Electron spin echo envelope modulation studies of the semiquinone anion radical of cholesterol oxidase from *Brevibacterium sterolicum*

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Abstract The electron spin echo envelope modulation (ESEEM) technique of pulsed EPR spectroscopy was applied to the anionic semiquinone of the cholesterol oxidase flavin cofactor, formed when the enzyme was photoreduced in the presence of 5-deazariboflavin and EDTA. Fourier transforms of the three-pulse ESEEM spectra showed the presence of ^{14}N nuclei magnetically coupled to the paramagnet. In $^{2}H_2O$ buffer the surroundings of the flavin ring were shown to be accessible to solvent exchange, with a deuterium population in close proximity to the paramagnetic centre. Upon binding of the pseudosubstrate, dehydroisoandrosterone, subtle changes were observed in the coupling to nitrogen nuclei, which are interpreted as changes in the electron density distribution of the flavin ring system. The results are discussed in terms of the three-dimensional structure reported for the protein and the flavin ring architecture.

Key words: Cholesterol oxidase, *Brevibacterium sterolicum*; Electron spin echo envelope modulation; Flavoprotein semiquinone; Hyperfine interaction; Pulsed EPR spectroscopy

1. Introduction

Electron spin echo (ESE) techniques have been shown to be useful for gaining qualitative and quantitative information on the structure around paramagnetic centres in biological systems. Structural data on paramagnetic centres in biological systems have been obtained by analysing electron spin echo envelope modulation (ESEEM) from various types of nuclei [1–3]. ESEEM is a pulsed EPR method which is particularly sensitive to weak hyperfine interactions between paramagnetic centres and quadrupolar nuclei such as ^{14}N or ^{2}H. Electron spin echoes are induced by the application of two or more resonant microwave pulses; the amplitude of the echo shows a periodic variation as a function of the interval between the pulses. Fourier transformation of the modulation pattern reveals the frequencies of nuclei coupled to the paramagnetic centre. These frequencies can then be interpreted to determine superhyperfine and electric quadrupolar couplings. The method has been particularly applied to transition-metal centres in proteins [4,5], and to some radicals such as in amine oxidases [6] and the neutral semiquinones of the flavoproteins ferredoxin:NADP reductase and flavodoxin [7].

Cholesterol oxidase (3β-hydroxysteroid oxidase, EC 1.1.3.6) is a flavin-dependent enzyme that catalyses the oxidation and isomerisation of steroids having a double bond at Δ^5–Δ^6 of the steroid ring backbone and a 3β-hydroxy group [8,9]. The enzyme from *Brevibacterium sterolicum* is a monomeric oxidase containing one molecule of FAD per molecule of protein [10]. Its three-dimensional structure has been determined at 1.8 Å resolution, in the presence and absence of a bound steroid [11,12]. It is related to the glucose-methanol-choline (GMC) oxidoreductases [13,14], which undergo the flavin-assisted oxidation of an alcohol to an aldehyde or ketone function. Based on structural observations of cholesterol oxidase bound to a steroid substrate, speculations have led to two possible mechanisms for flavin-assisted oxidation, a radical-mediated mechanism and a hydride-transfer mechanism [12]. Both mechanisms suggest a histidine residue, conserved in glucose oxidase and other GMC oxidoreductases, as the general base, accommodating one proton from the steroid substrate. Moreover, a transient flavin semiquinone would be expected in the case of a radical mechanism. The formation of the cholesterol oxidase anionic semiquinone from *B. sterolicum* has been studied and this redox state has been characterized using EPR and ENDOR spectroscopy [15]. It can be generated chemically or photochemically, but not by substrate addition.

A study of nuclear hyperfine couplings in protein-bound flavins is of interest to determine whether any correlation exists between the spin distribution about the isoalloxazine ring and the functional and oxidation-reduction properties of flavoproteins. ENDOR spectroscopy of cholesterol oxidase has already provided information regarding the coupling of the 8-CH_3 group and 6-C proton [15]. In the present paper we have used ESEEM to investigate the distribution of the unpaired electron density on the flavin ring, and its accessibility to the solvent and the substrate. ESEEM spectra were collected for enzyme samples in H_2O, $^{2}H_2O$ and H_2O in the presence of dehydroisoandrosterone, a steroid substrate which is more water-soluble than cholesterol.

