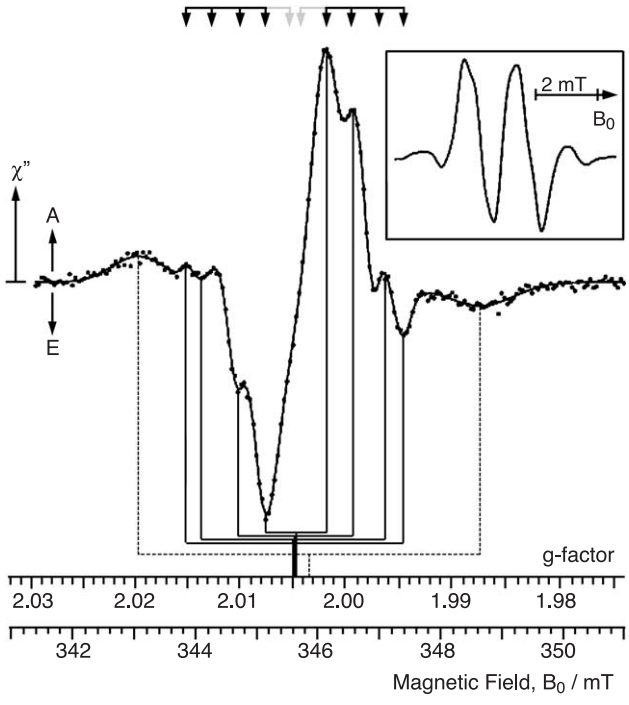

Fig. 4. Transient spin-polarized EPR signal generated by pulsed laser excitation of *Xenopus laevis* (6–4) photolyase at $T = 278$ K. The EPR data were recorded at 1.4 μs after laser-flash excitation in the direct-detection mode with A, enhanced absorption, and E, emission. The inset shows the corresponding light-initiated EPR signal observed in *E. coli* CPD photolyase. Figure taken from Ref. [64].

neutral (deprotonated) tyrosyl radical. Further evidence for a neutral tyrosyl radical being involved in (6–4) photolyase photoreduction is provided by the characteristic line shape of the central narrow feature. A repeating 0.45 mT hyperfine splitting pattern is observed which points to a paramagnetic species with two or more magnetically equivalent nuclei. Again this is consistent with a neutral tyrosine radical but not with a tryptophan radical, where many inequivalent nuclei contribute to an unresolved broad line. In tyrosyl neutral radicals, because of molecular symmetry, the protons H2 and H6, as well as H3 and H5 are pair-wise magnetically equivalent. The same applies for the β-protons in the special case when the phenoxy-radical residue is bound symmetrically to be protein backbone. Hence it was concluded, that the hyperfine splitting observed in Fig. 4 arises from the H3/H5 and β-protons, for which in an in vacuo model study almost identical hyperfine couplings of −0.57 and 0.5 mT, respectively, have been recently calculated using density functional theory [65].

Interestingly, the narrow spin-polarized transient EPR signals in photoreduction of CPD and (6–4) photolyases point to some motional dynamics being present in the protein. Overall motion of the 60 kDa photolyase enzyme is not expected to be sufficient for EPR line averaging. Hence, high flexibility of the loop region binding the terminal tyrosine acceptor might be responsible for a partial averaging of the anisotropies of the Zeeman, hyperfine and dipolar interactions [64]. Whether the role of such protein motion is of functional importance for flavin photoreduction is a question that needs to be examined in future experiments.

4. Summary and outlook

The direct investigation of transient RPs as working states of proteins by time-resolved EPR techniques is a valuable source of structural and functional information as shown above by examples of studies on photosystems and photolyase. The beauty of such experiments is that the protein does not need to be manipulated, be it by introduction of a spin probe via site-directed mutagenesis or by chemical oxidative or reductive treatment. Rather, the systems can be studied under ambient conditions in the wild-type. Transient EPR and echo-detected pulse methods therefore have great potential in the study of protein function in cases where the three-dimensional structure is not readily available or specific point mutants are only poorly expressed. One example is the photoreduction reaction of the flavin cofactor in photolyases, a reaction which is, however, also found in many other flavoproteins including cryptochromes and phototropins [66,67]. In both CPD and (6–4) photolyase, the transient EPR signals detected upon light excitation have not yet been assigned to a specific amino acid radical in the protein [62,64]. Hence, by measuring the distance between the flavin cofactor in its radical state and the counter-radical in the doublet-spin pair from an OOP-ESEEM experiment, a definite assignment can be accomplished. Such studies are currently being in progress in our laboratory.

