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Electron–nuclear double resonance from flavin free radicals in NADPH dehydrogenase ("old yellow enzyme")

Electron spin resonance (ESR) spectroscopy is a valuable method for studying paramagnetic species in enzymatic systems, although the spectra often are poorly resolved due to anisotropic broadening[1]. Electron–nuclear double resonance (ENDOR) is a useful technique for determining proton hyperfine couplings of organic free radicals in solution[2]. Recently it has been shown that meaningful ENDOR signals can be obtained from frozen radical solutions[3]. Especially favourable are methyl groups attached to α-carbons of π-electron radicals. In most rigid media, methyl groups rotate sufficiently rapidly at 77°K and higher so that all three protons are equivalent. The average methyl coupling is described by a tensor of cylindrical symmetry with anisotropy amounting to about 10% of the isotropic coupling[4]. We have found that couplings to methyl groups of flavin radicals and radical chelates in frozen solutions can be determined with ENDOR[5]. In this note we discuss the extension of these ideas to flavin coenzyme radicals in a flavoprotein.

NADPH dehydrogenase ("old yellow enzyme") (NADPH:(acceptor) oxidoreductase, EC 1.6.99.1) has a molecular weight of about 104 000 and contains two FMN per molecule, but no metals. Enzyme flavin radicals can be produced in several ways. We have here employed anaerobic illumination of the NADPH dehydrogenase solution in the presence of EDTA[6]. The optically clear sample contained about 0.5 mM enzyme and 10 mM EDTA in 20 mM Tris buffer (pH 9.0). A strong ESR absorption was detected at −150°C. No determination of the spin concentration was carried out. The spectrum showed no indication of shoulders but exhibited some asymmetry. No doubt some small amount of FMN is in solution. The dissociation constant of NADPH dehydrogenase into the apoenzyme and FMN, oxidized[7] and reduced[8], under the conditions applied here may be assumed to be smaller than 10^{-8} M. Moreover, at a pH of 9.0 the radical yield of FMN in solution is near a minimum[9], amounting to one percent. Hence we can neglect the contribution from FMN radicals not attached to the enzyme. The enzyme-bound FMN radicals are of the "red" type at pH 7 (ref. 6). From a light absorption study of model compounds it was inferred that this species is the anionic radical[9].

The ESR spectrum of the frozen enzyme sample saturates with microwave power somewhat more readily than frozen solutions of flavin free radicals. NADPH dehydrogenase and other metal-free flavoproteins should therefore be very suitable for ENDOR studies. The ENDOR spectrum of NADPH dehydrogenase at −160°C is shown in Fig. 1 together with the structure of the FMN anionic radical. The line at 19 MHz is a methyl signal of the type observed at this temperature for the 8-methyl group of flavin anionic radicals prepared both in water and in dimethylformamide. With ENDOR we have determined the isotropic coupling for this group in the lumiflavin anionic radical in dimethylformamide solution to be 4.1 *G*, corresponding to a frequency of 19.2 MHz (ref. 5). ESR measurements of the coupling constant of the 8-methyl group gave 4.0 *G* for the anionic[9] lumiflavin radical in dimethylformamide

Abbreviations: ESR, electron spin resonance; ENDOR, electron–nuclear double resonance.

