The Electronic State of Flavoproteins: Investigations with Proton Electron–Nuclear Double Resonance

Erik Schleicher · Ringo Wenzel · Margret Ahmad ·
Alfred Batschauer · Lars-Oliver Essen ·
Kenichi Hitomi · Elizabeth D. Getzoff · Robert Bittl ·
Stefan Weber · Asako Okafuji

Received: 26 June 2009 / Published online: 17 November 2009
© Springer 2009

Abstract Electron–nuclear double resonance (ENDOR) spectroscopy provides useful information on hyperfine interactions between nuclear magnetic moments and the magnetic moment of an unpaired electron spin. Because the hyperfine coupling constant reacts quite sensitively to polarity changes in the direct vicinity of the nucleus under consideration, ENDOR spectroscopy can be favorably used for the detection of subtle protein–cofactor interactions. A number of pulsed ENDOR studies on flavoproteins have been published during the past few years; most of them were designed to characterize the flavin cofactor by means of its protonation state, or to detect individual protein–cofactor interactions. The aim of this study is to compare the pulsed ENDOR spectra from different flavoproteins in terms of

E. Schleicher · S. Weber (✉) · A. Okafuji
Institut für Physikalische Chemie, Albert-Ludwigs-Universität Freiburg,
Albertstr.21, 79104 Freiburg, Germany
e-mail: Stefan.Weber@physchem.uni-freiburg.de

R. Wenzel · R. Bittl
Institut für Experimentalphysik, Fachbereich Physik, Freie Universität Berlin,
Arnimallee 14, 14195 Berlin, Germany

R. Bittl
e-mail: Robert.Bittl@physik.fu-berlin.de

M. Ahmad
CNRS, University of Paris VI, Paris, France

A. Batschauer
Fachbereich Biologie, Philipps-Universität Marburg, Marburg, Germany

L.-O. Essen
Fachbereich Chemie, Philipps-Universität Marburg, Marburg, Germany

K. Hitomi · E. D. Getzoff
Department of Molecular Biology, The Skaggs Institute for Chemical Biology,
The Scripps Research Institute, La Jolla, CA, USA

variations of characteristic proton hyperfine values. The general concept is to observe limits of possible influences on the cofactor's electronic state by surrounding amino acids. Furthermore, we compare ENDOR data obtained from in vivo experiments with in vitro data to emphasize the potential of the method for gaining molecular information in complex media.

1 Introduction

Since their discovery in the beginning of the last century, flavins have been recognized as exceptionally versatile protein cofactors, which are involved in the catalysis of a wide range of biological processes, mostly acting as redox-active molecules [1]. They participate in one-electron transfer as well as in two-electron transfer reactions due to their intrinsic ability in adopting three different redox states. As the one-electron-reduced flavin molecule, either in its anionic ($\text{Fl}^{\bullet-}$) or in its neutral ($\text{FlH}^\bullet$) form is an open-shell system, application of electron paramagnetic resonance (EPR) spectroscopy is a powerful and frequently applied tool to gain information on the protonation state of the flavin [2, 3] and on the mode of its binding to the protein surroundings; such information on flavin semiquinones is quite useful, because flavin radicals are often observed as transient reaction intermediates in flavoprotein-mediated catalysis. Traditionally, anionic and neutral flavin radicals are distinguished mainly based on their EPR line width. However, as hydrogen bonding of variable strength from surrounding amino acids to N(5) in $\text{Fl}^{\bullet-}$ and from H(5) in $\text{FlH}^\bullet$ contributes significantly to the spectral width, an unambiguous identification of anion and neutral flavin radicals is often difficult based on the EPR spectrum alone (see Fig. 1).

By the EPR-derived electron-nuclear double resonance (ENDOR) technique, hyperfine interactions between nuclear magnetic moments and the magnetic moment of the unpaired electron spin are detected. Via hyperfine coupling constant (hfcs), the unpaired electron-spin density at the specific positions of magnetic nuclei can be evaluated [4, 5]. Moreover, hfcs respond quite sensitively to changes in the flavin's immediate environment. The hfc of a particular nucleus can be directly read out from ENDOR spectra from pairs of resonance lines that are, according to the resonance condition $\nu_{\text{ENDOR}} = |\nu_n \pm A/2|$, either equally spaced about the magnetic field-dependent nuclear Larmor frequency ν_n and separated by the (orientation-dependent) hfc A (for the case of $\nu_n > |A/2|$), or about $|A|/2$ and separated by $2\nu_n$ (for

Fig. 1 International Union of Pure and Applied Chemistry (IUPAC) nomenclature of the 7,8-dimethyl isoalloxazine moiety of flavins. *R* denotes the ribityl side chain. ENDOR-visible protons are highlighted in red

$v_n < |A/2|$). Pulsed EPR and ENDOR methods became increasingly popular during the last few years in investigations of flavoproteins [6–14]. Here, we focus on a discussion of spectra obtained from pulsed ENDOR. In contrast to “conventional” continuous-wave (cw) ENDOR spectroscopy, the signal in the pulsed methodology is obtained by recording the electron spin echo intensity as a function of the frequency of a radio frequency pulse. Changes in the echo intensity occur when the radio frequency is on resonance with a nuclear magnetic resonance transition, thus, generating the ENDOR response [5, 15, 16]. Pulsed ENDOR generally yields more distortionless line shapes than cw-ENDOR, and is particularly useful when strongly anisotropic hyperfine interactions are of interest [7].