Most of the specific experiments discussed above involved spin-polarized RP states that occur during the light-driven ET reactions in those systems. Therefore, these systems show rather peculiar properties in EPR spectroscopy with emissive and absorptive spectral features. We assume that the methods discussed here on photosystems and photolyase can readily be applied to any biological process involving photoinduced ET. For other ET chains with transient paramagnetic pair states, e.g., the respiratory chain, these time-resolved methods are not immediately applicable because no synchronization of the reaction sequences is provided by a light stimulus. It should be noted however, that the electron spin polarization often only enhances the obtainable EPR signal intensity and in some cases renders data evaluation easier, but that light-induced spin polarization is no prerequisite to obtain orientation and distance data between pairs of paramagnetic states in proteins, nor is it indispensable for kinetic or other functional analysis. The orientation as well as distance information is contained in the anisotropic dipolar electron spin–spin coupling between a pair of paramagnetic states. The use of the same principles as established in Ref. [8] has been, e.g., discussed in the context of site-directed double spin-labeling [68], to obtain orientation information between two spin labels. The direct measurement of the dipolar coupling as shown for the RP states in photosystems [23,25–29,32,35] is now successfully applied for structural studies on proteins as well as polymers, also using the site-directed double spin-labeling technique in combination with pulsed electron–electron double resonance (ELDOR, also called DEER, for recent reviews see e.g. [69,70]). Similarly, structural and functional information can be obtained by EPR spectroscopy when pairs of paramagnetic species can be stabilized under specific conditions. This has been used for photosynthetic RCs, e.g., in Ref. [71], and for a protein of the respiratory chain, e.g., in Ref. [72].

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References

[1] P. Heathcote, Type I photosynthetic reaction centres, Biochim. Biophys. Acta 1507 (2001) 1–2.
[2] B.A. Diner, F. Rappaport, Structure, dynamics, and energetics of the primary photochemistry of photosystem II of oxygenic photosynthesis, Annu. Rev. Plant Biol. 53 (2002) 551–580.
[3] J. Barber, Photosystem II: a multisubunit membrane protein that oxidises water, Curr. Opin. Struct. Biol. 12 (2002) 523–530.

[4] A. Sancar, Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors, Chem. Rev. 103 (2003) 2203–2237.
[5] R. Blankenship, A. McGuire, K. Sauer, Chemically induced dynamic electron polarization in chloroplasts at room temperature: evidence for triplet state participation in photosynthesis, Proc. Natl. Acad. Sci. U. S. A. 72 (1975) 4943–4947.
[6] P.J. Hore, D.A. Hunter, C.D. McKie, A.J. Hoff, Electron paramagnetic resonance of spin-correlated radical pairs in photosynthetic reactions, Chem. Phys. Lett. 137 (1987) 495–500.
[7] G.L. Closs, M.D.E. Forbes, J.R. Norris, Spin-polarized electron paramagnetic resonance spectra of radical pairs in micelles. Observation of electron spin–spin interactions, J. Phys. Chem. 91 (1987) 3592–3599.
[8] D. Stehlik, C.H. Bock, J. Petersen, Anisotropic electron spin polarization of correlated spin pairs in photosynthetic reaction centers, J. Phys. Chem. 93 (1989) 1612–1619.
[9] J. Deisenhofer, O. Epp, K. Miki, R. Huber, H. Michel, X-ray structure analysis of a membrane protein complex: electron density map at 3 Å resolution and a model of the chromophores of the photosynthetic reaction center from *Rhodopseudomonas viridis*, J. Mol. Biol. 180 (1984) 385–398.
[10] J. Deisenhofer, O. Epp, K. Miki, R. Huber, H. Michel, Structure of the protein subunits in the photosynthetic reaction centre *Rhodopseudomonas viridis* at 3 Å resolution, Nature (London) 318 (1985) 618–624.
[11] G. Füchsle, R. Bittl, A. van der Est, W. Lubitz, D. Stehlik, Transient EPR spectroscopy of the charge separated state P^+Q^- in photosynthetic reaction centers. Comparison of Zn-substituted *Rhodobacter sphaeroides* R-26 and photosystem I, Biochim. Biophys. Acta 1142 (1993) 23–35.
[12] P.J. Hore, Analysis of polarized EPR spectra, in: A.J. Hoff (Ed.), Advanced EPR in Biology and Biochemistry, Elsevier, Amsterdam, 1989, pp. 405–440.
