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Effect of isotopic substitution on the electron spin resonance spectra of flavin and flavoprotein free radicals*

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SUMMARY

Electron spin resonance spectra of fully deuterated blue-green algal *(Synechococcus lividus)* flavin and flavoprotein radicals are presented and compared to spectra from ^1H radicals. The [^2H] flavoprotein spectrum indicates the utility of deuterated organisms for *in vivo* studies of this signal. Isotropic hyperfine coupling constants for [^2H] flavin mononucleotide radicals in water at neutral pH have been determined to a precision of ± 0.1 Gauss. They are (in Gauss): $A_5(N)$, 7.2; $A_{10}(N)$, 3.6; $A_5(^1H)$, 7.7; $A_5(^2H)$, 1.2.

It is now possible to isolate in pure form a number of interesting proteins of unusual isotopic composition from algae grown in heavy water containing 99.7 atom % ^2H (refs. 1 and 2). Some initial results of proton magnetic resonance (PMR) and electron spin resonance (ESR) examination of fully deuterated and isotope hybrid algal cytochrome *c*, ferredoxin, and flavoprotein have already been reported[3,4]. We wish to report here the results of ESR experiments using fully deuterated flavoprotein and the fully deuterated flavin mononucleotide ([^2H] FMN) that can be extracted from this protein. This work may be considered in part as an extension of the work of Müller *et al.*[5] in which ESR studies of neutral flavosemiquinone radicals were assisted by isotopic substitution at exchangeable positions with ^2H and with ^{15}N. We have found that the ESR spectra of the [^2H] FMN radical allows precise calculation of isotropic hyperfine coupling constants and that the [^2H] flavoprotein radical generates an unsaturated ESR spectrum with resolved hyperfine splitting whereas the unsaturated spectra of [^1H]-flavoprotein radicals are structureless[6]. This finding has important consequences for the use of [^2H] flavosemiquinone radicals as natural spin labels.

Both [^1H]- and [^2H] flavoprotein (mol. wt. 17 000) were isolated from the thermophilic blue-green alga *Synechococcus lividus* according to techniques previously

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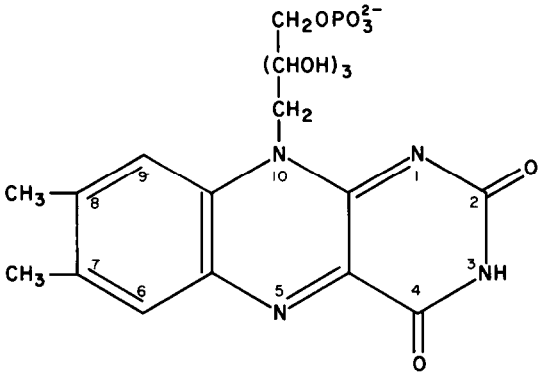
described[2,6]. The flavin prosthetic group, [^{2}H]FMN, was extracted from the pure [^{2}H]flavoprotein with aqueous trichloroacetic acid[7], neutralized, freeze-dried, desalted (Sephadex), and freeze-dried. These operations were performed in dim light. To generate the semiquinone radical, [^{1}H]- or [^{2}H]FMN was dissolved in 0.05 M sodium phosphate buffer (pH 7.0 to 7.5) and an appropriate amount of dithionite dissolved in 1 M dibasic potassium phosphate was added anaerobically (nitrogen box). Anaerobic neutral solutions of flavoprotein containing 0.05 M EDTA were irradiated with ultraviolet light to rapidly generate the flavoprotein free radical[8,9]. This reaction is completely reversible on exposure to atmospheric oxygen. ESR spectra were recorded with a Varian E-9 or V-4500 spectrometer equipped for repetitive scanning (Fabri-tek Model 1072 computer). Saturation studies were done in a rectangular TE 102 cavity (Varian) filled with a quartz dewar sleeve of the E-257 Varian variable temperature unit. The samples were contained in Scanlon S-808 aqueous solution quartz cells. Zero dB corresponds roughly to 200 mW of power and the field modulation frequency was 100 kHz. Coupling constants were stepped in increments of 0.1 Gauss to give the best fit to the experimental data. Deviations of 0.1 Gauss away from the values in Table I gave obviously poorer fits. The calculations were done using the Sigma V computer of the ANL Chemistry Division and a computer program similar to that of Stone and Maki[10].

