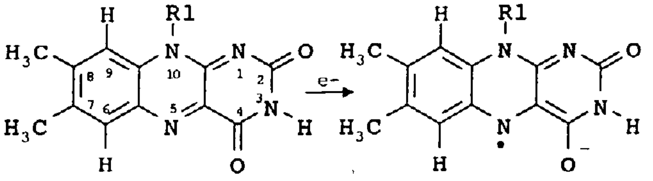
Analysis and Simulation of Anionic Riboflavin Free Radical Isotropic *EPR* *S*pectra

Boško Jovanović [1], Ljubica Vujisić [2], Ivan Juranić [3]
[1] *Center for Multidisciplinary Studies, P.O.Box 33 11030 Belgrade, Yugoslavia.*
[2] *Vinča Institute of Nuclear Sciences, P.O. Box 522, 11001 Belgrade, Yugoslavia.*
[3] *Faculty of Chemistry, University of Belgrade, 11000 Belgrade, Yugoslavia.*

Introduction

Some of the most important flavin coenzymes are derivatives of riboflavin (RF) (Fig. 1). Functioning of flavin containing enzymes is based on the changes of redox state of flavin coenzymes and very often different forms of flavin radicals are appearing as an intermediary stage (1,2). In this study we investigated anionic RF radical by the electron paramagnetic resonance spectroscopy (EPR). Isotropic EPR spectrum of riboflavin anion radical (Fig.2) is spectrum with a complex hyperfine pattern, but spectral resolution is not good enough to permit a complete analysis. Therefore, for the straightforward interpretation of the hyperfine structure of EPR spectrum of RF anion radical, which is the main goal of this study, we applied different computer methods of EPR spectra analysis and simulation. The result of such analysis is a set of possible values of spectral parameters with both false and true values presented in the set. Simulation of theoretical spectra based on the initial set and the comparison of such spectra with experimental ones, using mathematical iteration methods, is a way of arriving at true values of spectral parameters. For the simulation procedure we took into account results obtained previously (3) for the lumiflavin (LF) anionic radical. We also calculated spin densities by semiempirical methods to proof obtained values.



riboflavin (vitamin B_2):
R1=$CH_2(CHOH)_3CH_2OH$
lumiflavin: R1=CH_3
flavin mononucleotide:
R1=$H_2(CHOH)_3CH_2OPO(OH)_2$

Fig. 1. Oxidized and anionic radical forms of the some flavins.

Materials and Methods

RF was obtained from Sigma Chemical Co. and was not purified further. Preparation of alkaline aqueous solutions of RF anionic radical (pH 12) was described elsewhere (1,3). EPR spectra of anionic RF radical were recorded on a Varian E-104A EPR X- band spectrometer. Depending on the method of computer analysis applied experimental setup parameters were varied in the following range: micro wave power 2-10 mW (in this range no saturation occur); modulation amplitude 0.05-0.5 G; radical concentrations 5-10 mM. Acquisition of spectra was made on personal computer through A/D converter with scan range 100 G and acquisition time from 4 min to 16 min.

The autocorrelation, the cepstral and the CCE-SEARCH analysis of experimental spectra and simulation of theoretical one were performed using special program package PEST (4).

The methods of the molecular geometry and spin densities calculations and EPR spectra analysis and simulation for the anionic LF free radicals have been reported in our previous paper (3), results are presented in Table I. Molecular geometry and spin densities of the anionic RF free radicals have been calculated by semi-empirical AM1 method (5). Calculated values of spin densities, for both RF and LF anionic radicals were used for theoretical estimation of coupling constants.

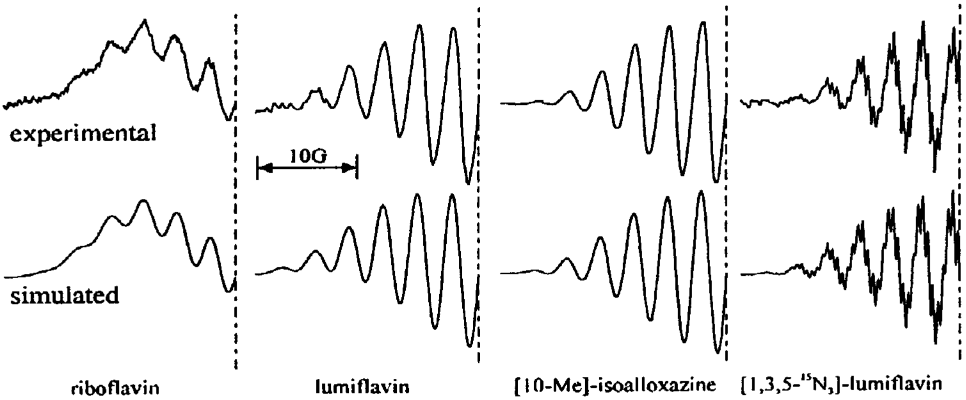


Fig.2. Left half of experimental (upper row) and simulated (lower row) EPR spectra of some investigated compounds. Spectra are in order of the increasing spectral resolution.

