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ENDOR and Related EMR Methods Applied to Flavoprotein Radicals

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Abstract. Flavoproteins are involved in a wide range of biological processes, owing to the versatility of the isoalloxazine moiety of flavin, which can undergo both one- and two-electron reactions with the formation of three oxidation states. Paramagnetic semiquinone radical states are stabilised in some flavoproteins and appear as transient intermediates in the reaction of many others. The apoprotein controls the reactivity of flavin semiquinones, including redox potentials, protonation states and access to substrates. Most flavoproteins are involved in oxidation-reduction processes, but some catalyze different types of reactions involving radical intermediates. Anionic and neutral flavin radicals are found in flavoproteins and are distinguished by their line widths in X-band electron paramagnetic resonance. Electron–nuclear double resonance, electron spin echo envelope modulation and hyperfine sublevel correlation spectroscopy make it possible to observe biological electron transfer and catalysis at the level of the electronic structure of the intermediate states. They provide information about the protein environment of flavin semiquinone radicals and their interactions with nearby nuclear and electron spins. Hyperfine couplings, particularly to the 8-methyl protons on the flavin ring, are a sensitive probe of perturbations of the flavin environment. They demonstrate differences in polarity of the flavin binding site and changes that occur in flavoenzymes during binding of substrates.

1 Introduction

The primary reactions of photosynthesis, so elegantly elucidated by Feher and others, involve electron transfers. The downstream biochemical reactions, such as carbon fixation, involve transfers of pairs of hydrogen atoms catalyzed by the dehydrogenases. At the interface between the two types of reactions are the flavoproteins, containing flavin mononucleotide (FMN) and/or flavin adenine dinucleotide (FAD). They can carry out the electron–hydrogen conversion due to their ability to form semiquinone radicals of the isoalloxazine ring. The three oxidation states: quinone (oxidized), semiquinone (one-electron-reduced, electron paramagnetic resonance [EPR]-detectable), and hydroquinone (two-electron-reduced) are depicted in Fig. 1. The semiquinone radical states of flavins were

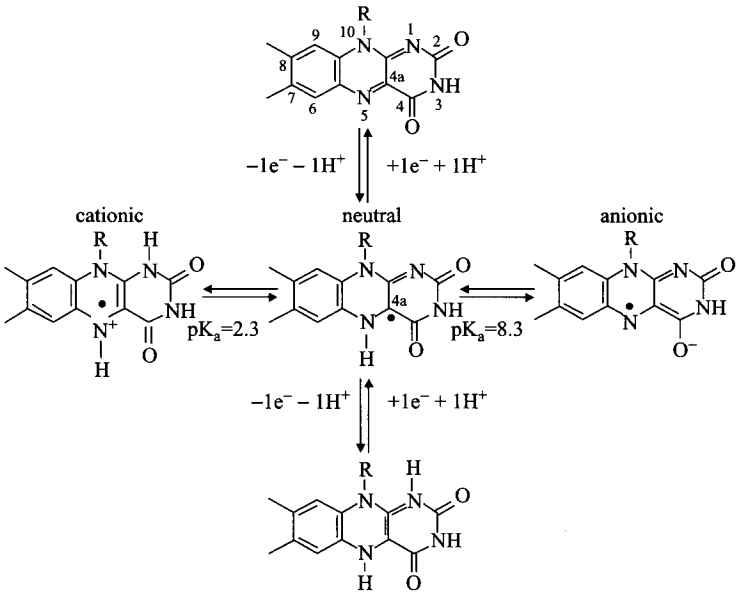

Fig. 1. Redox states of the isoalloxazine ring. Acid-base conjugates of the semiquinone state. In this paper the three rings of the isoalloxazine are described, from left to right, as the benzenoid, pyrazine and pyrimidine rings.

first discovered, by electrochemical methods, by Michaelis and colleagues [1], as long ago as 1936. Flavoproteins are now known to be versatile enzyme cofactors, involved in a wide variety of biological processes, from energy accumulation in respiratory and photosynthetic electron transfer chains to oxidation of toxic compounds [2]. Some reactions catalyzed by flavoproteins do not involve an overall oxidation or reduction at all [3].