TABLE I

ISOTROPIC HYPERFINE COUPLING CONSTANTS FOR [^{2}H]FMN* RADICALS IN WATER

System	*Group*	*Coupling constant (Gauss)*
[^{2}H]FMN in ^1H_2O	A_5(N)	7.2 ± 0.1
	A_{10}(N)	3.6 ± 0.1
	$A_5(^1H)$	7.7 ± 0.1
	$A_6(^2H)$	0.3**
	$A_8(C^2H_3)$	0.4**
	$A_{10}(C^2H_2)$	0.6**
[^{2}H]FMN in ^2H_2O	$A_5(^2H)$	1.2 ± 0.1

*The structural formula and numbering in FMN is as follows:



**From ref. 5.

In Fig. 1 we show ESR spectra of the [^{2}H] FMN semiquinone radical in ^1H_2O and ^2H_2O buffers. For comparison we also show the spectra obtained from the [^{1}H] FMN semiquinone radical[11]. The substitution of ^{2}H at all non-exchangeable proton positions results in such a large decrease in the hydrogen hyperfine coupling constants that the large coupling constants to N^5, N^{10}, and the exchangeable hydrogen at position 5 can be measured directly. Assignments are according to Müller *et al.*[5]. Table I summarizes our findings. The major coupling constants are calculated from our data, and the minor coupling constants are within the error limits given by Müller *et al.*[5], but our value for A_5^N is slightly smaller. The measured coupling constants of Table I for $A_5(^1H)$ and $A_5(^2H)$ differ precisely by the factor of 6.5 expected for deuterium replacement[5], which supports the validity of our measurements.

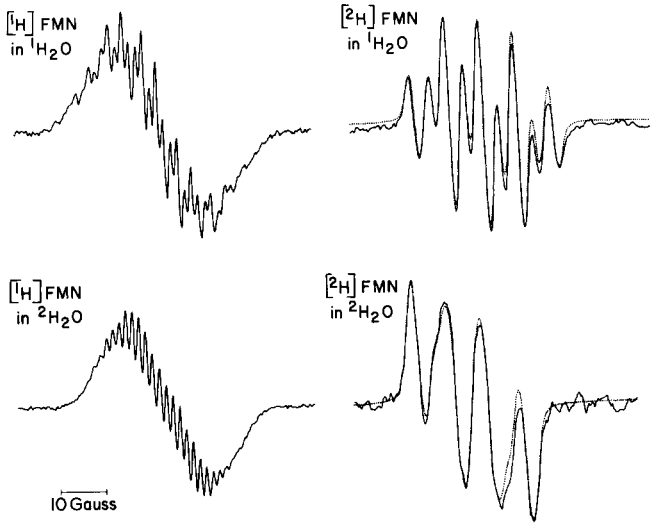


Fig. 1. ESR spectra at 25° of the flavosemiquinone radical generated by [^{1}H] FMN and [^{2}H] FMN dissolved in neutral ^1H_2O and ^2H_2O buffers. The dotted curves are the computer simulated fits to the spectra of the deuterated FMN. Although the calculated spectrum for [^{2}H] FMN in ^1H_2O has been placed slightly high, the agreement between experimental and calculated curves is excellent.

In Figs. 2 and 3 we show ESR spectra recorded at different power levels of [^{1}H]- and [^{2}H] flavoprotein in ^1H_2O and ^2H_2O. The [^{1}H] flavoprotein shows a spectrum typical of the blue, neutral semiquinone radicals described by Palmer *et al.*[12]. At low power levels, the line width is 19 Gauss, and, as the power level is increased, the radical exhibits the 'anomalous' saturation behavior described by Hyde *et al.*[13]. At high power levels the 'shoulders' of the spectrum become extremely pronounced. According to the explanation of Hyde *et al.*[13] the transitions represented by the outer spectral wings arising from the (+1, +1) or (−1, −1) nitrogen spin configurations in slowly tumbling proteins (with correlation times of 10^{-7}-10^{-8} sec) saturate less readily than the more nearly isotropic (0, 0) transitions at the central part of the spectrum. A quantitative treatment of this saturation behavior can best be investigated with [^{2}H] flavoprotein radicals.