[13] K.M. Salikhov, C.H. Bock, D. Stehlik, Time development of electron spin polarization in magnetically coupled, spin correlated radical pairs, Appl. Magn. Reson. 1 (1990) 195–211.
[14] R. Bittl, G. Kothe, Transient EPR of radical pairs in photosynthetic reaction centers: prediction of quantum beats, Chem. Phys. Lett. 177 (1991) 547–553.
[15] G. Kothe, S. Weber, R. Bittl, E. Ohmes, M.C. Thurnauer, J.R. Norris, Transient EPR of light-induced radical pairs in plant photosystem: I. Observation of quantum beats, Chem. Phys. Lett. 186 (1991) 474–480.
[16] G. Kothe, S. Weber, E. Ohmes, M.C. Thurnauer, J.R. Norris, Transient EPR of light-induced spin-correlated radical pairs: manifestation of zero quantum coherence, J. Phys. Chem. 98 (1994) 2706–2712.
[17] S.G. Zech, W. Hofbauer, A. Kamlowski, P. Fromme, D. Stehlik, W. Lubitz, R. Bittl, A structural model for the charge separated state $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ in photosystem I from the orientation of the magnetic interaction tensors, J. Phys. Chem. B 104 (2000) 9728–9739.
[18] G. Link, T. Berthold, M. Bechtold, J.-U. Weidner, E. Ohmes, J. Tang, O. Poluektov, L. Utschig, S.L. Schlesselman, M.C. Thurnauer, G. Kothe, Structure of P_{700}^{+} A_1^{-} radical pair intermediate in photosystem I by high time resolution multifrequency electron paramagnetic resonance: analysis of quantum beat oscillations, J. Am. Chem. Soc. 123 (2001) 4211–4222.
[19] P. Jordan, P. Fromme, H.-T. Witt, O. Klukas, W. Saenger, N. Krauß, Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution, Nature (London) 411 (2001) 909–917.
[20] P. Fromme, P. Jordan, N. Krauß, Structure of photosystem I, Biochim. Biophys. Acta 1507 (2001) 5–31.
[21] K.M. Salikhov, Y.E. Kandrashkin, A.K. Salikhov, Peculiarities of free induction and primary spin echo signals for spin-correlated radical pairs, Appl. Magn. Reson. 3 (1992) 199–216.
[22] J. Tang, M.C. Thurnauer, J.R. Norris, Electron spin echo envelope modulation due to exchange and dipolar interactions in a spin-correlated radical pair, Chem. Phys. Lett. 219 (1994) 283–290.
[23] S.A. Dzuba, P. Gast, A.J. Hoff, ESEEM study of spin–spin interactions in spin-polarised $P^+Q_A^-$ pairs in the photosynthetic purple bacterium *Rhodobacter sphaeroides* R26, Chem. Phys. Lett. 236 (1995) 595–602.
[24] G. Katona, U. Andréasson, E.M. Landau, L.-E. Andréasson, R. Neutze, Lipidic cubic phase crystal structure of the photosynthetic reaction centre from *Rhodobacter sphaeroides* at 2.35 Å resolution, J. Mol. Biol. 331 (2003) 671–692.
[25] S.G. Zech, W. Lubitz, R. Bittl, Pulsed EPR experiments on radical pairs in photosynthesis: comparison of the donor–acceptor distances in photosystem I and bacterial reaction centers, Ber. Bunsenges. Phys. Chem. 100 (1996) 2041–2044.
[26] S.A. Dzuba, H. Hara, A. Kawamori, M. Iwaki, S. Itoh, Y.D. Tsvetkov, Electron spin echo of spin-polarised radical pairs in intact and quinone-reconstituted plant photosystem I reaction centres, Chem. Phys. Lett. 264 (1997) 238–244.
[27] R. Bittl, S.G. Zech, Pulsed EPR study of spin-coupled radical pairs in photosynthetic reaction centers: measurement of the distance between $P_{700}^{\bullet+}$ and $A_1^{\bullet-}$ in photosystem I and between $P_{865}^{\bullet+}$ and $Q_A^{\bullet-}$ in bacterial reaction centers, J. Phys. Chem. B 101 (1997) 1429–1436.
[28] S.G. Zech, J. Kurreck, H.-J. Eckert, G. Renger, W. Lubitz, R. Bittl, Pulsed EPR measurement of the distance between $P_{680}^{+\bullet}$ and $Q_A^{-\bullet}$ in photosystem II, FEBS Lett. 414 (1997) 454–456.