2. Materials and methods

Cholesterol oxidase was isolated from *B. sterolicum* and purified using the methods described by Uwajima et al. [10]. Samples were transferred into the desired buffer (usually 10 mM Bicine, pH 8.6 in $^{1}H_2O$ or $^{2}H_2O$), by dilution and ultrafiltration through Centricon 30 microconcentrators (Amicon), at 4°C.

Cholesterol oxidase was reduced anaerobically by light irradiation as previously described [15]. Samples (400 μM protein) were prepared

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Abbreviations: EPR, electron paramagnetic resonance; ENDOR, electron-nuclear double resonance; ESEEM, electron spin echo envelope modulation; GMC, glucose-methanol-choline; NQR, nuclear quadrupole resonance; η, asymmetry parameter; e^2qQ, quadrupole coupling constant; A, hyperfine coupling constant; ν_i, nuclear Zeeman frequency

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in a sealed glass vessel under argon and transferred under argon with a gas-tight microsyringe into quartz EPR tubes, which were immediately frozen in liquid nitrogen. Samples for ESEEM were illuminated for 75 min when they were prepared in H_2O or for 240 min in ^2H_2O.

Pulsed EPR measurements were recorded at X-band on a Bruker ESP380 spectrometer, with a dielectric variable-Q resonator. ESEEM data were collected at 3.8 K by the three-pulse, stimulated echo procedure (90°-τ-90°-*T*-90° echo). τ was varied in the range 112–480 ns, so that the τ-suppression behaviour of the resolved modulation frequency components could be examined [16]. The time-domain spectra (echo amplitude vs. *T*) were linear phase corrected before Fourier transformation, and magnitude spectra were calculated.

3. Results

3.1. Electron spin echo envelope modulation of cholesterol oxidase in its semiquinone state

ESEEM was applied to the flavosemiquinone of cholesterol oxidase in H_2O, ^2H_2O and in H_2O in the presence of the substrate dehydroisoandrosterone (1.5 mM). Measurements on the enzyme in H_2O at a magnetic field corresponding to g=2.005, showed modulations due to the matrix protons (14.7 MHz) (not shown). The depth of these modulations was diminished in samples exchanged into ^2H_2O-enriched buffer, or in the presence of the substrate. These 'matrix' protons weakly coupled to the semiquinone have been previously studied by ENDOR [15]. Spectra recorded at τ values between 112 and 480 ns, revealed strong, low-frequency modulations between 0.6 and 4.8 MHz, the most prominent component being at 3.04 MHz (Fig. 1). At τ-values of 136 ns and 296 ns, modulations due to the weakly coupled protons were suppressed and amplitudes were obtained for the low frequency (<10 MHz) lines were more intense. In paramagnetic proteins, such low-frequency modulations are characteristically observed as the result of couplings to ^{14}N nuclei.

Fig. 1A shows a Fourier transform of the ESEEM of cholesterol oxidase semiquinone, recorded at a τ value of 136 ns. Narrow low-frequency components are observed at 0.55, 1, 2.19 and 3.04 MHz. There are also broader features in the region 3.9–4.9 MHz. At a τ value of 296 ns some of these features have decayed and are no longer detectable.

The spectrum from interaction with a ^{14}N nucleus is the result of the combined effects of electron-nuclear hyperfine, nuclear Zeeman and nuclear quadrupole interactions. A particularly pronounced ESEEM pattern occurs in the special case where the hyperfine and nuclear Zeeman terms are equal, when the quadrupolar peak frequencies and line widths reach minimum values, and their modulation amplitudes attain a maximum [16,17]. For strictly isotropic hyperfine interactions, this situation occurs when $\nu_i = |A/2|$, where ν_i is the nuclear Zeeman frequency at the observing magnetic field and A the hyperfine coupling constant. This is the condition of exact cancellation between the nuclear Zeeman and hyperfine terms

