OPTICAL AND ESR SPECTRA OF FLAVIN NEUTRAL AND CATION RADICALS IN UNPOLAR SOLVENTS

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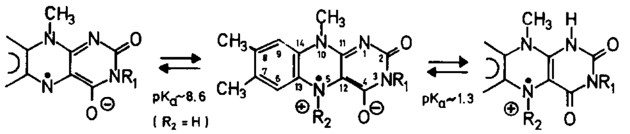
1. Introduction

Among the monomeric radical (semiquinoid) species of halfreduced flavin (=10-substituted isoalloxazine) three types have to be distinguished [1,2,3]. The two protonation stages have their pK_a at 8.6 [4] and about 1.3 [5], respectively, and each of the three radical species has its specific ESR and light absorption spectrum.



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Because of the unfavourable disproportionation equilibrium, the radical concentration in the physiological pH-range is not higher than 1–2% [4]. Under anhydrous, strongly basic conditions the flavin anion I is, however, formed quantitatively [4]. Under strongly acidic conditions, the cation radical III (R_2 = H) is formed in high yield. Using 5-substituted 1,5-dihydroflavins, obtained through light induced benzyl substitution with phenylacetate [6], we also obtain the neutral 5-substituted radical II (R_2 = $CH_2C_6H_5$) quantitatively.

We found that in unpolar solvents both the optical and ESR (electron spin resonance) spectra are particularly well resolved. Because of the low dielectric loss of these solvents we have been able to use large sample volumes in the ESR experiments thus giving a high signal-to-noise ratio. This will be of importance when studying these radicals with the ENDOR technique [7].

Detailed structures and optical spectra of the neutral radical in flavins and flavoproteins will be given elsewhere [8], as well as spin densities at some positions of the neutral [9], and the cation radical [10]. The spin density on carbon number 12 in all protonation stages will also be discussed [11].

2. Methods and Materials

Recording of the optical and ESR spectra was done as described [4]. For ESR, quartz tubes with 1 cm inner diameter were used. Syntheses of the following lumiflavin (LFI) derivatives were made: 3-ethylcarboxymethyl-LFI (IV) [12], 3-methyl-5-benzyl-1,5-dihydro-LFI (V) and 3-methyl-12-hydroxy-5-benzyl-12,5-dihydro-LFI (VI) [6]. The green colored neutral radical II (R_1 = methyl, R_2 = benzyl) was produced by dissolving anaerobically equivalent amounts of V and VI in the appropriate solvent (benzene or toluene, *pro analysi* quality). The 5-methyl or 5-deuteromethyl substituted flavin radical was obtained upon reductive alkylation of IV with CH_3I or CD_3I (Ciba AG) in analogy to description [13]. It will be shown later [8,9] that the N-5 and not the O-4 substituted dihydroflavin is formed upon this alkylation. The N-5 substituted 1,5-dihydroflavin is dissolved in the appropriate solvent and reoxidized with I_2 to maximum green color of the neutral radical. Upon addition of CF_3COOH to the neutral radical solutions, the cations are formed. The 5-unsubstituted cation radical III

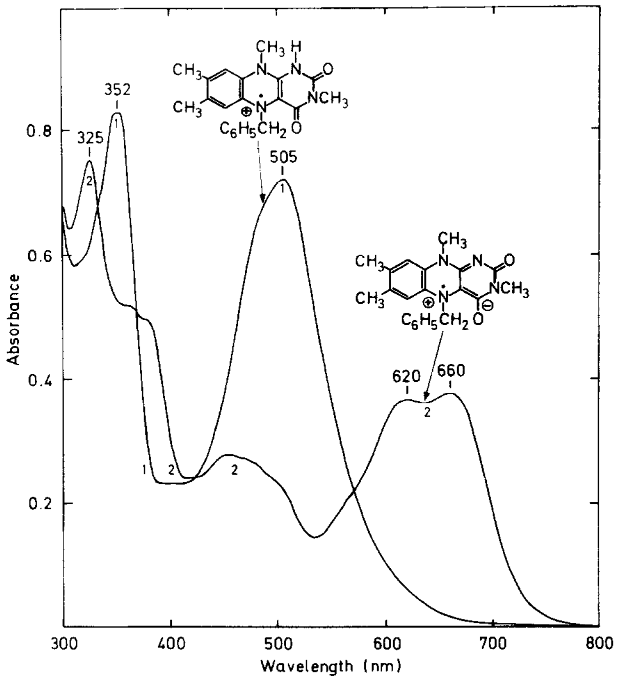

Fig. 1. Absorption spectrum of 5-benzylated lumiflavin cation and neutral radical. Curve 1: 3-methyl-5-benzyl-1,5-dihydrolumiflavin (V) and 3-methyl-12-hydroxy-5-benzyl-12, 5-dihydrolumiflavin (VI), 1 ml each in benzene were mixed with 0.2 ml CF_3COOH. End concentration 1 mM. Curve 2: Addition of 0.4 ml $(C_2H_5)_3N$ to the above solution yields the neutral radical. Light path 0.1 cm.