[29] H. Hara, S.A. Dzuba, A. Kawamori, K. Akabori, T. Tomo, K. Satoh, M. Iwaki, S. Itoh, The distance between P680 and QA in photosystem II determined by ESEEM spectroscopy, Biochim. Biophys. Acta 1322 (1997) 77–85.
[30] A. Zouni, H.-T. Witt, J. Kern, P. Fromme, N. Krauß, W. Saenger, P. Orth, Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution, Nature (London) 409 (2001) 739–743.
[31] B.A. Diner, E. Schlodder, P.J. Nixon, W.J. Coleman, F. Rappaport, J. Lavergne, W.F.J. Vermaas, D.A. Chisholm, Site-directed mutations at D1-His198 and D2-His197 of photosystem II in *Synechocystis* PCC 6803: sites of primary charge separation and cation and triplet stabilization, Biochemistry 40 (2001) 9265–9281.
[32] S.G. Zech, J. Kurreck, G. Renger, W. Lubitz, R. Bittl, Determination of the distance between $Y_Z^{ox\bullet}$ and $Q_A^{-\bullet}$ in photosystem II by pulsed EPR spectroscopy on light-induced radical pairs, FEBS Lett. 442 (1999) 79–82.
[33] N. Krauß, W. Hinrichs, I. Witt, P. Fromme, W. Pritzkow, Z. Dauter, C. Betzel, K.S. Wilson, H.T. Witt, W. Saenger, Three-dimensional structure of system I of photosynthesis at 6 Å resolution, Nature (London) 361 (1993) 326–331.
[34] N. Krauß, W.-D. Schubert, O. Klukas, P. Fromme, H.T. Witt, W. Saenger, Photosystem I at 4 Å resolution represents the first structural model of a joint photosynthetic reaction centre and core antenna system, Nat. Struct. Biol. 3 (1996) 965–973.
[35] R. Bittl, S.G. Zech, P. Fromme, H.T. Witt, W. Lubitz, Pulsed EPR structure analysis of photosystem I single crystals: localization of the phylloquinone acceptor, Biochemistry 36 (1997) 12001–12004.
[36] W.-D. Schubert, O. Klukas, N. Krauß, W. Saenger, P. Fromme, H.T. Witt, Photosystem I of *Synechococcus elongatus* at 4 Å resolution: comprehensive structure analysis, J. Mol. Biol. 272 (1997) 741–769.
[37] M.C.W. Evans, J.H.A. Nugent, Structure and function of the reaction center cofactors in oxygenic organisms, in: J. Deisenhofer, J.R. Norris (Eds.), The Photosynthetic Reaction Center, Academic Press, San Diego, 1993, pp. 391–415.
[38] J.H. Golbeck, Photosystem I in cyanobacteria, in: D.A. Bryant (Ed.), The Molecular Biology of Cyanobacteria, Kluwer Academic Publishing, Dordrecht, 1994, pp. 319–360.
[39] R.R. Rustandi, S.W. Snyder, L.L. Feezel, T.J. Michalski, J.R. Norris, M.C. Thurnauer, Contribution of vitamin K_1 to the electron spin polarization in spinach photosystem I, Biochemistry 29 (1990) 8030–8032.
[40] I. Sieckmann, A. van der Est, H. Bottin, P. Sétif, D. Stehlik, Nanosecond electron transfer kinetics in photosystem I following substitu-

tion of quinones for vitamin K_1 as studied by time resolved EPR, FEBS Lett. 284 (1991) 98–102.
[41] S.W. Snyder, R.R. Rustandi, J. Biggins, J.R. Norris, M.C. Thurnauer, Direct assignment of vitamin K_1 as the secondary acceptor A_1 in photosystem I, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 9895–9896.
[42] P. Moënne-Loccoz, P. Heathcote, D.J. Maclachlan, M.C. Berry, I.H. Davis, M.C.W. Evans, Path of electron transfer in photosystem I: direct evidence of forward electron transfer from A_1 to Fe-S_X, Biochemistry 33 (1994) 10037–10042.
[43] A. van der Est, C.H. Bock, J.H. Golbeck, K. Brettel, P. Sétif, D. Stehlik, Electron transfer from the acceptor A_1 to the iron–sulfur centers in photosystem I as studied by transient EPR spectroscopy, Biochemistry 33 (1994) 11789–11797.