2 ENDOR Studies of Flavoproteins

Kay and Weber [4] have reviewed the studies of flavoproteins made by a wide range of electron magnetic resonance (EMR) techniques between 1992 and 2002. Flavoprotein semiquinones were among the first radicals involved in enzyme catalysis to be studied by EMR methods [5–9]. These studies produced some general observations about flavoproteins. Of the three possible oxidation states of the flavin ring system, only the neutral and anionic radical species have been found in flavoproteins. These are sometimes termed the "blue neutral" and "red anionic" semiquinones, respectively, on the basis of the optical absorption spectra of the free flavins in these states [10]. At pH of about 7, it is not possible to obtain more than a small fraction of the semiquinone state of free flavins, in thermodynamic equilibrium with the quinone and hydroquinone states, be-

cause the reduction potential for reduction of the quinone to the semiquinone is more negative than the value for the reduction of the semiquinone to the hydroquinone [11–14]. However in many flavoproteins, binding of FAD or FMN to the apoprotein modifies the flavin midpoint reduction potentials, with $E_{ox/sq}$ being displaced to a less negative value, while $E_{sq/rd}$ is shifted to a more negative one [15–17]. These changes often promote the stabilization of the intermediate flavin semiquinone by the protein.

Conventional continuous-wave (CW) EPR detects the presence of a flavoprotein semiquinone. The neutral and anionic semiquinone can be distinguished by the increased line width, approximately 1.9 mT for the neutral radical compared with 1.5 mT for the anionic form [18]. This is due to the strong hyperfine coupling to the N5 proton in the neutral semiquinone [19], which is abolished by transferring from aqueous solution to deuterium oxide. The pK_a of protonation at N5 is determined by the protein; hence a particular flavoprotein at pH 7 may characteristically have either a neutral or anionic semiquinone, or occasionally both. The *g*-factors of the flavoprotein semiquinone radicals do not depend on the ionization state, since the unpaired electron in both forms is largely delocalized.

The advent of the Bruker commercial CW electron–nuclear double resonance (ENDOR) and pulsed EPR instruments has facilitated the routine measurement of high-resolution spectra of frozen samples. The distribution of electron density over the atoms of the flavin ring has been assigned by ENDOR and electron spin echo techniques (electron spin echo envelope modulation [ESEEM], hyperfine sublevel correlation spectroscopy [HYSCORE], etc.). In most flavoproteins the cofactor is noncovalently bound to the protein and can be dissociated and reinserted, so the features of the ENDOR spectra have been assigned by reconstitution of flavoproteins with isotopically labelled derivatives and flavin analogs, supported by molecular orbital calculations [20, 21].

The CW-ENDOR spectra of flavin semiquinones have a number of characteristic features (see Fig. 2, Table 1). Signals with small couplings in the "matrix region" of the spectrum are the sum of numerous contributions: "distant ENDOR" from many protons in the protein and solvent; from water molecules around the flavin; from hydrogens of nearby amino acid residues; and from hydrogens 7-CH_3, H9 and H3 of the isoalloxazine ring. A prominent signal with two components, $A_\perp$ and $A_\parallel$, of an axial tensor with a hyperfine coupling typically around 8–10 MHz has been assigned to protons of the 8-CH_3 group of the flavin ring [21]. Rotating methyl groups are more favorable for ENDOR in frozen solution than methylene protons [19]. Another, more isotropic hyperfine coupling of about 4–6 MHz has been assigned to hydrogen C6H.

In the X-band ESEEM spectra of flavin semiquinones, the most prominent features are usually ^{14}N couplings to N1 and N3 (see Fig. 3). Thus while ENDOR provides more information about the spin density on the benzenoid ring of the flavin, ESEEM provides more information about the pyrimidine ring. This makes it possible to identify changes in the electron density distribution in any flavosemiquinone caused, for example, by addition of substrate to an enzyme.