The aim of this study is to compare pulsed ENDOR spectra from different flavoproteins in terms of variations of characteristic proton hfc values. The idea is to unravel the bandwidth of possible influences on the cofactor’s electronic state by surrounding amino acids. Furthermore, we compare these data to find general rules for distinguishing between protein-bound neutral and anionic flavin radical states. Here, we restrict ourselves on ENDOR data from chemically unmodified flavins as only very few data from chemically modified flavins is available up to now (see, e.g., Refs. [17–19]).

2 Materials and Methods

The investigated protein samples were recombinantly expressed and purified according to the published procedures [10, 13, 20–24]. To prepare the radical state of the flavin cofactor, purified protein samples were supplemented with 5 mM ethylenediaminetetraacetic acid or 5 mM dithiothreitol, illuminated with blue light (Halolux 30HL, Streppel, Wermelskirchen-Tente, Germany) filtered by a 420–470-nm band filter (Schott, Mainz) at 4°C for times adequate for radical generation. The concentration of the resultant blue flavin radical (FIH[•]) was estimated on the basis of its strong absorbance at 580 nm (an ϵ_{580} of $0.48 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was assumed). Samples comprising intact cells were prepared as described previously [12, 20].

The protein preparations were transferred into EPR quartz tubes (inner diameter, 3 mm) under an argon inert gas atmosphere in the dark. The enzymes were frozen rapidly in liquid nitrogen and stored therein. No changes in the signal line shape and intensity have been observed over a storage period of several months.

X-band pulsed ENDOR spectra were recorded on a commercial pulse EPR spectrometer Bruker E580 (Bruker BioSpin GmbH, Rheinstetten, Germany) in conjunction with a dielectric ring ENDOR resonator Bruker ER 4118X-MD5-EN. For Davies-type ENDOR, a microwave pulse sequence π – t – $\pi/2$ – τ – π using 64-ns and 128-ns $\pi/2$ - and π -pulses, respectively, and a radio frequency pulse of 11 μ s duration starting 1 μ s after the first microwave pulse were used. The separation times t and τ between the microwave pulses were set to 13 μ s and 500 ns, respectively. To avoid the saturation effects due to long relaxation times of flavin radicals, the entire pulse pattern was repeated with a frequency of only 200 Hz. All ENDOR spectra were recorded at the center-field position of the EPR spectrum, respectively.

For ENDOR spectral simulations, the signal amplitudes were normalized to the integral of the signal arising from the hfc of H(6) in FIH[•]. This proton is non-exchangeable, and hence, the intensity of its ENDOR signal is expected to remain practically constant throughout different buffer conditions. To precisely extract the principal values of the hfcs of the H(8 α), H(5) and H(1') protons from the ENDOR data, spectral simulations were performed using the EPR spectral simulation software toolbox EasySpin in conjunction with the MATLAB routine lsqcurvefit (The MathWorks, Natick, MA) [25]. This program calculates ENDOR powder spectra arising from overlapping hyperfine tensors and fits their principal values to achieve the best possible agreement with the experimental data.

3 Results

In Fig. 2, pulsed proton X-band ENDOR spectra from different flavoproteins are presented. More specifically, data from *Xenopus laevis* (6–4) photolyase (Xl64PL) [26], *Synechocystis* species cryptochrome-DASH (SsCry), *Thermus thermophilus* cyclobutane pyrimidine dimer (CPD) photolyase (TlCPDPL), *Arabidopsis thaliana* CPD photolyase (AtCPDPL), *Arabidopsis thaliana* cryptochrome 1 (AtCry1) [20], *Drosophila melanogaster* cryptochrome (DmCry) [13], *Desulfovibrio vulgaris* flavodoxin (DvFD) and *Aspergillus niger* glucose oxidase (AnGO) [10], the latter measured at two proton concentrations, at pH 5 and 10, have been compiled. On the left side of Fig. 2, the radio frequency range from 7.2 to 22.7 MHz, where most of the proton resonances of flavin radicals are expected, is shown, whereas on the right side of Fig. 2, the scaled-up ENDOR signals arising from the hfc of H(5) in neutral flavin radicals are displayed. This hfc from a flavin bound to a rigid protein environment is typically quite anisotropic and, therefore, of rather low intensity (see below).

ENDOR resonances from protons in a flavoprotein are observed in a range between 0 and nearly 36 MHz. Hfcs in the weak-coupling limit, $\nu_H > |A/2|$, are symmetrically arranged in pairs centered around the free proton Larmor frequency, ν_H . Riboflavin in its neutral radical state comprises 21 hydrogens, 10 of which are located in the redox-active 7,8-dimethyl isoalloxazine ring that carries most of the unpaired electron spin density (see Fig. 1). Eleven protons are found in the ribityl chain. However, only two protons of this side chain experience sufficient spin density to exhibit hfcs of appreciable strength. These are the two magnetically inequivalent β -protons, H(1'), attached to C(1'). Owing to their proximity to N(10), which, according to quantum-chemical calculations, carries rather high electron spin density [27], the isotropic hfcs of these two protons can attain values of up to about 10–12 MHz [28]. In proton ENDOR spectra recorded at X-band, the resonances of the two H(1') protons are normally found at around 9–10 and 19–20 MHz. The relative size of their hfcs depends, according to the Heller–McConnell relation [29], on the dihedral angle between the two H(1')–C(1') bonds and the direction defined by the p_z orbitals of the isoalloxazine's π -electron system. Depending on the geometry at C(1'), very often the signal of only one of the two protons is observed in this region, and the one from the second H(1') proton overlaps with signals from

