ENDOR STUDIES ON THE COVALENTLY BOUND FLAVIN AT THE ACTIVE CENTER OF SUCCINATE DEHYDROGENASE

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1. Introduction

Hemmerich et al. [1] recently reported that the probable site of attachment of the covalently bound FAD [2] to the peptide chain at the active center of succinate dehydrogenase (SD) is through the 8α group of the isoalloxazine ring system. This conclusion was based on a systematic comparison of acid hydrolyzed SD flavin with 8α-substituted and other model compounds in terms of EPR and optical spectroscopy, pH-fluorescence, and chemical reactivity and stability. The strongest evidence that the peptide is attached at this position comes from the nearly identical EPR hyperfine structure of the cation radical and hyposochromic optical shifts shown by SD flavin and 8α-substituted model compounds.

It was, nervertheless, desirable to confirm this conclusion by an independent method. This was done with a flavin peptide preparation [2]. In our previous studies [1] acid hydrolyzed peptides were used, which lack the amino acids but still contain a terminal group attached to the 8α carbon (cf fig. 5A for structure). It has been pointed out [3] that ENDOR (electron nuclear double resonance) is particularly suited to investigate methyl groups bonded to carbon atoms where there is a net electron spin density in the $2p_z$ orbital [4]. Hence, the ENDOR technique appears ideal for the investigation of 8α-substituted flavins.

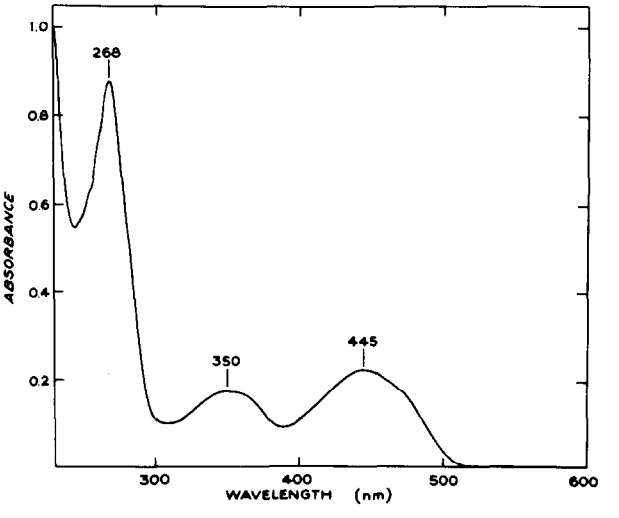

Fig. 1. Absorption spectrum of SD flavin peptide (neutral, FMN level).

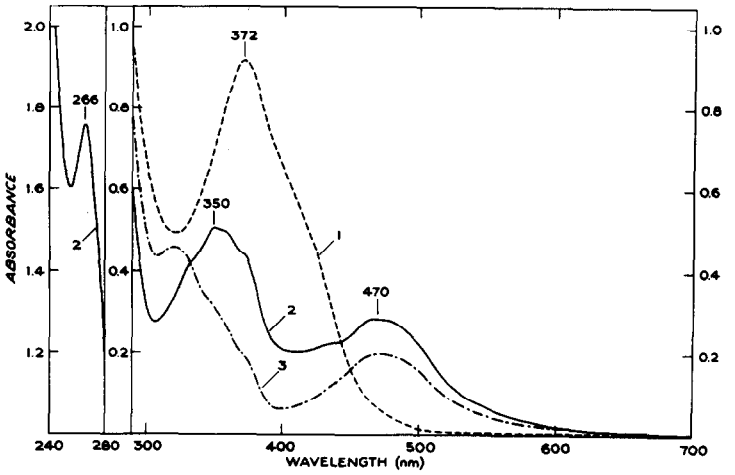

Fig. 2. Absorption spectrum of SD flavin peptide in protonated form at various stages of reduction. Curve I, oxidized (in 6 N HCl); Curve II, after addition of slightly less than I e equivalent of Ti^{+3} (radical cation not yet completely formed); Curve III, after further addition of Ti^{+3} (radical cation slightly overreduced).

2. Materials and methods

In order to avoid the breakdown of covalently bound flavin to normal flavin noted in our earlier work [1] in which SD flavin was directly extracted from submitochondrial particles, in the present study purified, soluble enzyme was the source of SD flavin. In these preparations the SD flavin is very stable [2]. SD was extracted by the method of Bernath and Singer [5] but ETP, rather than mitochondria, was used as the starting material and precipitated with 50% saturated $(NH_4)_2SO_4$. SD flavin peptides were extracted by the method of Kearney [2], hydrolyzed to the FMN level (5% trichloroacetic acid, 38°, 12 hr), and purified on Florisil and Sephadex G-25, followed by chromatography on Dowex-50, NH_4^+ cycle and DEAE cellulose, acetate cycle [2]. Details of this revised procedure will be published later.