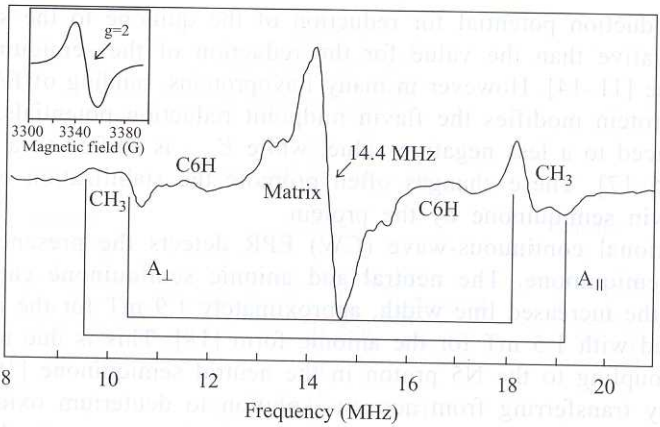

Fig. 2. ^{1}H-ENDOR spectrum of the semiquinone of Y308W pea ferredoxin:NADP$^+$ reductase. The magnetic field was centered at 336 mT, corresponding to $g = 2.005$ of the EPR signal (inset). Other conditions: microwave power, 6.7 mV; microwave frequency, 9.45 MHz; modulation depth, 112 kHz; radiofrequency power, 150 W; temperature, 120 K. Usually, a number of pronounced ENDOR line pairs symmetrically spaced around the proton Larmor frequency are detected in flavoprotein semiquinones and hence assigned to hydrogen hyperfine couplings. The ^{1}H resonances with the largest ENDOR splittings have been attributed to the two hyperfine components, $A_\perp$ and $A_\parallel$, of a tensor having axial symmetry from a freely rotating methyl group. These two values allow the determination of the isotropic (a) and anisotropic (T) contributions.

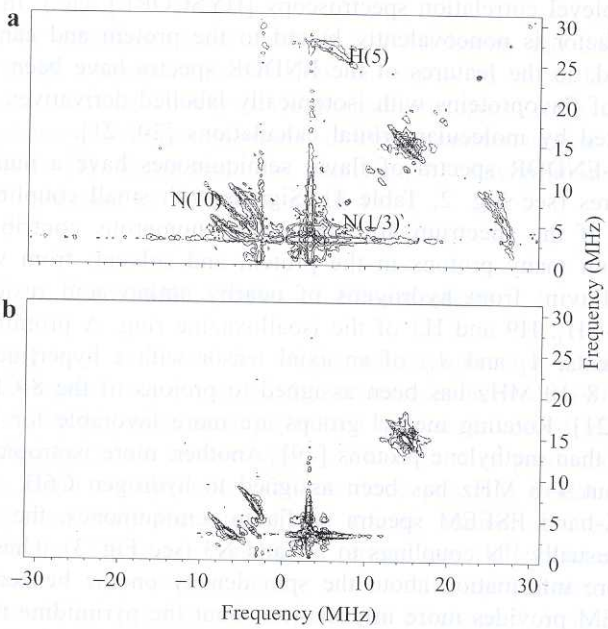

Fig. 3. Two-dimensional HYSCORE spectra of flavodoxin neutral semiquinone (**a**) and cholesterol oxidase anionic semiquinone (**b**). The main coupling features are marked. Figure adapted from ref. 34.

Table 1. Hyperfine parameters for the interaction of several nuclei of the ixoalloxazine flavin ring with the semiquinone radical in different flavoprotein forms. Parameters for N1, N3, 5H and N10 have been obtained by HYSCORE. Error in the determination of these parameters is ±0.2 MHz. Parameters for C6H and 8-CH_3 have been obtained by ENDOR. Error in the determination of these parameters is ±0.1 MHz.

Semiquinone form	Hyperfine parameter value (MHz) at:														
	N1[a,b]	N3[a,b]	5H[a]				C6H	8-CH_3				N10[a]			
	$\|a\|$	$\|a\|$	$A_{\parallel}$	$A_{\perp}$	a	T	$\|a\|$	$A_{\parallel}$	$A_{\perp}$	$\|a\|$	T	$A_{\parallel}$	$A_{\perp}$	a	T
Flavodoxin[c]	0.8	1.3	−0.4	−27.4	−18.4	9.0	5.7	9.5	7.9	8.4	1.5	29.7	2.7	11.7	9.0
Tyr94Ala[c]			−0.6	−26.7	−18.0	8.7	5.3	9.2	7.7	8.2	1.5				
Trp57Ala[c]			−0.6	−27.6	−18.6	9.0	6.2	10.0	8.4	8.9	1.6				
FNR[d]	0.7	1.2	−2.3	−26.9	−18.7	8.2	5.11	9.41	7.47	8.12		29.7	2.7	11.7	9.0
+ NADP[d]							5.11	9.04	7.06	7.72					
4-Hydroxy-butyryl-CoA dehydratase[e]							6.23	9.69	7.9						
+ 4-hydroxy-butyryl-CoA[e]							6.23	10.24	8.38						
Cholesterol oxidase[f]	1.9	0.7					9.0	12.11	10.3			29.7	2.7	11.7	9.0
+ dehydroiso-androsterone[f]							9.0	11.12	9.29						

[a] Parameters derived from ref. 34.
[b] HYSCORE experimental measurements was not able to unequivocally assign the values for N1 and N3. The assignment here reported is a tentative one taking into account DFT calculations [38, 39].
[c] Ref. 33.
[d] Ref. 31.
[e] Ref. 41.
[f] Ref. 37.