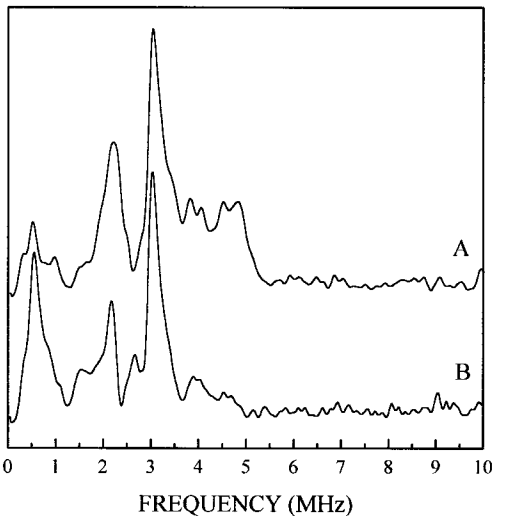

Fig. 1. Fourier-transform ESEEM spectra of cholesterol oxidase from *Brevibacterium sterolicum* in its semiquinone state at 346 mT magnetic field (corresponding to $g_x = g_y = g_z = 2.005$). Measurement conditions were: temperature, 3.7 K; microwave frequency, 9.71 GHz; τ-value, (A) 136 ns, (B) 296 ns; pulse width (for a 90° pulse) 16 ns; shot repetition time, 31 ms.

in one manifold of the nuclear hyperfine interaction. The observable spectral features in such a case comprise three quadrupole frequencies ν_o, ν_-, ν_+ ($\nu_+ = \nu_o + \nu_-$) from the compensated manifold and the double-quantum peak, which is often broader, from the other manifold [17]. The frequencies of the pure quadrupolar peaks ($\nu_o = e^2qQ\eta/2$, $\nu_- = e^2qQ(3-\eta)/4$, $\nu_+ = e^2qQ(3+\eta)/4$) permit a straightforward and direct determination of the asymmetry parameter (η) and the quadrupole coupling constant (e^2qQ). The double-quantum peak would permit the calculation of the hyperfine coupling, A_{iso}, since its frequency is given by

$$\nu_{dq} = 2[(\nu_i + |A_{iso}|/2)^2 + (e^2qQ/4)^2(3+\eta^2)]^{1/2}$$

The lineshape of the ν_{dq} component is a function of the anisotropy in the superhyperfine tensor. Model calculations of ESEEM spectra from nucleus I = 1 have shown that the quadrupole frequencies can be estimated, within 10% accuracy, if $|\nu_i - A/2|$ does not exceed 25% of ν_i [18]. Upon going away from the exact cancellation conditions for cases where $\nu_i > |A/2|$, the lines corresponding to ν_o and ν_- broaden out and decrease in amplitude, while a less pronounced effect is observed for the ν_+.

In Fig. 1B, four lines are well resolved which fit the pattern expected for the condition of near cancellation for a nitrogen nucleus. From the frequencies determined from this and simi-

Table 1
Hyperfine (A) and quadrupolar (e^2qQ) coupling parameter for the *B. sterolicum* cholesterol oxidase semiquinone

		Transition					
		ν_-	ν_+	ν_{dq}	η[a]	A_{iso}[b]	e^2qQ/h
Semiquinone	N-1	2.16	3.03	4.67	0.5 ±0.03	1.48±0.1	3.28±0.1
	N-2	1.54	2.67	3.95	0.5 ±0.05	1.0 ±0.1	2.68±0.1
±DHA		2.33	3.02	5.12	0.38±0.03	2.0 ±0.1	3.40±0.1

[a]The asymmetry parameter was calculated from a range of the ν_- and the ν_+ frequencies assuming they are near cancellation conditions.
[b]The hyperfine coupling constant was determined under the assumption that the coupling is isotropic (i.e., $A_x = A_y = A_z$).

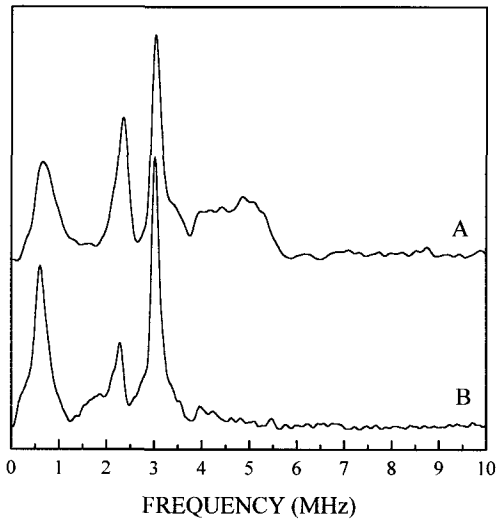

Fig. 2. Stimulated echo ESEEM spectra of cholesterol oxidase from *Brevibacterium sterolicum* in its semiquinone state in the presence of dehydroisoandrosterone. τ-value (A) 136 ns, (B) 296 ns. Measurement conditions as in Fig. 1.

lar spectra at longer values of τ, it is possible to estimate the hyperfine and quadrupole parameters of a nitrogen nucleus (N-1) (Table 1). The spectra at shorter values of τ reveal further features which appear to result from a second nitrogen nucleus (N-2). A tentative assignment of the parameters for this component is calculated on the basis of features at 0.55–1, 1.54, 2.67 and 3.95 MHz. The values obtained, as well as their variability, are presented in Table 1.