Fig. 2 X-band frozen-solution pulsed ENDOR spectra of FADH/FMN (flavin mononucleotide) in their semiquinone states bound to various flavoproteins measured under comparable experimental conditions. *Left* pulsed proton ENDOR spectra showing the main part of the flavin's hfcs between 7.3 and 22.7 MHz. *Right* pulsed ENDOR spectra showing the H(5) part of the flavin's hfcs between 21 and 40 MHz. *a* Xl64PL, *b* SsCry, *c* TtCPDPL, *d* AnGO (pH 5), *e* AtCPDPL, *f* AtCry1, *g* DvFD, *h* AnGO (pH 10), *i* DmCry. For better visibility, resonances from the hyperfine couplings arising from protons H(8 α), H(6) and H(1') are highlighted in (a). All spectra were measured at 80 K

other protons with smaller hfcs. An analysis of the hfcs of both β -protons can yield accurate structural information on the geometry of the ribityl chain attached to N(10). This has, for example, been achieved for the neutral flavin radical of *Escherichia coli* CPD photolyase [28].

H(3), H(9) and the three methyl group protons at C(7 α) are only weakly coupled to the unpaired, delocalized π -electron-spin system. Therefore, these protons give rise to ENDOR signals in the central, so-called “matrix region” of the spectrum,

which extends at X-band from about 13–16 MHz. This region comprises also hfc from other weakly coupled protons, e.g., from the protein backbone in the cofactor-binding pocket and from protons of water molecules surrounding the flavin radical. In principle, the matrix-ENDOR region carries substantial structural information on the flavin-binding pocket, but unfortunately no general method has been developed for an efficient unambiguous deconvolution of its signal contributions so far. Therefore, these overlapping hyperfine resonances remain unassigned.

When recording a proton-ENDOR spectrum of a flavin neutral radical, two prominent features in the 10–12 and 17–19-MHz radio frequency ranges are always apparent. The line shapes are frequently of nearly axial symmetry and arise from the hfc of the three β -protons of the methyl group attached to C(8 α). Even at very low temperatures, methyl groups usually rotate freely about their C–C bond. Consequently, an averaged hyperfine tensor for all three protons of the methyl group is observed, if this rotation is fast on the time scale of the ENDOR experiment. The signals of the H(8 α) hyperfine tensor are considered to be sensitive probes of the electron spin density on the outer xylene ring of the flavin's isoalloxazine ring. This is because the isotropic hfc of a β -proton is related to the spin density of the neighboring carbon or nitrogen atom in the π -plane [29–32]. Furthermore, electronic structure calculations show that the size of this hfc responds sensitively to polarity changes of the cofactor's protein surrounding [33].

Features arising from the hfc of the H(6) proton are found at around 12 and 17 MHz in proton ENDOR experiments at X-band. Unexpectedly, the H(6) hfc shows only a small hyperfine anisotropy when compared with other α -protons, such as H(5) (see below). It seems to be very insensitive towards changes in the protein environment and the electron spin density distribution [33].

One broad feature extending from the matrix-ENDOR region up to about 35 MHz is observed in pulsed ENDOR spectra, but escapes observation by the related cw-ENDOR technique [7]. By means of an H $\rightarrow$ D buffer exchange, the signal can be assigned to the H(5) proton. Its counterpart spanning the corresponding low radio frequency range is usually not resolved due to the overlap with ENDOR signals from nitrogens and other protons. The symmetry of the underlying hfc is clearly rhombic ($A_x \neq A_y \neq A_z$). Its contribution to the overall spectrum can be easily discriminated from that of other protons in the isoalloxazine ring due to the exchangeability of H(5) in a deuterated buffer. The only other exchangeable proton in the isoalloxazine moiety of flavins is that attached to N(3). However, its hfc is considerably smaller than that of H(5) as stated earlier [27]. The amplitude and the anisotropy of the H(5) hfc has recently been shown to yield information on the strength of the hydrogen bonding of neighboring amino acids to H(5) [7]. The A_z and A_y components of this coupling are easily extracted from the proton-ENDOR signal's peak position and the outer inflection point of the tensorial powder pattern, respectively. The value of the A_x component is more difficult to determine. It can be obtained from deuteron-ENDOR experiments performed at high magnetic fields, as has been shown by us previously [34].

For a comparison of proton hfc from different flavoproteins, spectral simulations of parts of the individual ENDOR spectra were performed. The results are summarized in Table 1 and will be discussed for the specific proton positions below.