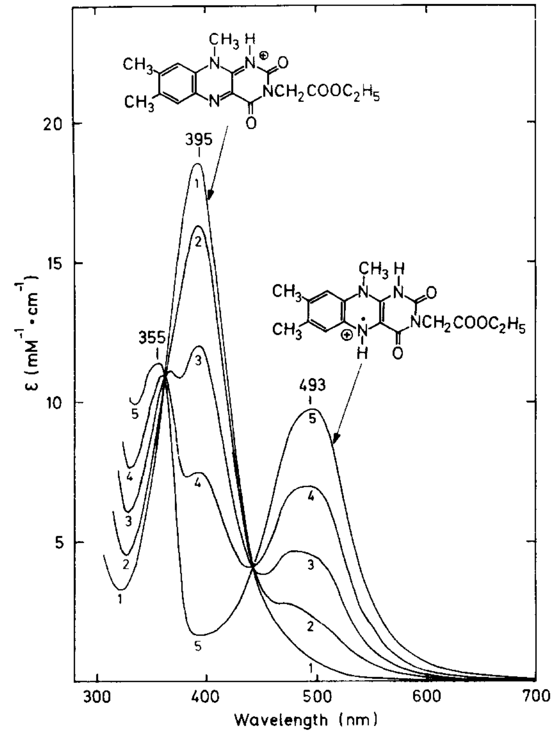

Fig. 2. Absorption spectra of the course of reduction of 3-ethyl-carboxy-methyl-lumiflavin cation in benzene + 10% CF_3COOH (curve 1) with solid $Na_2S_2O_4$ to the radical cation form (curve 5). Flavin concentration 0.44 mM, light path 0.1 cm.

(R_2 = H/D) is prepared by anaerobic reduction of IV with solid $Na_2S_2O_4$ in benzene + CF_3COOH/ CF_3COOD (Merck AG).

3. Results and discussion

The absorption spectrum of the neutral radical II ($R_1 = CH_3$, R_2 = benzyl) is shown in fig. 1 (curve 2). The solvent affects the absorption spectrum of this radical drastically. The radical is blue in water and green in benzene. The long wavelength doublet band at 675 and 623 nm in pure benzene shifts to shorter wavelength and collapses under extinction lowering with higher solvent polarity. No absorption in the near IR (800–1200 nm) was found. The 3,5-disubstituted cation radical in benzene shows two pronounced absorption bands at 505 and 352 nm (fig. 1, curve 1). Anaerobic reduction of the pale yellow oxidized flavin cation (fig. 2, curve 1) with solid dithionite gave rise to a slow change in the light absorption (fig. 2, curves 2–4). Isosbestic points are found at 362 and 444 nm. The reduction was stopped when the extinction at 493 nm reached its maximum (curve 5). The absorption at 395 nm reaches at the same time a minimum. Further reduction resulted in a colorless precipitate. The intensity of the absorption band at 493 nm and the ESR signal show a linear relationship. Therefore this band is due to the semiquinone. In contrast, the reduction of the flavin in the same solvent with Zn does not result in isosbestic points. 5-benzylation of the cation radical results in a bathochromic shift for the long wavelength absorption as well as in a decrease of extinction. By comparison with values determined for half-reduced FMN in dil. HCl [14] we found that the solvent polarity has only