2.1 Practical Aspects of Work on Flavoproteins: Production and Trapping of the Semiquinone State

Most flavoproteins will form flavin semiquinones under specific conditions. Usually this is achieved by partial reduction of the flavoprotein. Stable semiquinone forms can be observed at ambient temperature. For most radicals, EPR spectrometers are able to detect micromolar concentrations in 100–200 µl volumes. However, in the case of flavoproteins, the yield of the protein containing the semiquinone form determines the amount of protein required. The maximal amount and type of semiquinone formed in flavoproteins depend on the relative values of $E_{ox/sq}$ and $E_{sq/rd}$, which in turn depend on the relative binding affinities of the different flavin redox states to the apoprotein, due to electrostatic and hydrophobic interactions and solvent accessibility [17, 22].

The semiquinone oxidation states of a large number of flavoproteins are often readily oxidized by oxygen. In these cases, EPR samples must be prepared,

and the spectra recorded, under anaerobic conditions. Oxygen from the solution is removed by repeated degassing under vacuum and flushed with oxygen-free gas (N_2 or Ar), before the flavoprotein is directly treated to induce its semiquinone formation. Semiquinone formation can be achieved either by photoreduction of protein-bound flavin in buffer containing 1 mM EDTA and 2 μM 5-deazariboflavin to initiate reduction via the highly reductive deazariboflavin radical [14, 22, 23], or by anaerobic addition of substoichiometric amounts of a reducing agent such as sodium dithionite, sodium borohydride, or enzyme substrate [24, 25].

Transient kinetics of flavoprotein semiquinones can be studied by using rapid freezing. In this case, the solutions to be combined to produce semiquinone formation [26] are delivered on the millisecond timescale to a mixing chamber by two motor-driven syringes. The mixed solution is pushed out of the chamber to the reaction tubes. Variation of the length of these tubes allows different reaction times. For rapid freezing, the mixture is collected into isopentane at −150 °C [26, 27]. The ice particles formed are packed into the EPR tubes and analyzed at cryogenic temperature. It is usually necessary to freeze the samples in order to stabilize the semiquinone. Measurement of frozen samples at cryogenic temperatures has the advantage of enhancing the sensitivity of EPR and is usually essential for ENDOR or ESEEM.

3 Examples of the Application of ENDOR and Other EMR Techniques to Flavoproteins

3.1 Photosynthetic Flavodoxin and Ferredoxin:NADP Reductase

We began ENDOR studies on the of flavoproteins with two proteins involved in oxygenic photosynthesis: flavodoxin and ferredoxin:NADP reductase (FNR) from the cyanobacterium *Anabaena* sp. PCC 7119 [28]. Flavodoxin is an electron-transferring flavoprotein containing FMN which is produced by algae and cyanobacteria under conditions of iron deficiency as a substitute for the two-iron ferredoxin in photosynthesis. Since the growth of such organisms is limited by availability of iron in many parts of the oceans [29], this protein plays a central role in global photosynthetic productivity. FNR is the enzyme that converts the one-electron chemistry of ferredoxins and flavodoxin to the hydride or two-hydrogen transfer chemistry required for carbon fixation. In general, in higher plants and photosynthetic organisms two ferredoxin molecules, previously reduced by a type I photosynthetic reaction center, deliver one electron each to one molecule of FNR. The FAD cofactor of FNR is in this way first reduced to the semiquinone state by the first ferredoxin molecule and subsequently to the hydroquinone state, which finally reduces $NADP^+$ to NADPH.