3.2. *Electron spin echo envelope modulation of cholesterol oxidase semiquinone in the presence of dehydroisoandrosterone*

Fourier transforms were obtained for data collected for cholesterol oxidase semiquinone in the presence of its substrate, dehydroisoandrosterone (Fig. 2A,B), are similar to those obtained in the isolated enzyme (Fig. 1A,B), but with some differences. The three lowest-frequency components shifted slightly to 0.62, 2.13 and 3.02 MHz, and the highest-frequency features at $\tau = 136$ ns showed a shift to higher frequencies, (between 3.95 and 5.3 MHz). The second component, N-2, was not discernible. Similar results were obtained at different τ values (not shown). The isotropic hyperfine coupling estimated for N-1 was slightly higher than the one obtained in the absence of substrate (1.95 vs 1.3 MHz), consistent with a change in the electron density distribution of the flavin ring upon substrate binding.

3.3. *Deuterium effect of the electron spin echo envelope modulation of cholesterol oxidase in its semiquinone state*

A three-pulse ESEEM study of cholesterol oxidase semiquinone which had been exchanged into ^2H_2O showed additional deep modulations, centred around the deuterium larmor frequency, 2.26 MHz (Fig. 3). This result, attributed to interaction with exchangeable deuterons, is in agreement with previous ENDOR studies which showed the presence of exchangeable protons on protein groups, and also water molecules, surrounding the FAD [15]. From the structure of cholesterol oxidase it is known that the amino acid residues that line the active site are hydrophilic near the pyrimidine ring, and that a lattice of 13 water molecules occupies the substrate cavity of the free enzyme [12].

4. Discussion

It is unlikely that ^{14}N nuclei in a protein environment would have transitions at a frequency as high as 4.8 MHz without magnetic contributions from hyperfine contact interactions [19]. Such a contact interaction could result from a nitrogen of the flavin ring. The observed hyperfine couplings (A_{iso} of 0.8–2 MHz) are less than those expected for nitrogens at position 5 or 10 of the flavin ring, typically 20 and 9 MHz respectively for an anionic flavin semiquinone [20–22]. Couplings of this magnitude are too large for detection by conventional ESEEM [23].

Another possibility is an amino acid residue, either hydrogen-bonded or in close proximity. The crystallographic three-dimensional structure reported for cholesterol oxidase from *B. sterolicum* shows that, besides the nitrogens on the flavin ring, there are several nitrogen atoms in the neighbourhood of the ribityl chain, the flavin ring system and the steroid binding domain [11,12]. The flavin ring has hydrogen-bonding interactions with a number of conserved residues, including Asn-119, Phe-487 and Met-122. There is a water molecule, H_2O-541, within hydrogen-bonding distance of ND_2-Asn-485 and NE_2-His-447, N5 of the flavin moiety and O-1 of the substrate (Fig. 4). The isotropic hyperfine coupling through a hydrogen bond would be expected to change significantly upon exchange in ^2H_2O, as ^2H in general forms weaker hydrogen bonds [19]. The ^{14}N ESEEM frequencies in the cholesterol oxidase semiquinone remain unchanged upon exchange in ^2H_2O (Fig. 3). Because acidic protons are expected to exchange readily, this argues against an amide or amino group which is hydrogen bonded to the flavin radical. A more likely source of the nitrogen interaction is a superhyperfine coupling between a nitrogen ligand from an amino acid, such as the imidazole ring of His447. The position of His-447 in the active site of cholesterol oxidase is near to that of the flavin ring system [12,24]. His-447 is believed to be involved in the oxidation reaction of the substrate, accommodating one proton from the steroid substrate. In addition this histidine is thought to release the proton to a 4α flavin-peroxide adduct in the reoxidation step of the enzyme.