The 3-methyl-lumiflavin, 10-methyl-isoalloxazine, 8α-morpholino-3-methyl-lumiflavin and 8-nor-8-chloro-lumiflavin were gifts of Prof. P.Hemmerich. Radical cations were produced by reduction under He with Ti^{+3} in 6 N HCl. Upon freezing, these solutions formed a glass. The microwave dielectric loss of frozen HCl is greater than, for example, that of frozen solutions of proteins in ice [4]. This required the use of somewhat smaller sample tubes in order that the microwave cavity Q not be degraded too much. The X band cavity oscillated in the cylindrical TE_{013} mode, the sample tubes were 5 mm I.D., except for the riboflavin sample where a 7 mm I.D. tube was used. The flavin concentrations were about 1 mM, and the operating temperature for the ENDOR spectra was -160°.

Optical spectra were recorded in Thunberg type cuvettes with a Cary 14 spectrophotometer and ENDOR spectra with a Varian E-700 System.

3. Results and discussion

In order to permit comparison of the material employed in our EPR studies [1] with the pure hexapeptide [2], the absorption spectrum of the flavin peptide used here is shown in fig. 1 (neutral oxidized form) and fig. 2 (oxidized cation and radical cation forms). Although the sample was not completely free from extraneous peptides, their content was relatively low as judged by the absorption at below 400 nm wave lengths. These peptides do not interfere with ENDOR studies. The fluorescence emission ratio of the sample at pH 7/pH 3.4 was 0.05, indicating the complete absence of normal flavins. As seen in fig. 2, on titration of the oxidized cation with Ti^{+3} the band at 372 nm disappears and a new band at 470 nm, characteristic of the radical cation of SD flavin, appears, in accord with previous observations of Hemmerich [6]. From comparison

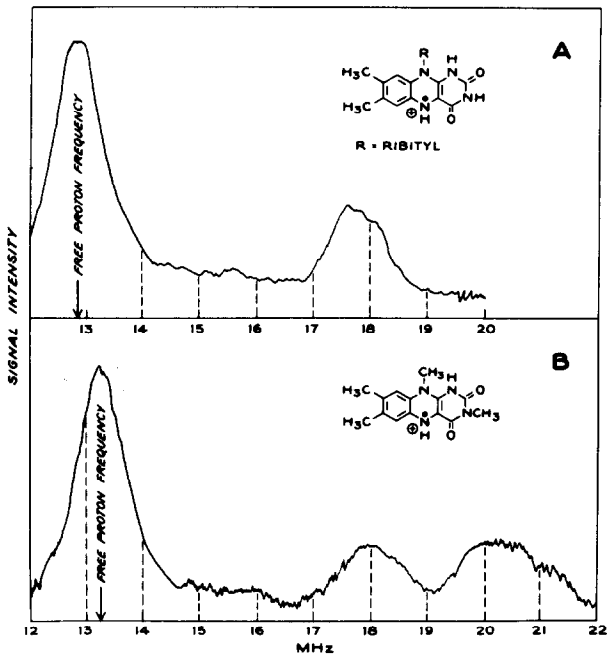

Fig. 3. ENDOR spectra of riboflavin (A) and 3-methyl-lumiflavin (B) in 6 N HCl.

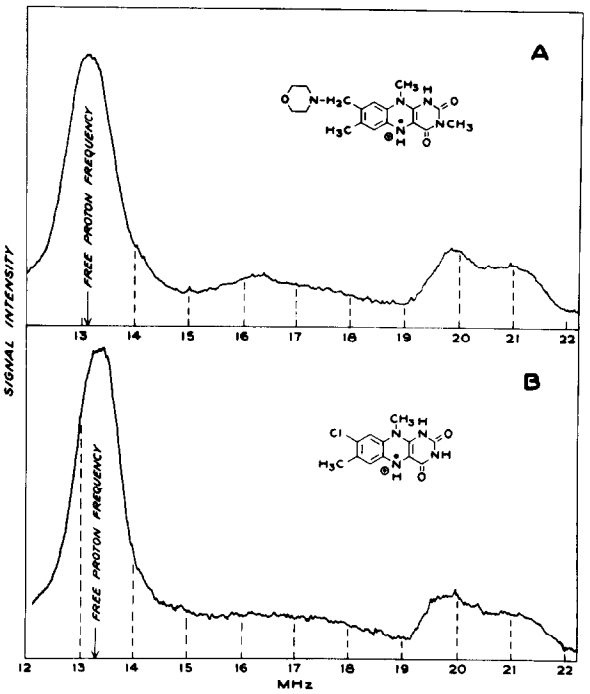

Fig. 4. ENDOR spectra of 8α-morpholino-3-methyl-lumiflavin (A) and 8-nor-8-chloro-lumiflavin (B) in 6 N HCl.

of the absorbance of the oxidized cation and of the cation radical, the radical yield of the SD flavin in 6 N HCl appears to be about the same as of riboflavin in the same solvent.