Results and Discussion

Substituent at the N(10) of the isoalloxazine structure is the major difference between LF and RF. In the case of RF free rotation of the ribityl chain is satirically hindered, so the two (1')-hydrogen atoms are not magnetically equivalent as in the case of the LF where all three protons of freely rotating methyl group have the same coupling constant. Calculated spin densities for LF and RF anionic radicals show that the nature of substituent at the N(10) is altering the spin distribution of the unpaired electron to the minor extent (unpaired electron is predominantly localized at the isoalloxazine structure). Thus, correct simulation and proper determination of spectral parameters of the RF's spectra can be based on the set of coupling constants obtained from the LF. In simulation procedures, we performed, the three magnetically equivalent hydrogen atoms of LF's methyl group were replaced by two nonequivalent (1')-hydrogen atoms (with unknown coupling constants) of the ribityl chain.

Results of the computer analysis and farther simulation of experimental spectra for considered free radicals are summarized on Fig.2 and in the Table I. It has been shown previously (3) that only the [1,3,..- ^{15}N] substituted lumiflavin anionic radicals have satisfactory resolved spectra for the precise determination of hyperfine couplings. Based on the set of the hyperfine couplings established from

[1,3,...- ^{15}N] substituted LFs spectra of all other lumiflavin anionic radicals (^{2}H substituted LF's and isoalloxazine derivatives) (3) and spectrum of RF were well fitted, approving estimated values. This values are in a good agreement with the literature data (Table I). Unfortunately, error of their determination is relatively high due to undetectable small hyperfine couplings (<0.2 G) from N on positions 1 and 3 which are not considered in spectra simulation. Moreover, coupling constants estimated from calculated spin densities (Table I) are in very good agreement with literature and computer analysis and stimulation data. All values of calculated coupling constants are determined from p_z spin densities, accept the values for the RF (1')-protons which are obtained from the LF AM1-UHFQ H(1s) spin densities (* in Table I). This can be assumed while the results of stationary calculated molecular geometry for both RF and LF show that the part of ribityl chain replaces one LF methyl hydrogen while the other two are on the same position comparing isoalloxazine structure. The range of values for the calculated coupling constants (Table I) is determined by the limits of spin polarization parameters (Q) found in the literature (2). Q is proportionality constant connecting hyperfine couplings with an adequate p_z spin densities.

Table I. Coupling constants of the lumiflavin and riboflavin anion radicals.

Source of data	radica	The values of the hyperfine coupling constants in Gauss [G] and assignment						
		$a^N{}_5$	$a^H{}_6$	$a^H{}_{7(CH_3)}$	$a^H{}_{8(CH_3)}$	$a^H{}_9$	$a^N{}_{10}$	$a^H{}_{10(CH_3\ or CH_2)}$
computer analysis	LF	7.0±0.2	3.45±0.3	0.5±0.1	3.75±0.2	0.8±0.2	3.2±0.2	(2.7±0.2) x 3
and simulation	RF	6.8±0.2	3.2±0.4	0.5±0.1	3.7±0.2	0.8±0.2	3.3±0.2	(3.1 & 0.3) ±0.3
calculated from	LF	6.2-8.3	2.5-3.3	0.3-0.4	2.3-3	0.2-0.3	2.5-2.8	(2.4-2.7) x 3
p_z spin densities	RF	4.5-6.6	2.6-3.4	0.4-0.6	2.8-4.1	0.5-0.7	1.5-2.2	3.3 & 0.1 *
literature data(1,2)	LF	7.0-7.7	2.9-3.9	< 0.3	3.7-4.2	0-1.5	2.6-3.6	(2.8-3.5) x 3

References:

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Apstrakt

Izotropni EPR spektar anion radikala riboflavina analiziran je i simuliran različitim kompjuterskim metodama. Semiempirijskom AM1 metodom izračunata je raspodela spinskih gustina u molekulu. Na osnovu dobijenih rezultata i rezultata proračuna spinskih gustina i analize i simulacije spektara anion radikala lumiflavina određeni su spektralni parametri koji uspešno opisuju eksperimentalni spektar anjon radikala riboflavina.