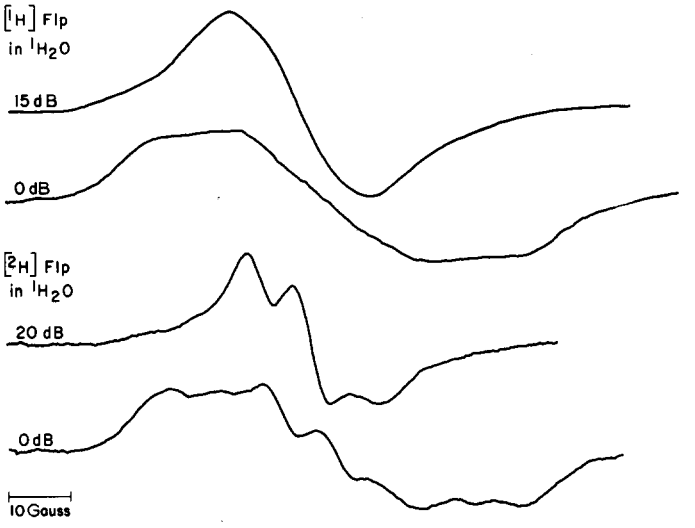


Fig. 2. ESR spectra of ^{1}H- and ^{2}H- labeled algal flavoprotein (Flp) taken in $^{1}H_2O$ buffer, pH 7, 25°. Both protein radicals exhibit anomalous saturation properties, but the saturation effect is much more amenable to quantitative study with the deuterated protein radical.

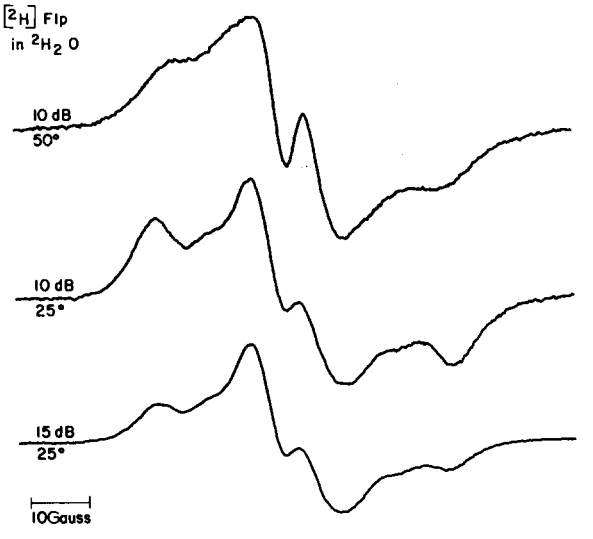


Fig. 3. ESR spectra of the [^{2}H] flavoprotein semiquinone radical in $^{2}H_2O$ at 25° and 50°. The width of this signal is 13 Gauss (peak to peak, using maximum and minimum peaks) and almost entirely due to the nitrogen splittings. At 50° a characteristic spectrum is still observed, but the saturation effect is less intense at the higher temperature. With respect to the observed saturation effects, the spectra shown above are meant to be illustrative, not quantitative.

As shown clearly by Fig. 3, the ESR spectrum of the [^{2}H] flavoprotein radical in $^{2}H_2O$ exhibits resolved hyperfine interactions in the wings of the spectrum rather than the ill-defined shoulders observed with [^{1}H] flavoprotein. The line separations approximat those expected from the model of Hyde *et al.*[13], *i.e.* $2.5\ (A_5(N) + A_{10}(N))\cdot 2$. The

coupling due to $A_5(H)$ does not readily appear in the spectra taken in ^2H_2O, as this hydrogen is easily exchangeable with the solvent water, an effect that has also been observed by others[12]. Also given in Fig. 3 is the ESR spectrum of the deuterated radical at 50°. At the higher temperature the 'anomalous' saturation effect is less intense than at room temperature probably because of the decreased correlation time of the protein. This temperature effect is not due to denaturation of the protein, as this flavoprotein is stable to temperatures as high as 80°. (The source organism grows optimally at 50°.)

The ESR properties of the fully deuterated flavoprotein radical described here make possible a number of experiments not otherwise feasible. Thus, quantitative information concerning anisotropic coupling constants can be directly extracted. The flavosemiquinone free radical is a natural spin label and can yield information on the molecular motions of the protein. Of particular interest is the fact that whole organisms and particularly photosynthetic organisms, that generate *in vivo* flavoprotein radicals are now open to direct observation when the fully deuterated cell is used because of the structure and line width due to the nitrogen couplings[14,15].

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