Alternatively, good candidates to explain the ^{14}N ESEEM pattern are the nitrogens at positions N-1 and N-3 of the

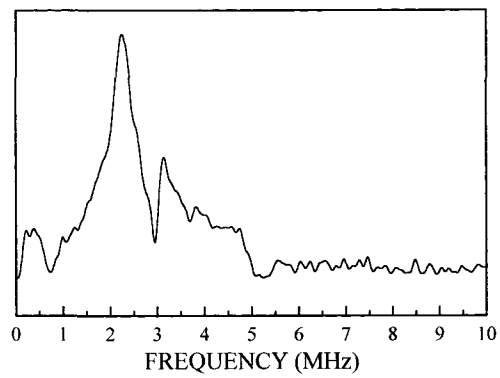

Fig. 3. Stimulated echo ESEEM spectra of cholesterol oxidase from *Brevibacterium sterolicum* in its semiquinone state in ^2H_2O; τ-value 136 ns. Other measurement conditions as in Fig. 1.

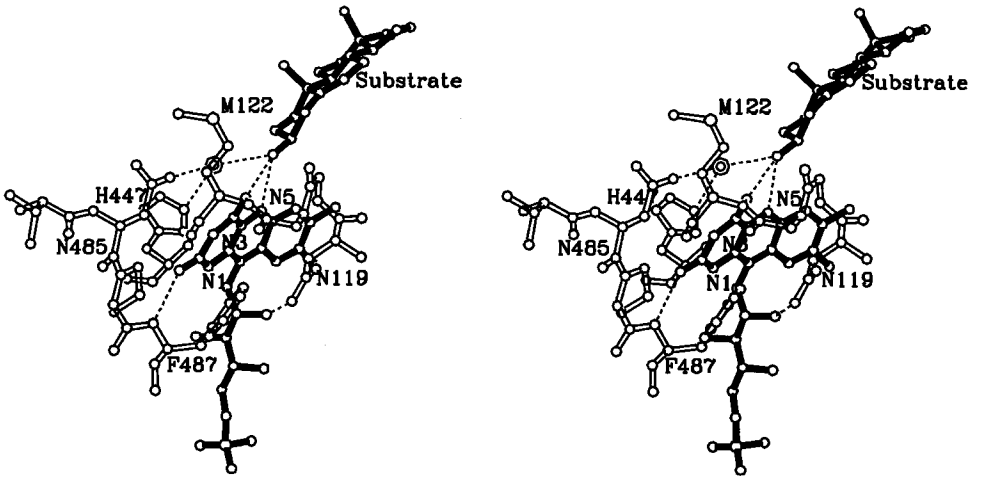

Fig. 4. Stereo diagram showing the active site of cholesterol oxidase with the bound steroid substrate, dehydroisoandrosterone. The protein residues are shown with open bonds and the substrate and riboflavin portion of the FAD cofactor are shown in closed bonds. The bound water molecule, H_2O-541 is shown as a double circle. Hydrogen bonds are shown by dotted lines.

flavin ring. So far, no hyperfine couplings have been reported for these nitrogens in model compounds. The values are expected to be very small (less than 2 MHz), owing to the almost inexistent spin density estimated for anionic flavin semiquinone models [25,26]. Unpublished electron spin echo data on flavodoxin show that the N-1 and/or N-3 nitrogens exhibit a weak coupling of the order of 1 MHz [21]. Our recent ESEEM spectra of the neutral semiquinones of the flavoproteins flavodoxin and ferredoxin-NADP$^+$ reductase from *Anabaena* have also comprised sharp lines around 3–3.4 MHz, corresponding to ^{14}N hyperfine coupling constants of 1.3 MHz [7].

Upon substrate binding, an increase of the hyperfine coupling constant was observed for N-1, while the asymmetry parameter and the quadrupolar coupling constant were not significantly altered. This result is consistent with an increased hyperfine coupling for one or more nitrogen nuclei, arising from an electronic distribution in the flavin ring. ENDOR studies on this sample have demonstrated that binding of the hydroxyl group of the steroid has a polarizing effect on the flavin ring system, decreasing the electron spin density of the benzene ring [15]. This is consistent also with our ESEEM results, if the nitrogen we are detecting magnetically coupled to the electron unpaired spin is N-1 and/or N-3 of the flavin ring, or an imidazole nitrogen atom of His-447, each of which would experience an increase in spin density in the pyrimidine ring.