So far the only published and analyzed ENDOR spectrum of a flavin cation radical is that of lumiflavin in formic acid (Eriksson et al. [4]). Although the hyperfine coupling of the methyl group is slightly shifted in 6 N HCl to higher frequencies, the assignment of the various signals of 3-methyl-lumiflavin (fig. 3B) is unambiguous. The intense signal at 13.25 MHz is called the matrix-ENDOR [3] and is due to protons in the vicinity of the radical. The signals centered at about 18 and 20 MHz, which are 4.75 and 6.75 MHz from the free proton frequency, correspond to the 8-CH_3 and 10-CH_3 groups, with couplings of 3.4 G and 4.8 G, respectively. These coupling constants are in fair agreement with those previously determined by EPR [7]. The 3- and 7-CH_3 groups contribute to the shoulders of the matrix-ENDOR since they have very small hyperfine couplings. Riboflavin shows only the intense signal at 4.75 MHz from the free proton frequency, corresponding to the 8-CH_3 group (fig. 3A). Ehrenberg et al. [4] also observed this strong ENDOR signal from the 8-CH_3 group of the FMN radical in photoreduced NADPH dehydrogenase, where, of course, the flavin is not covalently bonded to the apoenzyme. Comparison of the ENDOR spectra of riboflavin (fig. 3A), 3-methyl-lumiflavin (fig. 3B), 8α-morpholino-3-methyl-lumiflavin (fig. 4A), 8-nor-8-chloro-lumiflavin (fig. 4B) and 10-methyl-isoalloxazine (fig. 5B) reveals that the methylene protons in 10- and 8- position of riboflavin and 8-morpholino-lumiflavin, respectively, do not give a strong ENDOR signal. The hyperfine coupling to the methylene protons is very dependent on their orientation with respect to the molecular framework, and if this orientation is not well defined the resulting ENDOR signal broadens and is usually not discernible at low temperature. Hyperfine couplings to methylene protons which are

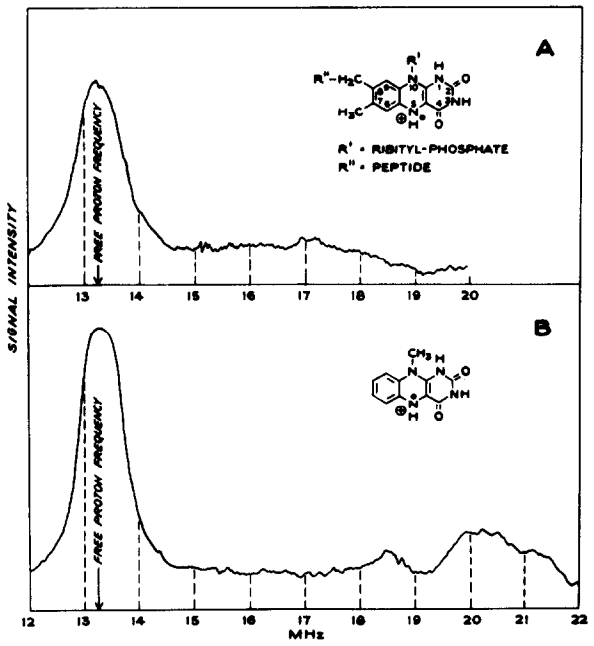

Fig. 5. ENDOR spectra of SD flavin peptide (A) and of 10-methyl-isoalloxazine (B) in 6 N HCl.

fixed with respect to the molecular framework are described by tensors of fairly low anisotropy and might in principle be detectable in frozen solution. Indeed, there is some evidence for this in the work of Eriksson et al. [4]. Nevertheless, methylene protons appear always to be less favorable for ENDOR in frozen solution than rotating methyl groups. By comparison riboflavin (fig. 3A) and 8α-morpholino-3-methyl-lumiflavin (fig. 4A) with 8-nor-8-chloro-lumiflavin (fig. 4B) we can however assume that these methylene protons contribute to the broad shoulder between 3 and 5 MHz from the free proton frequency. It has been shown [8] by EPR of riboflavin that even at room temperature the two protons in the 10α position are not equivalent: one gives a strong coupling, the other considerably smaller.

With these facts in mind ENDOR spectrum of the SD flavin peptide (fig. 5A) is readily interpretable. The signal at 6.75 MHz distance from the free proton frequency is missing because of the attachment of ribityl phosphate to the 10 position. The signal at 4.75 MHz from the free proton frequency is missing because of the attachment of the bulky peptide to the 8-CH_3 group. The broad signals between about 16 and 18 MHz *might* be due to the strongly coupled protons in the 10α and 8α positions.

These results fully confirm the assignment [1] of the peptide attachment of SD to the 8α position of riboflavin. Studies of the ENDOR spectrum of the acid hydrolyzed SD flavin are in progress.

Acknowledgements

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