Table 1 H(8 α), H(5) and H(1') proton hfcs of the FADH[•] cofactors obtained from simulations of the pulsed ENDOR spectra of various flavoproteins from the photolyase/cryptochrome class

H(8 <i>α</i>)			H(1')			H(5)	
<i>ArCry1</i>							
<i>A</i> ₁	6.85	<i>A</i> _{iso} = 7.91	<i>A</i> ₁	8.99	<i>A</i> _{iso} = 9.82	<i>A</i> ₁	–
<i>A</i> ₂	7.90		<i>A</i> ₂	8.99		<i>A</i> ₂	–25.00
<i>A</i> ₃	9.00		<i>A</i> ₃	11.49		<i>A</i> ₃	–37.02
<i>DmCry</i>							
<i>A</i> ₁	9.93	<i>A</i> _{iso} = 10.84	<i>A</i> ₁	nd	<i>A</i> _{iso} = nd	<i>A</i> ₁	–
<i>A</i> ₂	10.40		<i>A</i> ₂	nd		<i>A</i> ₂	–
<i>A</i> ₃	12.19		<i>A</i> ₃	nd		<i>A</i> ₃	–
<i>SsCry</i>							
<i>A</i> ₁	6.41	<i>A</i> _{iso} = 7.23	<i>A</i> ₁	7.84	<i>A</i> _{iso} = 9.26	<i>A</i> ₁	–
<i>A</i> ₂	7.01		<i>A</i> ₂	8.55		<i>A</i> ₂	–25.53
<i>A</i> ₃	8.26		<i>A</i> ₃	11.39		<i>A</i> ₃	–38.36
<i>TrCPDPL</i>							
<i>A</i> ₁	6.61	<i>A</i> _{iso} = 7.36	<i>A</i> ₁	6.41	<i>A</i> _{iso} = 8.62	<i>A</i> ₁	–
<i>A</i> ₂	6.99		<i>A</i> ₂	8.70		<i>A</i> ₂	–25.30
<i>A</i> ₃	8.47		<i>A</i> ₃	10.75		<i>A</i> ₃	–37.49
<i>ArCPDPL</i>							
<i>A</i> ₁	6.54	<i>A</i> _{iso} = 7.38	<i>A</i> ₁	8.41	<i>A</i> _{iso} = 9.42	<i>A</i> ₁	–
<i>A</i> ₂	7.21		<i>A</i> ₂	8.50		<i>A</i> ₂	–24.60
<i>A</i> ₃	8.40		<i>A</i> ₃	11.34		<i>A</i> ₃	–37.02
<i>Xf64PL</i>							
<i>A</i> ₁	6.24	<i>A</i> _{iso} = 6.96	<i>A</i> ₁	7.05	<i>A</i> _{iso} = 8.85	<i>A</i> ₁	–
<i>A</i> ₂	6.59		<i>A</i> ₂	8.01		<i>A</i> ₂	–25.90
<i>A</i> ₃	8.04		<i>A</i> ₃	11.50		<i>A</i> ₃	–38.36

All values in MHz; signs of hfcs have not been determined

The experimental errors are ± 0.04 MHz for all components

nd Not determined

4 Discussion

4.1 Comparison of the Hyperfine Couplings from the 8 α Methyl Protons in Neutral and Anionic Flavin Radicals

The axial tensorial line shapes arising from the methyl protons attached to C(8 α) dominate proton ENDOR spectra of flavins and have been detected and assigned in numerous studies before [18, 26, 35–37]. The hfc of this methyl group reacts sensitively to changes in the electron spin density on the outer xylene ring and to micropolarity changes of the protein surroundings [33]. Moreover, as has been shown in a number of experimental [18, 19] and theoretical [38] studies on flavin radicals, the H(8 α) hfcs of anion radicals, are in general, significantly larger when compared with those in neutral radicals. This is because deprotonation of N(5)

results in a significant redistribution of the unpaired electron spin density from the pyrazine and pyrimidine rings of the isoalloxazine moiety towards the less polar xylene ring.

The flavin radical in *AnGO* can be converted from its anionic to its neutral form by changing the buffer pH from pH 9 to 5. Therefore, this enzyme can serve as a model system for rationalizing how changes in the protonation state are reflected by the hfc values. Conformational changes of the binding pocket, which may also affect the spin density distribution, are supposed to be negligible. Dramatic changes in hfc values of the H(8 α) methyl group are detected and are clearly visible in panels d and h of Fig. 2. The isotropic hfc increases from 7.36 to 10.45 MHz on generation of the anionic flavin radical. In addition, the values arising from the H(6) proton are nearly doubled in the anion radical when compared with those of the neutral radical (see Fig. 2, panels d and h). These findings are consistent with a significant increase in spin density on the xylene ring of the isoalloxazine moiety upon deprotonation of the flavin radical [38].

Deoxyribonucleic acid (DNA) photolyases and cryptochromes are a class of structurally conserved flavoproteins where some ENDOR studies have been published recently [26, 28]. This protein class seems to be a good choice to evaluate ENDOR data to unravel the variability of their hyperfine values. When comparing the A_{iso} values from different DNA photolyases and cryptochromes, it becomes obvious that the smallest value investigated in this study is the one from FADH $^{\bullet}$ of Xf64PL (see Table 1; Fig. 2a) [26]. Recent structural data revealed a substrate-binding pocket, which is small but deep, when compared with other photolyases. It contains a high proportion of aromatic amino acids, including two histidines located close to the FAD cofactor [39]. These render the environment of the 8 α methyl protons less polar, thus, leading to this unique small hfc [26].