ESEEM studies on *B. sterolicum* cholesterol oxidase semiquinone have provided additional information on the differences in environment and electron-density distribution in the free enzyme and the substrate-bound semiquinone. To the best of our knowledge, this is the first report of an ESEEM characterization of an anionic flavin semiquinone in a protein molecule, which complements the recent characterization of the neutral semiquinones of flavodoxin and ferredoxin-NADP$^+$ reductase. It shows that ESEEM may be used for observation of changes in electron density of flavoprotein semiquinones.

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References

[1] Mims, W.B. and Peisach, J. (1989) in: Advanced EPR: Applications in Biology and Biochemistry (Hoff, A.J., Ed.) pp. 1–57, Elsevier, Amsterdam.
[2] Dikanov, S.A. and Tsvetkov, Y.D. (1992) Electron Spin Echo Envelope Modulation (ESEEM) Spectroscopy, CRC Press, Boca Raton, FL.
[3] Britt, R.D. (1993) Curr. Opin. Struct. Biol. 3, 774–779.
[4] Britt, R.D., Zimmermann, J.-L., Suer, K. and Klein, M.P. (1989) J. Am. Chem. Soc. 111, 3522–3523.
[5] Tipton, P.A., McCracken, J., Cornelius, J.B. and Peisach, J. (1989) Biochemistry 28, 5720–5728.
[6] McCracken, J., Peisach, J., Cote, C.E., McGuirl, M.A. and Dooley, D.M. (1992) J. Am. Chem. Soc. 114, 3715–3720.
[7] Medina, M. and Cammack, R. (1996) J. Chem. Soc. Perkin Trans. 2, 633–638.
[8] Inouye, Y., Taguchi, K., Fuji, A., Ishimaru, K., Nakamura, S. and Nomi, R. (1982) Chem. Pharm. Bull. 30, 951–958.
[9] Kamei, T., Takiguchi, Y. Suzuki, H., Matsuzaki, M. and Nakamura, S. (1978) Chem. Pharm. Bull. 26, 2799–2804.
[10] Uwajima, T., Yagi, H. and Terada, O. (1974) Agric. Biol. Chem. 38, 1149–1156.
[11] Vrielink, A., Lloyd, L.F. and Blow, D.M. (1991) J. Mol. Biol. 219, 533–554.
[12] Li, J., Vrielink, A., Brick, P. and Blow, D.M. (1993) Biochemistry 32, 11507–11515.
[13] Cavener, D.R. (1992) J. Mol. Biol. 223, 811–814.
[14] Hecht, H.J., Kalisz, H.M., Hendle, J., Schmid, R.D. and Schomburg, D. (1993) J. Mol. Biol. 229, 153–172.
[15] Medina, M., Vrielink, A. and Cammack, R. (1994) Eur. J. Biochem. 222, 941–947.
[16] Peisach, J., Mims, W.B. and Davis, J.L. (1979) J. Biol. Chem. 254, 12379–12389.
[17] Flanagan, H.L. and Singel, D.J. (1987) J. Chem. Phys. 87, 5606–5616.
[18] Reijerse, E.J. and Keijzers, C.P. (1987) J. Magn. Reson. 71, 83–96.
[19] DeRose, V.J., Yachandra, V.K., McDermott, A.E., Britt, R.D., Sauer, K. and Klein, M.P. (1991) Biochemistry 30, 1335–1341.
[20] Edmondson, D.E. (1985) Biochem. Soc. Trans. 13, 593–600.
[21] Edmondson, D.E., Müller, F., Schaub, F. and Nisimoto, Y. (1990) in: Flavins and Flavoproteins (Curti, B., Ronchi, S. and Zanetti, G., Eds.) pp. 67–72, Walter de Gruyter, Berlin.

[22] Kurreck, H., Bock, M., Bretz, N., Elsner, M., Kraus, H., Lubitz, W., Müller, F., Geissler, J. and Kroneck, P.M.H. (1984) J. Am. Chem. Soc. 106, 737–746.
[23] Mims, W.B. and Peisach, J. (1978) J. Chem. Phys. 69, 4921–4930.
[24] Vrielink, A., Li, J., Brick, P. and Blow, D.M. (1994) in: Flavins and Flavoproteins (Yagi, K., Ed.) pp. 175–184, Walter de Gruyter, Berlin.
[25] Ehrenberg, A., Müller, F. and Hemmerich, P. (1967) Eur. J. Biochem. 2, 286–293.
[26] Müller, F., Hemmerich, P., Ehrenberg, A., Palmer, G. and Massey, V. (1970) Eur. J. Biochem. 14, 185–196.