On binding of flavin to the apoflavodoxin, its midpoint reduction potentials are drastically altered and the semiquinone radical becomes much more stable [22]. This property allows flavodoxin to function as a one-electron transfer redox center in which the flavin cycles between the semiquinone and fully reduced oxidation states. In FNR by contrast the flavin behaves as a one-electron acceptor from

the electron carrier protein and as a two-electron donor to $NADP^+$ [22, 28, 30]. Therefore, during electron flow from photosystem I to $NADP^+$ via flavodoxin, formation of both semiquinones is expected. The flavin semiquinones of flavodoxin and FNR from *Anabaena* PCC 7119, and of some of their mutants, were studied by EPR at X- and S-band and by ENDOR, ESEEM and HYSCORE at X-band [13, 23, 31–33]. In each case the semiquinone state was produced by anaerobic photoreduction in the presence of EDTA and 5-deazariboflavin. Both EPR signals were centered at $g = 2.005$ and had a line width of 2.0 mT that decreased to 1.48 mT in deuterated buffer, consistent with neutral flavin semiquinones [31]. The line widths were virtually the same at X- and S-band, which immediately indicates that line broadening is not due to *g*-factor anisotropy but to unresolved nuclear hyperfine couplings, and moreover that the broadening is partly due to exchangeable hydrons. No changes in the shape or line width of the spectra of the semiquinones were detected upon formation of the complex between the proteins, or in the case of FNR, addition of substrate [31].

ENDOR of each semiquinone produced a typical flavin powder type spectrum (Fig. 2). The most prominent features were symmetrically located around the proton Larmor frequency and hence were assigned to ^1H hyperfine couplings. No changes in these hyperfine couplings were detected when the proteins were studied in ^2H_2O, nor when FNR or flavodoxin semiquinones were complexed with each other. However, in the case of FNR the coupling attributed to hydrogens on 8-CH_3 decreased from 8.12 to 7.72 MHz in the presence of $NADP^+$. The decrease in the electron spin density distribution on this part of the flavin ring system was attributed to binding of the substrate, polarising the electron density towards the pyrimidine ring of the flavin [31]. Some of the hyperfine couplings in matrix region (less than 3 MHz) disappeared when the proteins were transferred into ^2H_2O and therefore represent exchangeable protons. In the case of FNR, binding of $NADP^+$ caused some of these weak couplings to disappear; these signals were attributed to displaced water hydrogens or possibly to exchangeable hydrogens from amino acid residues on the protein near the flavin-binding site involved in substrate stabilization.

One- and two-dimensional ESEEM spectroscopy were also applied to the neutral semiquinone states of flavodoxin and FNR [32, 34]. Three-pulse ESEEM showed the presence of prominent nuclear modulation frequencies consistent with the presence of one or more ^{14}N nuclei magnetically coupled to the paramagnet; these were interpreted as arising from N1 and/or N3 of the flavin ring system [32]. In FNR, distortion of the frequency-domain spectrum was also observed when $NADP^+$ was bound to the enzyme, again indicating specific interaction of the flavosemiquinone with the substrate nicotinamide ring. The presence of exchangeable hydrogens and/or water molecules in the vicinity of the FNR flavin ring was confirmed by substituting deuterated buffer. In both flavodoxin and FNR, HYSCORE (two-dimensional ESEEM) spectra showed correlations between transitions resulting from interaction of the electron spins on the isoalloxazine radicals with N and H nuclei [34]. The use of isotopically labelled samples ([^{15}N]FMN-flavodoxin and [^2H]-flavodoxin) allowed the assignment of all the detected transitions to nuclei belonging to the flavin ring. Isotropic hyperfine

coupling constants in the 1–2 and 0.5–1 MHz ranges were determined for N1 and N3 of the flavosemiquinones (Table 1). An intense correlation in the negative quadrant was associated to the strongly interacting N10 nucleus. The complete hyperfine term parameters were obtained, including the quadrupolar parameters [34]. Finally, a ridge-shaped correlation in the HYSCORE spectra, corresponding to hydrogen N5H in neutral flavin semiquinones, was also detected and its parameters characterized (Fig. 3, Table 1).