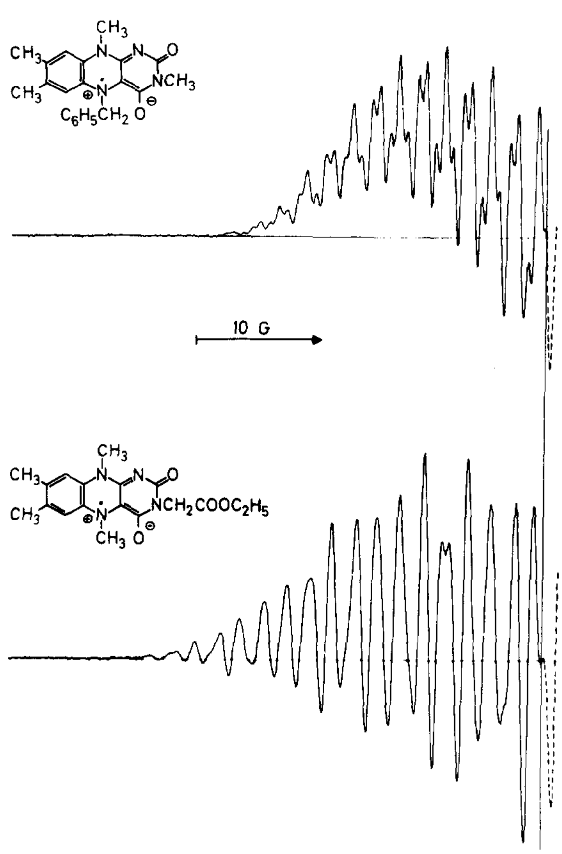

Fig. 3. ESR recordings of 3- and 5-substituted lumiflavin neutral radicals at room temperature. Radical concentration 0.5 mM, in benzene. Only the low field part of the spectrum is shown.

a minor influence on wavelength and extinction of the cation radical.

ESR spectra of neutral radicals II and cation radicals III are shown in figs. 3 and 4. The coupling constant of the α-proton attached to N-5 for the cation radical is 11.5 G. That of the β-protons of the 5-N-methyl group for the neutral radical is 8.5 G. These couplings are calculated from differences between total widths of pairs of spectra of H/D exchanged flavins, as described [4]. For the 5-benzylated cation radical (fig. 4, bottom) the outermost line is not seen at the conditions used for recording the spectrum. The proton on N-1 will not affect the ESR spectrum, since the spin density on this nitrogen is very small [15]. Comparison of the ESR spectra of the two cations in fig. 4 indicate, that only one methylene proton of the 5-benzyl group is strongly coupling, whereas the other one has a much weaker interaction. This was also found in the solvent $CHCl_3/HCOOH$[10]. The NMR spectrum of V shows also that the rotation of the methylene group is restricted [6]. The same is found to hold for the methylene group in the neutral radical from comparison of the spectra of fig. 3.

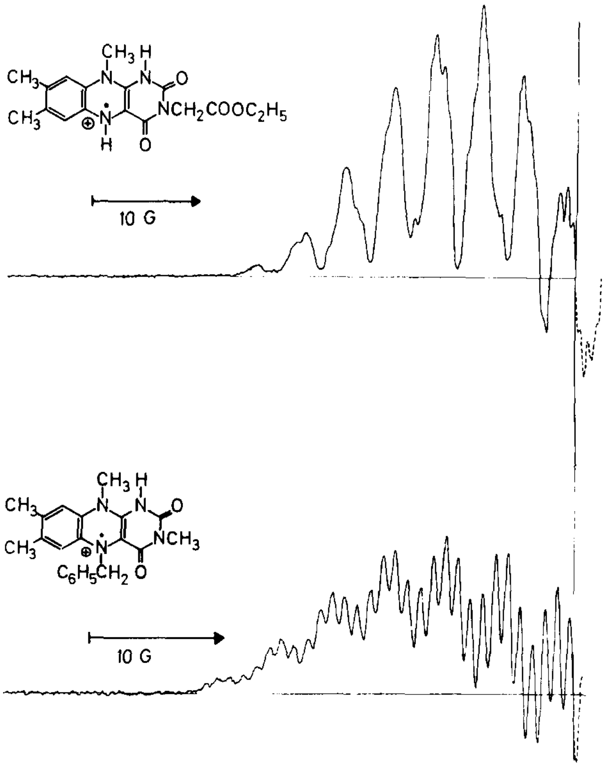

Fig. 4. ESR recordings of 3- and 5-substituted lumiflavin cation radicals at room temperature. Radical concentration 0.5 mM in benzene + 10% CF_3COOH. Only the low field part of the spectrum is shown.

Using toluene as solvent we studied the temperature effect on the hyperfine structure (hfs) of the neutral radicals in the range between −120 and +60°. Both radicals of fig. 3 show upon freezing of the solvent at −95° abolishing of the hfs due to anisotropic broadening. At about −10° there is a temperature optimum for the resolution of the hfs. Between −10 and

60° the hfs of the 5-methylated radicals were affected to a small extent. In contrast, the substructure of the ESR spectrum of the 5-benzylated radical disappeared reversibly. Since we worked with both radicals under the same conditions we can exclude a spin exchange as the cause of the change of the hfs resolution but interprete the observed effect as a result of the changed mobility of the methylene protons. The subline spacing of 0.95 G for the cation and of 0.73 G for the neutral 5-benzylated radical observed might be coincident with the coupling constant of the weakly coupling proton.

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