[44] M. Guergova-Kuras, B. Boudreaux, A. Joliot, P. Joliot, K. Redding, Evidence for two active branches for electron transfer in photosystem I, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 4437–4442.
[45] B. Boudreaux, F. MacMillan, C. Teutloff, R. Agalarov, F. Gu, S. Grimaldi, R. Bittl, K. Brettel, K. Redding, Mutations in both sides of the photosystem I reaction center identify the phylloquinone observed by electron paramagnetic resonance spectroscopy, J. Biol. Chem. 276 (2001) 37299–37306.
[46] W. Xu, P.R. Chitnis, A. Valieva, A. van der Est, Y.N. Pushkar, M. Krzystyniak, C. Teutloff, S.G. Zech, R. Bittl, D. Stehlik, B. Zybailov, G. Shen, J.H. Golbeck, Electron transfer in cyanobacterial photosystem I: I. Physiological and spectroscopic characterization of site-directed mutants in a putative electron transfer pathway from A_0 through A_1 to F_X, J. Biol. Chem. 278 (2003) 27864–27875.
[47] W. Xu, P.R. Chitnis, A. Valieva, A. van der Est, K. Brettel, M. Guergova-Kuras, Y.N. Pushkar, S.G. Zech, D. Stehlik, G. Shen, B. Zybailov, J.H. Golbeck, Electron transfer in cyanobacterial photosystem I: II. Determination of forward electron transfer rates of site-directed mutants in a putative electron transfer pathway from A_0 through A_1 to F_X, J. Biol. Chem. 278 (2003) 27876–27887.
[48] I.P. Muhiuddin, P. Heathcote, S. Carter, S. Purton, S.E.J. Rigby, M.C.W. Evans, Evidence from time resolved studies of the $P_{700}^{\bullet+}/A_1^{\bullet-}$ radical pair for photosynthetic electron transfer on both the PsaA and PsaB branches of the photosystem I reaction centre, FEBS Lett. 503 (2001) 56–60.
[49] T.W. Johnson, G. Shen, B. Zybailov, D. Kolling, R. Reategui, S. Beauparlant, I.R. Vassiliev, D.A. Bryant, A.D. Jones, J.H. Golbeck, P.R. Chitnis, Recruitment of a foreign quinone into the A_1 site of photosystem I: I. Genetic and physiological characterization of phylloquinone biosynthetic pathway mutants in *Synechocystis* sp. PCC 6803, J. Biol. Chem. 275 (2000) 8523–8530.
[50] B. Zybailov, A. van der Est, S.G. Zech, C. Teutloff, T.W. Johnson, G. Shen, R. Bittl, D. Stehlik, P. Chitnis, J.H. Golbeck, Recruitment of a foreign quinone into the A_1 site of photosystem I: II. Structural and functional characterization of phylloquinone biosynthetic pathway mutants by electron paramagnetic resonance and electron-nuclear double resonance spectroscopy, J. Biol. Chem. 275 (2000) 8531–8539.
[51] T. Langenbacher, X. Zhao, G. Bieser, P.F. Heelis, A. Sancar, M.E. Michel-Beyerle, Substrate and temperature dependence of DNA photolyase repair activity examined with ultrafast spectroscopy, J. Am. Chem. Soc. 119 (1997) 10532–10536.
[52] S.-T. Kim, A. Sancar, C. Essenmacher, G.T. Babcock, Evidence from photoinduced EPR for a radical intermediate during photolysis of cyclobutane thymine dimer by DNA photolyase, J. Am. Chem. Soc. 114 (1992) 4442–4443.
[53] R.R. Rustandi, M.S. Jorns, Photoinduced spin-polarized radical pair formation in a DNA photolyase–substrate complex at low temperature, Biochemistry 34 (1995) 2284–2288.
[54] V. Massey, G. Palmer, On the existence of spectrally distinct classes of flavoprotein semiquinones. A new method for the quantitative production of flavoprotein semiquinones, Biochemistry 5 (1966) 3181–3189.
[55] Y.F. Li, P.F. Heelis, A. Sancar, Active site of DNA photolyase: tryptophan-306 is the intrinsic hydrogen atom donor essential for flavin radical photoreduction and DNA repair in vitro, Biochemistry 30 (1991) 6322–6329.
[56] H.-W. Park, S.-T. Kim, A. Sancar, J. Deisenhofer, Crystal structure of DNA photolyase from *Escherichia coli*, Science 268 (1995) 1866–1872.