The structures of a considerable number of flavoproteins have now been determined. This information may be combined with complementary information from EMR techniques to obtain a better understanding of the functions of the flavin and mechanisms of catalysis. EMR can observe protons, and follow changes in electron spin density, which are not resolved in the crystal structure. The proteins can be trapped and frozen in states that are too unstable for crystallography. Site-directed mutagenesis makes it possible to examine the effects that specific amino acid residues have on the properties of the flavin. In many flavoproteins in general, and in the flavodoxin and FNR families in particular, the flavin isoalloxazine ring is stacked between two aromatic residues [22, 30]. The influence of the amino acid residues surrounding the flavin ring in flavodoxin and FNR on the electron spin density distribution of the flavin semiquinone was examined using different protein mutants [33, 35]. Recombinant forms of *Anabaena* flavodoxin were prepared with mutations at residues that were considered likely to have a role in controlling the properties of the semiquinones. In fact, most of them gave CW-EPR, ENDOR and HYSCORE spectra similar to those reported for the wild-type flavodoxin, indicating no significant perturbation of the electronic structure of the FMN. However significant differences were observed when either of the two aromatic residues (Tyr94 and Trp57) sandwiching the isoalloxazine ring was substituted by alanine (Table 1). These studies suggested that the presence of a bulky residue (either aromatic or aliphatic) at position 57, as compared with an Ala, decreased the electron spin density in the benzene flavin ring, while an aromatic residue at position 94 increased the electron spin density at positions N5 and C6 of the flavin ring. In FNR, where the flavin ring is sandwiched between two tyrosyl residues, numbered Tyr89 and Tyr308 (C-terminal) in the pea enzyme, the latter residue was substituted by the other aromatic residues, by Gly and by Ser [36]. Once again the EPR spectra were not significantly changed by these substitutions, but in the ENDOR spectra subtle changes were observed in the isotropic and anisotropic hyperfine coupling constants for the 8-CH_3 and C6 hydrogens [35] (Fig. 2, Table 2). The presence of any residue other than Tyr (aromatic or aliphatic, bulky or small) at the C-terminal position increased the electron spin density at the nuclei of the benzene flavin ring in the semiquinone state. The net effect of this Tyr on the FNR semiquinone seems to be the withdrawal of electron density from the benzene ring, probably moving it into the pyrazine ring, and this may displace the electron density entering the oxidized flavin ring nearer to a site where it can best be neutralized by protonation of the N5 position. Binding of $NADP^+$ also appears to displace the electron spin density distribution away from the benzene ring, as seen in the *Anabaena* FNR.

Table 2. Hyperfine coupling constants determined by ENDOR spectroscopy for 8-CH_3 and C6H hydrogens of the flavin ring in the semiquinones of different pea FNR forms where the C-terminal Tyr has been mutated. Error in the determination of these parameters is ±0.1 MHz.

FNR form	Hyperfine parameter value (MHz) at:				
	8-CH_3				C6H, A
	$A_{\parallel}$	$A_{\perp}$	$\lvert a \rvert$	T	
Wild-type	9.2	7.6	8.1	0.8	5.0
Wild-type + $NADP^+$	9.0	7.5	8.0	0.7	5.0
Y308F	9.4	7.7	8.3	0.8	5.3
Y308F + $NADP^+$	9.4	7.7	8.3	0.8	5.3
Y308W	9.5	7.8	8.4	0.8	5.3
Y308W + $NADP^+$	9.4	7.8	8.3	0.8	5.6
Y308S + $NADP^+$	9.1	7.6	8.2	0.7	4.8
Y308G + $NADP^+$	9.2	7.8	8.3	0.7	5.0

The changes observed for the isotropic hyperfine coupling constant in the FNR mutants, although small, are similar in magnitude to those described in equivalent positions of flavosemiquinones upon substrate binding and also to those above described upon modification of the aromatic residues in the close environment of the isoalloxazine ring in *Anabaena* flavodoxin. Finally, no changes were detected in the interaction parameters when flavodoxin was reconstituted with either lumiflavin or riboflavin, suggesting that the electron density distribution in the semiquinone form is not influenced by the phosphate or ribityl portion of the flavin [35].

3.2 Cholesterol Oxidase

Cholesterol oxidase from *Brevibacterium sterolicum* is an enzyme that catalyzes the oxidation of 3β-hydroxysteroids having a double bond at Δ5–Δ6 of the steroid ring backbone [23, 37]. The anionic semiquinone, obtained by reduction of cholesterol oxidase by sodium dithionite or by light irradiation, was characterized by EMR techniques. The EPR spectrum centred at $g = 2.004$ and with a line width of 1.48 mT, either in H_2O or ^2H_2O, was characteristic of a flavin anionic semiquinone. The line width decreased to 1.43 mT when the pseudosubstrate dehydroisoandrosterone was added. ENDOR spectroscopy provided more detailed information about the interactions of the flavin radical with protons. A group of signals, with couplings of 9–12 MHz, was attributed to protons in 8-CH_3 ($|a| = 10.9$ MHz) and on C6H ($|a| = 9$ MHz) of the flavin ring. When the protein was studied in ^2H_2O, the 8-CH_3 hyperfine couplings decreased by 0.98 MHz in the presence of dehydroisoandrosterone ($|a| = 9.92$ MHz). Hyperfine couplings were also detected in the matrix region of the ENDOR spectrum, some of which disappeared when the protein was transferred to ^2H_2O or when