Other members of the photolyase/cryptochrome protein family, namely *AtCPDPL*, *TtCPDPL* [21] and *SsCry* show quite similar hfc values $A_{\text{iso}}(8\alpha)$ (see Table 1). The latter protein is a member of the so-called cryptochrome-DASH (CRY-DASH) subfamily. The function of CRY-DASH proteins has not been finally established; confirmed is its capacity in repairing ultraviolet light-generated CPD in single-stranded DNA and loop structures of duplex DNA [23, 40, 41]. *AtCPDPL* belongs to the yet incompletely characterized class-II subclass of CPD photolyases; its classification is based on an amino acid similarity alignment [42]. Detailed structural information is available for the *TtCPDPL* [43], and the *SsCry* protein [23]. Only conserved aromatic amino acids are found in the close surroundings of the 8 α protons in these two proteins (the pocket around the 8 α methyl group consists of a Phe, one Trp, an Ala and one Arg residue). Although the binding pocket in *SsCry* is slightly wider and shallower than that in class-I CPD photolyases (*TtCPDPL* and *E. coli* CPD photolyase), the effect of this structural feature seems not to be reflected in the H(8 α) hfc. As a further result, it can be concluded that the amino acids located close to the FAD cofactor in *AtCPDPL* should be very similar to the one in *TtCPDPL* and *SsCry*.

AtCry1, a member of the plant cryptochrome class [44], differs in terms of its $A_{\text{iso}}(8\alpha)$ value, which was determined to be 7.9 MHz. Structural data show distinct changes of the cavity when compared with other members of this protein class [45].

The FAD-access cavity of *AtCry1* is larger and has a unique chemical environment when compared with the cavities of the other structurally characterized members of the photolyase/cryptochrome superfamily. Moreover, the surface of *AtCry1* is mostly negatively charged, with a small concentration of positive charge near to the FAD-access cavity, which is in clear contrast to all CPD photolyases. The lack of thymine-dimer repair activity of cryptochromes was explained with this finding. Besides, a different amino acid, an aspartate (compared with an asparagine in CPD photolyases), is located close to N(5) of the 7,8-dimethyl isoalloxazine moiety and is suggested to donate the proton forming H(5) in the neutral radical. It has been speculated that this alters the strength of the N(5)–H(5) bond, which is as well visible in optical spectroscopy as an approximately 50-nm downshift of the 600-nm radical band in the neutral flavin radical [46, 47]. As a consequence, the electron spin distribution in the isoalloxazine moiety should be altered to a certain extend.

DvFD, on the other hand, shows a completely different hyperfine pattern although optical spectroscopy confirms the generation of a neutral flavin radical upon photoreduction. This can be explained in terms of a different flavin-binding situation. Whereas the flavin cofactor is deeply buried in the protein in photolyases, cryptochromes and in GO samples, it is located near the surface of the protein in flavodoxins, with the xylene ring protruding into the solvent [48]. Therefore, no amino acids are in close contact with the xylene ring and in particular, with the three methyl protons at C(8 α). Therefore, the hfc of these protons reflects the extremely polar aqueous environment.

The *DmCry* protein, at last, shows again a significantly different coupling pattern: its isotropic H(8 α) hfc value is 10.8 MHz and differs from all other proteins of the photolyase/cryptochrome family investigated in this study. Following the discussion outlined previously [13, 49], *DmCry* forms an anion radical upon photoreduction. As no structural data and, therefore, no detailed information regarding the exact amino acid composition surrounding the FAD cofactor have been published as yet, no exact classification of *DmCry* with respect to other examples from the photolyase/cryptochrome class is possible. It has to be mentioned, however, that secondary structure models predict a conserved binding pocket, however, with a cysteine residue facing the N(5) position.

As a first summary, a scheme with all measured A_{iso} values for the 8 α -methyl protons extracted from spectral simulations is shown in Fig. 3. It is apparent that most members of the photolyase/cryptochrome class show hfc's clustering in a range between 7.2 and 7.4 MHz. Exceptions are the *XI/64PL* on the lower end and *AtCRY1* at the opposite side with a significantly larger hfc. The A_{iso} values of the two anion radicals in *DmCRY* and *AnGO* differ significantly as their spin density on the xylene ring is increased. Their values are found at 10.8 and 10.5 MHz, respectively. *DvFD*, on the other hand, shows an intermediate hfc value for A_{iso} of H(8 α), although it has been demonstrated in a number of publications [35, 50] that a neutral flavin radical is formed upon (photo)reduction. Therefore, we conclude that a comparison of H(8 α) A_{iso} values alone is insufficient for an unambiguous assignment of the resonances to a neutral or an anion flavin radical; such an approach appears only valid when flavoproteins are compared that bind their cofactor in a way so that it is shielded from the aqueous surroundings at the protein surface. On the other hand,

Fig. 3 Isotropic hfc of H(8z) protons obtained from spectral simulations of ENDOR data (for details, see Tables 1, 2)

Table 2 H(8z), H(5) and H(1') proton hyperfine couplings of the FADH[•]/FMN[•] cofactors obtained from simulations of the pulsed ENDOR spectra of various flavoproteins