[57] T. Tamada, K. Kitadokoro, Y. Higuchi, K. Inaka, A. Yasui, P.E. de Ruiter, A.P.M. Eker, K. Miki, Crystal structure of DNA photolyase from *Anacystis nidulans*, Nat. Struct. Biol. 4 (1997) 887–891.
[58] H. Komori, R. Masui, S. Kuramitsu, S. Yokoyama, T. Shibata, Y. Inoue, K. Miki, Crystal structure of thermostable DNA photolyase: pyrimidine-dimer recognition mechanism, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 13560–13565.
[59] 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, Identification of a new cryptochrome class: structure, function, and evolution, Mol. Cell 11 (2003) 59–67.
[60] C. Essenmacher, S.-T. Kim, M. Atamian, G.T. Babcock, A. Sancar, Tryptophan radical formation in DNA photolyase: electron-spin polarization arising from photoexcitation of a doublet ground state, J. Am. Chem. Soc. 115 (1993) 1602–1603.
[61] S.-T. Kim, A. Sancar, C. Essenmacher, G.T. Babcock, Time-resolved EPR studies with DNA photolyase: excited-state $FADH^0$ abstracts an electron from Trp-306 to generate $FADH^-$, the catalytically active form of the cofactor, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 8023–8027.
[62] Y.M. Gindt, E. Vollenbroek, K. Westphal, H. Sackett, A. Sancar, G.T. Babcock, Origin of the transient electron paramagnetic resonance signals in DNA photolyase, Biochemistry 38 (1999) 3857–3866.
[63] M. Fuchs, E. Schleicher, A. Schnegg, C.W.M. Kay, J.T. Törring, R. Bittl, A. Bacher, G. Richter, K. Möbius, S. Weber, The g-tensor of the neutral flavin radical cofactor of DNA photolyase revealed by 360-GHz electron paramagnetic resonance spectroscopy, J. Phys. Chem. B 106 (2002) 8885–8890.
[64] S. Weber, C.W.M. Kay, H. Mögling, K. Möbius, K. Hitomi, T. Todo, Photoactivation of the flavin cofactor in *Xenopus laevis* (6–4) photolyase: observation of a transient tyrosyl radical by time-resolved electron paramagnetic resonance, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 1319–1322.
[65] F. Himo, A. Gräslund, L.A. Eriksson, Density functional calculations on model tyrosyl radicals, Biophys. J. 72 (1997) 1556–1567.
[66] C.W.M. Kay, E. Schleicher, A. Kuppig, H. Hofner, W. Rüdiger, M. Schleicher, M. Fischer, A. Bacher, S. Weber, G. Richter, Blue light perception in plants. Detection and characterization of a light-induced neutral flavin radical in a C450A mutant of phototropin, J. Biol. Chem. 278 (2003) 10973–10982.
[67] R. Bittl, C.W.M. Kay, S. Weber, P. Hegemann, Characterization of a flavin radical product in a C57M mutant of a LOV1 domain by electron paramagnetic resonance, Biochemistry 42 (2003) 8506–8512.
[68] E.J. Hustedt, A.H. Beth, Nitroxide spin–spin interactions: applications to protein structure and dynamics, Annu. Rev. Biophys. Biomol. Struct. 28 (1999) 129–153.
[69] G. Jeschke, Distance measurements in the nanometer range by pulse EPR, Chem. Phys. Chem. 3 (2002) 927–932.
[70] G. Jeschke, Determination of the nanostructure of polymer materials by electron paramagnetic resonance spectroscopy, Macromol. Rapid Commun. 23 (2002) 227–246.
[71] R. Calvo, E.C. Abresch, R. Bittl, G. Feher, W. Hofbauer, R.A. Isaacson, W. Lubitz, M.Y. Okamura, M.L. Paddock, EPR study of the molecular and electronic structure of the semiquinone biradical $Q_A^{-\bullet}Q_B^{-\bullet}$ in photosynthetic reaction centers from *Rhodobacter sphaeroides*, J. Am. Chem. Soc. 122 (2000) 7327–7341.
[72] H. Käss, F. MacMillan, B. Ludwig, T.F. Prisner, Investigation of the Mn binding site in cytochrome *c* oxidase from *Paracossus denitrificans* by high-frequency EPR, J. Phys. Chem. B 104 (2000) 5362–5371.
[73] W. Lubitz, F. Lendzian, R. Bittl, Radicals, radical pairs and triplet states in photosynthesis, Acc. Chem. Res. 35 (2002) 313–320.