the substrate was present [37]. These signals are attributed to displaced water hydrogens, or to exchangeable hydrogens from amino acid residues on the protein near the flavin binding site, involved in accommodation of substrate. ESEEM and HYSCORE spectra showed the presence of ^{14}N nuclei magnetically coupled to the paramagnet [23, 34]. A strong coupling was shown to be produced by N10 of the flavin ring, and two weaker interactions were assigned to either N1 or N3 of the flavin ring. Density-functional theory calculations allowed a more confident assignment [38, 39]. The surroundings of the flavin ring were shown to be accessible to solvent and to pseudosubstrate binding, which produced changes in the coupling to nitrogen nuclei [23, 34]. These changes were interpreted as changes in the electron density distribution of the flavin ring system, involving an increase in electron density on the pyrimidine ring at the expense of the benzenoid ring upon substrate binding. This is in good agreement with the X-ray structures reported for cholesterol oxidase in the presence and in the absence of a bound steroid [40]. These structures show that the binding pocket contains 13 water molecules, 12 of which are lost when the substrate binds, and indicate that the flavin is involved in substrate binding through H bonds from the hydroxyl group of the steroid to O4 and N5 of the flavin ring, producing an electron-withdrawing effect on the isoalloxazine ring.

3.3 4-Hydroxybutyryl-CoA Dehydratase

The neutral semiquinone of the 4-hydroxybutyryl-CoA dehydratase from *Clostridium aminobutyricum* was also investigated by EMR techniques [41]. It catalyzes the reversible dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA involving the cleavage of an unactivated C–H bond at the β-carbon. This enzyme is unusual in that, although the overall reaction catalyzed is not an oxidation–reduction, it contains FAD and a [4Fe-4S] iron-sulfur cluster. It has been proposed that the very difficult abstraction of the C3-proS hydrogen of the substrate would be facilitated by transient oxidation to an enoxy radical by FAD. The flavin semiquinone radical of the enzyme was produced by partial reduction with dithionite. Its EPR spectrum showed rapid electron-spin relaxation, indicating a magnetic interaction with the [4Fe-4S] cluster, which has zero spin in the ground state but magnetic excited states. ENDOR spectroscopy revealed the typical couplings to the 8-CH_3 and C6H protons of the flavin ring. On addition of the substrate, a significant electron density increase was observed at the 8-position. This observation indicated an interaction of 4-hydroxybutyryl-CoA with the flavin, as required by the currently proposed mechanism [41]. The crystal structure of 4-hydroxybutyryl-CoA dehydratase has recently been determined and a mechanism proposed [42]. The binding site for the substrate is predicted to lie between the flavin and the [4Fe-4S] cluster. The cluster has a histidine ligand which might act as a base to abstract the substrate proton. Oxidation of the substrate by FAD during the catalytic cycle would produce the flavosemiquinone anion, which is consistent with the formation of this species in the ENDOR experiments.

4 Other Flavoenzymes

4.1 DNA Photolyase

Some of the most advanced EMR studies on flavin semiquinones have been described by Kay, Weber and colleagues on the flavin-dependent DNA photolyases [38, 43–47]. These enzymes catalyze the photochemical repair of pyrimidine dimers in DNA, which are themselves induced by UV/visible irradiation. The neutral semiquinone radical in photolyase from *Escherichia coli* was studied by ENDOR at X- and W-band. The coupling to the 8-CH_3 protons was unusually small, indicating a nonpolar environment for the flavin. At W-band, the *g*-factors of the flavin radical could be resolved, allowing the components of the hyperfine tensor to be obtained by orientation selection. The *z*-axis was assumed to be perpendicular to the plane of the flavin ring. Also couplings to the two ribityl CH_2 protons at the 1-position in the ring could be detected, and the orientation relative to the ring plane was calculated using the McConnell relation. All the couplings to protons in the ring were assigned, and their signs were determined by TRIPLE resonance [43]. The results were supported by density functional calculations [38]. Binding of the substrate cyclobutane pyrimidine dimer caused small shifts in the proton couplings to the ring protons [38]. By comparison between couplings at the N5 position in the flavins in ^1H_2O and in ^2H_2O it was possible to discern small differences in dipolar couplings, attributed to the very small differences between the N–^{1}H and N–^{2}H bond distances [45, 47]. Similar results were obtained with another enzyme of this class, the (6–4) photolyase from the frog *Xenopus laevis* [46].