H(8z)			H(1')			H(5)	
<i>DvFD</i>							
<i>A</i> ₁	7.63	<i>A</i> _{iso} = 8.70	<i>A</i> ₁	7.98	<i>A</i> _{iso} = 11.10	<i>A</i> ₁	–
<i>A</i> ₂	8.40		<i>A</i> ₂	11.11		<i>A</i> ₂	–25.96
<i>A</i> ₃	10.08		<i>A</i> ₃	14.20		<i>A</i> ₃	–38.68
<i>AnGO (neutral)</i>							
<i>A</i> ₁	6.82	<i>A</i> _{iso} = 7.36	<i>A</i> ₁	nd	<i>A</i> _{iso} = nd	<i>A</i> ₁	–
<i>A</i> ₂	6.82		<i>A</i> ₂	nd		<i>A</i> ₂	–24.1
<i>A</i> ₃	8.45		<i>A</i> ₃	nd		<i>A</i> ₃	–34.7
<i>AnGO (anionic)</i>							
<i>A</i> ₁	9.95	<i>A</i> _{iso} = 10.45	<i>A</i> ₁	nd	<i>A</i> _{iso} = nd	<i>A</i> ₁	–
<i>A</i> ₂	9.95		<i>A</i> ₂	nd		<i>A</i> ₂	–
<i>A</i> ₃	11.45		<i>A</i> ₃	nd		<i>A</i> ₃	–

All values in MHz; signs of hyperfine couplings have not been determined experimentally, but were taken from theoretical calculations

The experimental errors are ± 0.04 MHz for all components

nd Not determined

the results from *DvFD* undoubtedly show the potential of ENDOR spectroscopy in correlating hyperfine data to structural information.

4.2 Hyperfine Couplings from the H(5) Proton in Neutral Flavin Radicals

Hfcs from protons bound directly to π -conjugated systems, so-called α -protons, are typically strongly anisotropic ($A_x \neq A_y \neq A_z$). If one subtracts the isotropic coupling, $A_{\text{iso}} = (A_x + A_y + A_z)/3$, from the tensor principal values, the traceless anisotropic hyperfine contributions remain: $T_i = A_i - A_{\text{iso}}$, with $i \in \{x, y, z\}$. These depend on the electron–nuclear dipolar interaction. Short N(5)–H(5) bond lengths lead, according to density functional theory (DFT) calculations, to a symmetric rhombic hyperfine tensor with $T_z \rightarrow 0$ and $T_x = -T_y$ [7]. When the N–H bond length is increased, e.g., as a result of hydrogen bonding of H(5) to a nearby amino acid in the protein backbone, the hyperfine tensor is expected to become more

axially symmetric. Only the two large hfc components of the H(5) tensor can be extracted from proton ENDOR spectra at X-band. The third component is small and is, therefore, overlapping with other signals in the matrix region of the spectra. An exact determination of the A_x component is possible from the investigation of deuterium-exchanged samples by deuterium-ENDOR, but this is beyond the scope of this contribution. All samples with a proposed neutral flavin radical clearly show the A_y and A_z resonances from the H(5) hfc. On the other hand, the *DmCry* and *AnGO* samples (measured at pH 10) show virtually no signal in the spectral region between 22 and 40 MHz. The presence of this hfc, therefore, provides unambiguous evidence for a neutral flavin radical.

4.3 Proton ENDOR Spectroscopy on Cryptochromes in Intact Cell Samples

Recently, we have investigated different cryptochrome blue-light photoreceptors under in vivo conditions and in comparison with purified samples [12, 13, 20]. The idea behind these experiments was to obtain information on the flavin cofactor redox state in vivo, and how this redox state is modulated under different light conditions. To this end, ENDOR spectroscopy turned out to be a very valuable method for obtaining such information in complex media, such as intact cells, because (1) other cell components, such as lipids or DNA, do not give rise to EPR or ENDOR signals as they are diamagnetic, (2) ENDOR signals arising from other paramagnetic cell compartments such as iron–sulfur clusters can be ruled out via a control of the temperature, and (3) the signal-to-noise ratios can be directly controlled via the expression rate of the protein under investigation. In Fig. 4, *ArCRY* samples in intact cells (spectra a) are compared with in vitro purified protein samples (spectra b). In contrast to recently published data [20], the quality of the ENDOR spectra has been significantly improved such that a direct comparison of in-cell samples with in vitro samples is now feasible. Qualitatively, both spectra look quite similar; the hyperfine values of the protons from H(8 α) and H(6) are almost identical. Moreover, resonances from H(5) are clearly visible in both spectra.

Fig. 4 X-band frozen-solution (80 K) pulsed proton ENDOR spectra of $\text{FADH}^\bullet$ bound to *ArCry1* samples measured under comparable experimental conditions. *a* Frozen intact *Sf21* cells expressing *ArCry1*, *b* frozen purified *ArCry1* protein solution

It can finally be concluded that a neutral flavin radical is formed under illumination under physiological conditions. Interestingly, close inspection reveals small differences between the two ENDOR spectra. Although the signal-to-noise ratio obtained for the cell sample is still not as good as that for the protein preparation in Fig. 4 left, it seems that the A_y and A_z components of the H(5) coupling are smaller by about 1 MHz in the cell sample. Moreover, the resonance at 26 MHz is considerably broadened. This could be caused by an increased conformational flexibility of the Asp...H(5)–N(5) hydrogen bond in the cell sample when compared with the purified protein *in vitro*. The observed broadening of the H(5) hfc would then be a result of hyperfine strain.