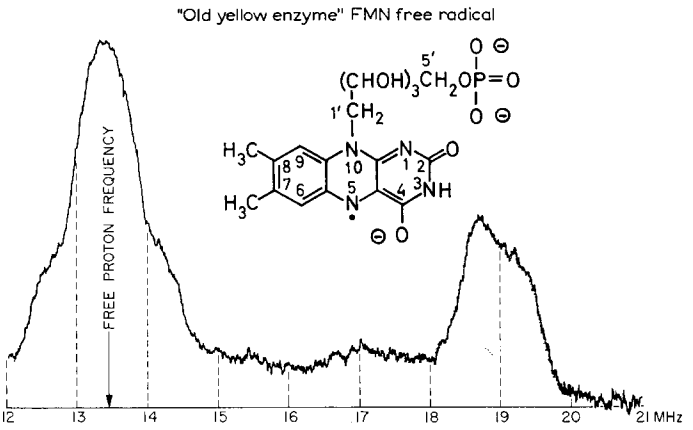

Fig. 1. ENDOR recording of FMN radicals in NADPH dehydrogenase at −160° C with about 4.7-mW microwave power incident on the matched cavity (Q approx. 10^4). 0.5 mM enzyme in 20 mM Tris buffer (pH 9.0), anaerobically photoreduced in the presence of 10 mM EDTA. The structure of the anionic FMN free radical is also shown.

and 3.0 G for the neutral* radical in chloroform. An attempt will be made to obtain direct proof for the correctness of ou r8-methyl assignment by combining NADPH dehydrogenase apoenzyme and isotopically substituted FMN. The compound [8-Me-$^2H_3$]FMN can be prepared[10]. From the shape of the ENDOR methyl signal, we conclude that the methyl group rotates with a frequency estimated to be faster than 10^7 Hz at −160°C (see refs. 11 and 12 for discussions of methyl group rotation).

A strong ENDOR signal is seen at the free proton frequency. We have observed a signal of this type in numerous frozen radical solutions and identified it as matrix-ENDOR[3]. The absorption is probably due to dipole–dipole coupling between the unpaired electron and the surrounding protons. Calculations indicate that this signal arises substantially from protons within a radius of 6 Å from the radical[3]. For flavin radicals in frozen aqueous solution, the intensity of the matrix-ENDOR is about the same as that of the methyl-ENDOR. In organic solvents the matrix-ENDOR is two to three times stronger than the methyl signal and somewhat narrower than in ice. The intensity and width of the matrix-ENDOR from NADPH dehydrogenase is similar to that found for samples of flavin radicals in polycrystalline dimethylformamide. We postulate that the matrix-ENDOR originates from the protein itself and not from solvation water. In order to elucidate this we intend to study the enzyme in ^2H_2O buffer.

The weakly coupled protons contribute to the intensity of the shoulders of the matrix signal. On the basis of studies on substituted anionic radicals and radical chelates, we assign the shoulders to the ring proton at position 9, the 7-methyl group, and the weakly coupled 1′-methylene proton.

The broad intensity in the low-frequency vicinity (centred around 17 MHz) of the methyl signal has been found only for riboflavin radicals. The exact shape depends critically on the setting of the d.c. magnetic field. We assign it to the strongly coupled

* A. Ehrenberg, P. Hemmerich, V. Massey, F. Müller and G. Palmer, unpublished results.

1'-methylene proton in a preferential orientation with respect to the g-tensor, which of course, is fixed in the flavin framework.

The ENDOR results give further proof that the radical species in NADPH dehydrogenase is the FMN coenzyme. At present there is no evidence from ENDOR for the existence of more than one type of FMN radical in this flavoprotein. For many flavoproteins the ESR spectra exhibit shoulders at room temperature. An ENDOR study of these shoulders would be of interest. We hope to extend the investigation of flavin radicals to include neutral radicals, both in solution and bound to enzymes.

NADPH dehydrogenase was purified from brewers' yeast[13]. 1 ml of sample was carefully deoxygenated at 0°C in a 6-mm internal diameter quartz sample tube. The sample was kept in an ice bath and irradiated for a total of 38 min with the parallel beam from a $f/3.5$ lens and a 500-W projector lamp. The sample tube was circulated and shaken. It was found that already 33 min were sufficient to more than half reduce the FMN. ESR spectra were recorded with a Varian V-4502 spectrometer and a V-4535 large sample access cavity at −160°C. The ENDOR spectrometer has been described previously[14]. A modified V-4535 cavity also was used for ENDOR.

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