4.2 Chorismate Synthase

Chorismate synthase catalyzes the 1,4-elimination of phosphate and the C-(6-pro-R) hydrogen from 5-enolpyruvylshikimate 3-phosphate (EPSP) to generate chorismate, an intermediate in the shikimic acid pathway for synthesis of aromatic amino acids [48]. The enzyme needs FMN for activity, which is weakly bound to the free enzyme. Addition of substrate or the substrate analogue (6R)-6-fluoro-EPSP enhances binding of FMN, and the formation of a neutral flavin radical [49]. The ENDOR spectrum is typical of a neutral semiquinone and changes in response to addition of the substrate chorismate.

4.3 Dihydroorotate Dehydrogenase

Studies have recently been reported on the neutral flavin radical in the iron-sulfur flavoprotein dihydroorotate dehydrogenase B from *Lactococcus lactis* [50]. This enzyme contains FAD, FMN and a [2Fe-2S] cluster, which have the appropriate redox potentials to form an intramolecular electron transport chain. The

semiquinone of one of the flavins in the dithionite-reduced protein showed a strong spin–spin interaction with the reduced [2Fe-2S] cluster, as seen in the EPR spectrum, which precluded ENDOR analysis. The other flavin produced a semiquinone with the properties of a neutral radical. The similarity of the hyperfine parameters of this semiquinone to that of a free flavin in aqueous solution suggested that the flavin is hydrogen-bonded to the protein.

5 Conclusions

The detailed information that may be obtained about the electronic structure of flavin radicals in flavoproteins and flavoenzymes (Fig. 4) has wide implications in understanding their biochemical functions [4]. Biochemists would like to know about any effects of substrate binding on the interaction between the flavin and the protein; and, for flavins involved in oxidation–reduction reactions, the mechanisms of electron transfer, and the sites at which electrons and protons enter and leave the flavin molecule. Higher electron density at particular positions in the flavin ring, in particular the N5 and 8-CH_3 positions, may indicate a propensity for electron transfer at these positions. Water protons are important in flavoprotein mechanisms. For example, in order to maintain charge neutrality, reduction of the flavin by electrons usually involves transfer of protons.

For flavin-containing enzymes, it would be extremely interesting to observe hyperfine interactions between the flavosemiquinone and nuclear spins in the sub-

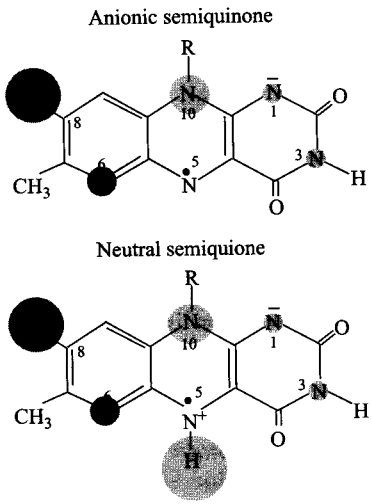

Fig. 4. Structures of the neutral and anionic flavin semiquinone states. Numbers indicate the positions on the isoalloxazine ring where nuclear hyperfine interaction parameters with the unpaired spin have been identified either by ENDOR (dark grey circles) or 2D-HYSCORE (light grey circles) in flavoprotein semiquinones.

strates. So far, with the exception of water protons, such interactions have been difficult to observe. However the binding of substrate manifests itself in a number of other ways. Hyperfine couplings to water molecules in the substrate-binding site can be observed in the matrix region of the ENDOR spectrum (Fig. 2) and they may be displaced when substrate binds. Binding of substrates near the flavin has also been observed to alter the hyperfine coupling to nuclei in the flavin ring. This has been interpreted as a shift in electron density across the flavin ring. Alternatively, it may indicate a change in solvent polarity, a smaller hyperfine coupling indicating a less polar environment [43]; a possible explanation in some cases may be that it indicates a shift between the anion and neutral radical, i.e., a shift in the pK_a of N5. In general the magnitude of the C6H and N5H coupling constants is greater for the radical anions than for the neutral radicals [21].

ENDOR may be used to explore other catalytic functions of flavins. There are a variety of flavoproteins for which the overall reaction catalyzed is not an oxidation or reduction, although in some cases redox processes may be involved in the mechanism [3]. The semiquinone flavin radical may be generated in these proteins and used as a probe of the active site, even when the semiquinone may not normally take part in the catalytic cycle.

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