Furthermore, the integral of the matrix region differs between both samples when normalized with respect to the H(8 α) signal. As both samples have been recorded under exactly the same spectroscopic conditions, these differences can be rationalized in terms of the content of hydrogens in the flavin environment. The integral of the matrix region reflects the sum of all weakly coupled protons. It seems, therefore, as if the total number of protons interacting with the flavin radical under in-cell conditions is decreased. As resonances from close amino acids should not be altered, the number of protons must therefore be lowered. This could be explained if AtCry was membrane bound and not freely located in the cytosol of the cell. Another possibility is that due to an interaction with an as yet to be identified interaction partner that is bound via the pocket close to the 8 α methyl group, the number of interacting water molecules is decreased, thus attenuating the matrix-ENDOR signal. Moreover, small but clearly visible additional features are observed in the 12–18 MHz region. Although no clear band can be observed, the minimum between H(8 α) and H(6) coupling is less pronounced. At this stage, no conclusions can be drawn if this signal arises from a second, yet unidentified radical species in the cell, or if subtle changes in the protein-cofactor geometry are responsible for this observation. In conclusion, measuring ENDOR under biological “correct” conditions is a major improvement for assigning electronic structures of cofactors.

5 Conclusions

In the present study, we demonstrate that by determination of the hyperfine interactions of both the H(8 α) and H(5) protons by pulsed ENDOR, the neutral radical form of a flavin semiquinone can be distinguished from its anionic radical form, as long as the cofactor is deeply buried in the protein. Partial exposure of the redox-active isoalloxazine ring, that carries the majority of the unpaired electron spin, to the aqueous protein surroundings can obscure the simple assignment based on the H(8 α) hyperfine coupling alone. In such cases, unambiguous evidence for the neutral radical form is only obtained from a direct detection of the H(5) ENDOR resonance. Given the high detection sensitivity of modern pulsed EPR/ENDOR instrumentation that is commercially available, such experiments can nowadays be performed even on flavoproteins in their native cell environment.

Acknowledgments This work was supported by the Deutsche Forschungsgemeinschaft (SFB-498, projects A2 and B7, and the Research Group “Blue light photoreceptors”, FOR-526). We thank Prof. Stephen G. Mayhew and Dr. Mary Gallagher (both University College Dublin, Ireland) for providing us with *Desulfovibrio vulgaris* flavodoxin. It is a pleasure to thank Dr. Chris Kay (University College of London) for assistance during the initial experiments and for helpful discussions. We thank Gebhard Kaiser and Tobias Klar for ArCry1 and TrCPDPL protein preparations.

References

1. V. Massey, *Biochem. Soc. Trans.* **28**, 283–296 (2000)
2. D.E. Edmondson, *Biochem. Soc. Trans.* **13**, 593–600 (1985)
3. C.W.M. Kay, S. Weber, in *Electron Paramagnetic Resonance*, ed. by B.C. Gilbert, M.J. Davies, D.M. Murphy (Royal Society of Chemistry, Cambridge, UK, 2002), pp. 222–253
4. D.M. Murphy, R.D. Farley, *Chem. Soc. Rev.* **35**, 249–268 (2006)
5. S. van Doorslaer, E. Vinck, *Phys. Chem. Chem. Phys.* **9**, 4620–4638 (2007)
6. R. Bittl, C.W.M. Kay, S. Weber, P. Hegemann, *Biochemistry* **42**, 8506–8512 (2003)
7. S. Weber, C.W.M. Kay, A. Bacher, G. Richter, R. Bittl, *ChemPhysChem* **6**, 292–299 (2005)
8. B. Barquera, L. Ramirez-Silva, J.E. Morgan, M.J. Nilges, *J. Biol. Chem.* **281**, 36482–36491 (2006)
9. C.W.M. Kay, H. El Mkami, G. Molla, L. Pollegioni, R.R. Ramsay, *J. Am. Chem. Soc.* **129**, 16091–16097 (2007)
10. A. Okafuji, A. Schnegg, E. Schleicher, K. Möbius, S. Weber, *J. Phys. Chem. B* **112**, 3568–3574 (2008)
11. H. Nagai, Y. Fukushima, K. Okajima, M. Ikeuchi, H. Mino, *Biochemistry* **47**, 12574–12582 (2008)
12. R. Banerjee, E. Schleicher, S. Meier, R. Muñoz Viana, R. Pokorny, M. Ahmad, R. Bittl, A. Batschauer, *J. Biol. Chem.* **282**, 14916–14922 (2007)
13. N. Hoang, E. Schleicher, S. Kacprzak, J.-P. Bouly, M. Picot, W. Wu, A. Berndt, E. Wolf, R. Bittl, M. Ahmad, *PLoS Biol.* **6**, e160.1559–e160.1569 (2008)
14. M. Medina, R. Cammack, *Appl. Magn. Reson.* **31**, 457–470 (2007)
15. D. Goldfarb, D. Arieli, *Annu. Rev. Biophys. Biomol. Struct.* **33**, 441–468 (2004)
16. D. Goldfarb, *Phys. Chem. Chem. Phys.* **8**, 2325–2343 (2006)
17. N. Bretz, N. Henzel, H. Kurreck, F. Müller, *Isr. J. Chem.* **29**, 49–55 (1989)
18. H. Kurreck, M. Bock, N. Bretz, M. Elsner, H. Kraus, W. Lubitz, F. Müller, J. Geissler, P.M.H. Kroneck, *J. Am. Chem. Soc.* **106**, 737–746 (1984)
19. H. Kurreck, N.H. Bretz, N. Helle, N. Henzel, E. Weilbacher, *J. Chem. Soc. Faraday Trans. 1* **84**, 3293–3306 (1988)
20. J.-P. Bouly, E. Schleicher, M. Dionisio-Sese, F. Vandenbussche, M. Ahmad, R. Bittl, P. Galland, A. Batschauer, S. Meier, N. Bakrim, D. Van der Straeten, *J. Biol. Chem.* **282**, 9383–9391 (2007)
21. T. Klar, G. Kaiser, U. Hennecke, T. Carell, A. Batschauer, L.-O. Essen, *Chem. Biol. Chem.* **7**, 1798–1806 (2006)
22. K. Hitomi, S.-T. Kim, S. Iwai, N. Harima, E. Otsoshi, M. Ikenaga, T. Todo, *J. Biol. Chem.* **272**, 32591–32598 (1997)
23. 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* **11**, 59–67 (2003)
24. O. Kleiner, J. Butenandt, T. Carell, A. Batschauer, *Eur. J. Biochem.* **264**, 161–167 (1999)
25. S. Stoll, A. Schweiger, *J. Magn. Reson.* **178**, 42–55 (2006)
26. E. Schleicher, K. Hitomi, C.W.M. Kay, E.D. Getzoff, T. Todo, S. Weber, *J. Biol. Chem.* **282**, 4738–4747 (2007)
27. S. Weber, K. Möbius, G. Richter, C.W.M. Kay, *J. Am. Chem. Soc.* **123**, 3790–3798 (2001)
28. C.W.M. Kay, R. Feicht, K. Schulz, P. Sadewater, A. Sancar, A. Bacher, K. Möbius, G. Richter, S. Weber, *Biochemistry* **38**, 16740–16748 (1999)
29. C. Heller, H.M. McConnell, *J. Chem. Phys.* **32**, 1535–1539 (1960)
30. A. Carrington, A.D. McLachlan, *Introduction to Magnetic Resonance* (Harper & Row, New York, 1967)
31. E.W. Stone, A.H. Maki, *J. Chem. Phys.* **37**, 1326–1333 (1962)
32. E.L. Fasanella, W. Gordy, *Proc. Natl. Acad. Sci. USA* **62**, 299–304 (1969)

33. S. Weber, G. Richter, E. Schleicher, A. Bacher, K. Möbius, C.W.M. Kay, *Biophys. J.* **81**, 1195–1204 (2001)
34. C.W.M. Kay, E. Schleicher, K. Hitomi, T. Todo, R. Bittl, S. Weber, *Magn. Reson. Chem.* **43**, S96–S102 (2005)
35. I. Çinkaya, W. Buckel, M. Medina, C. Gómez-Moreno, R. Cammack, *Biol. Chem.* **378**, 843–849 (1997)
36. M. Medina, A. Lostao, J. Sancho, C. Gómez-Moreno, R. Cammack, P.J. Alonso, J.I. Martínez, *Biophys. J.* **77**, 1712–1720 (1999)
37. B. Barquera, J.E. Morgan, D. Lukoyanov, C.P. Scholes, R.B. Gennis, M.J. Nilges, *J. Am. Chem. Soc.* **125**, 265–275 (2003)
38. J.I. García, M. Medina, J. Sancho, P.J. Alonso, C. Gómez-Moreno, J.A. Mayoral, J.I. Martínez, *J. Phys. Chem. A* **106**, 4729–4735 (2002)
39. M.J. Maul, T.R.M. Barends, A.F. Glas, M.J. Cryle, T. Domratcheva, S. Schneider, I. Schlichting, T. Carell, *Angew. Chem. Int. Ed.* **47**, 10076–10080 (2008)
40. C.P. Selby, A. Sancar, *Proc. Natl. Acad. Sci. USA* **103**, 17696–17700 (2006)
41. R. Pokorny, T. Klar, U. Hennecke, T. Carell, A. Batschauer, L.-O. Essen, *Proc. Natl. Acad. Sci. USA* **105**, 21023–21027 (2008)
42. S. Kanai, R. Kikuno, H. Toh, H. Ryo, T. Todo, *J. Mol. Evol.* **45**, 535–548 (1997)
43. H. Komori, R. Masui, S. Kuramitsu, S. Yokoyama, T. Shibata, Y. Inoue, K. Miki, *Proc. Natl. Acad. Sci. USA* **98**, 13560–13565 (2001)
44. M. Ahmad, A.R. Cashmore, *Nature* **366**, 162–166 (1993)
45. C.A. Brautigam, B.S. Smith, Z. Ma, M. Palnitkar, D.R. Tomchick, M. Machius, J. Deisenhofer, *Proc. Natl. Acad. Sci. USA* **101**, 12142–12147 (2004)
46. T. Kottke, A. Batschauer, M. Ahmad, J. Heberle, *Biochemistry* **45**, 2472–2479 (2006)
47. C. Lin, D.E. Robertson, M. Ahmad, A.A. Raibekas, M.S. Jorns, P.L. Dutton, A.R. Cashmore, *Science* **269**, 968–970 (1995)
48. W. Watt, A. Tulinsky, R.P. Swenson, K.D. Watenpaugh, *J. Mol. Biol.* **218**, 195–208 (1991)
49. A. Berndt, T. Kottke, H. Breitkreuz, R. Dvorsky, S. Hennig, M. Alexander, E. Wolf, *J. Biol. Chem.* **282**, 13011–13021 (2007)
50. M. Medina, C. Gomez-Moreno, R. Cammack, *Eur. J. Biochem.* **227**, 529–536 (1995)