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Dedicated to the memory of
Professor Mircea D. Banciu (1941–2005)

THE OXIDATIVE ACTIVITY OF RIBOFLAVIN STUDIED BY LUMINESCENCE METHODS: THE EFFECT OF CYSTEINE, ARGININE, LYSINE AND HISTIDINE AMINO ACIDS

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Riboflavin (Vitamin B_2), one of the most important hydrosoluble vitamins, is a constitutive part of two coenzymes: flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), being involved in redox processes occuring in humans. Using chemilluminescence system luminol - hydrogen peroxide, in Tris-HCl buffer, pH=8.5, in the present study the action of different concentrations of Cys, Arg, Lys and His upon the antioxidative activity of the riboflavin has been evidenced. It was found that Cys, Arg, Lys increase while, Arg decreases the riboflavin antioxidative activity. The effect of these amino acids upon the riboflavin fluorescence has also been investigated. A reaction mechanism is also proposed and the results are discussed with relevance to the redox processes of riboflavin.

INTRODUCTION

Studies regarding fluorescence quantum yield and lifetime of riboflavin fluorescence in methanol, DMSO, water and aqueous solutions containing Methionine (Met) and Cysteine (Cys) amino acids are known.[1] It was found that in methanol, DMSO and water (pH = 4 - 8), the decrease of riboflavin fluorescence takes place due to the internal conversion and intersystem crossing processes. In aqueous solutions of Met, the static and dynamic riboflavin fluorescence quenching takes place because riboflavin anion/Met cation is formed. In aqueous solutions of Cys, one notices riboflavin fluorescence quenching with pH increase because Cys thiolat is formed. The thiol - Cys form which exists at low pH does not react with riboflavin in neutral form. When pH increases, thiol-Cys reacts with riboflavin in its neutral form causing riboflavin deprotonation (anion formation).[1] Studies were also undertaken of complex between plasm - blood and primary ligands like the following amino acids: Lysine, L-ornithine, L-threonine, L-serine, L-phenylglicine, L-phenylalanine, L-glutamic acid and L-aspartic acid and riboflavin as a secondary ligand.[2]

Riboflavin itself and its derivatives, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), are involved in redox processes and recently, studies are focused on the interaction between amino acid residues and flavinic enzymes.[3-9] In this regard an interest is granted to amino acids determination by the chemiluminescence method.[10-18]

Previously, the riboflavin oxidative activity in the presence of KI, KBr and thioureea, using chemiluminescence and fluorescence methods[19] was studied. At high values of time, I^- and Br^- intensify the chemiluminescence intensity of luminol-hydrogen peroxide in Tris-HCl buffer, pH = 8.5, and when riboflavin is present in the system its antioxidant activity is annihilated by those ions. In the presence of thioureea an opposite effect was observed. KI, KBr and thiourea quench riboflavin fluorescence. The Stern-Volmer quenching constant (K_{SV}) and the rate constant of the bimolecular quenching process (k_q) have been determined. An increase of the fluorescence polarization has been observed, as a consequence of fluorescence

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lifetime decrease and a dynamic quenching mechanism by an intermolecular electron transfer process was suggested.[19] Using the same chemiluminescence reference system, the action of different molar ratios of α, β or γ-cyclodextrin has been evidenced.[20] It was found that α, β, and γ- cyclodextrin have an antioxidative effect, probably due to the free radicals (generated in the system) encapsulation in their cavity. This behaviour depends on α, β, γ-cyclodextrin molar ratio; α-cyclodextrin and γ-cyclodextrin protect more efficiently against free radicals than β-cyclodextrin.[20] The effect of different molar ratio of cyclodextrins upon the antioxidative activity of riboflavin has also been evidenced.[21] It was found that α, β or γ-Cyclodextrin present in the luminol-hydrogen peroxide-riboflavin system, in alkaline solution, leads to the increase of the antioxidative activity of riboflavin. The behaviour depends on the reaction time and on the riboflavin:cyclodextrin molar ratio, the order of improving the antioxidative activity of riboflavin being: γ -CD> α-CD>β-CD.

This work followed the previous papers[19-21] and deals with the investigation of the effect of Lys, Arg, His and Cys amino acids on the riboflavin antioxidative activity studied by chemiluminescence and fluorescence methods, in order to get more details about the redox processes of the riboflavin.

EXPERIMENTAL

Materials. The system luminol (LH_2)-hydrogen peroxide (H_2O_2) at concentrations of LH_2 =2.5 × 10^{-5} M and H_2O_2 = 30 mM in 0.2 M Tris-HCl buffer, pH 8.5 was considered the reference system. LH_2 and H_2O_2 are from Merck and the Tris buffer from Sigma. The amino acids His, Cys, Lys, Arg are from Aldrich. Solutions were prepared in distilled water. 5, 5-dimethyl-1-pyrolin-N-oxide (DMPO) is from Sigma and it was dissolved at a final concentration of 0.2 M.

Methods and apparatus. The chemilluminescence (CL) measurements were undertaken with a chemilluminescence measuring device TD 20/20 Turner Design, USA. The points on the plot were obtained by integrating the light signal on periods of 4 seconds. Five measurements were made and an average value calculated, obtaining a maximum 10% relative scattering of the results from the mean value. The working volum was 1000 µl.

The extinction of the CL emission, S, was calculated according to the equation:

$$S\ (\%) = \frac{(I_0 - I)}{I_0} \times 100$$

where I_0 and I represent CL intensity measured for the reference system and for the reference system in the presence of His, Lys, Cys and Arg, respectively; both values were measured 5s after the beginning of the reaction.

The fluorescence spectra (emission and excitation) were recorded with Perkin Elmer 204 spectrofluorimeter, interfaced to a computer, permitting a prestabilized reading time of the data. Usually the range between two measurements is 550 ms.

The ESR spectra were recorded with a JEOL, JES-FA 100 spectrometer (X band frequencies). The spectrometer settings used for the experiments in Figure 3b were as follows: microwave power, 0.998 mW; modulation amplitude, 0.7 G; the sweep width, 150 G; field modulation frequency, 100 KHz; time constant, 0.3 s; sweep time 480 s, receiver gain 2×10^5, number of data points, 8192.

RESULTS AND DISCUSSION

Chemilluminescence studies

The experiments followed the riboflavin (RF) antioxidative activity in the presence of Cysteine (Cys), Lysine (Lys), Arginine (Arg) and Histidine (His), using the chemiluminescence generating system luminol-hydrogen peroxide in alkaline solution, Tris-HCl buffer, pH = 8.5. For this purpose, two aminoacid types were chosen: one with a polar hydrocarbonated fragment (R), Cys aminoacid, and another with a positively charged fragment at a physiological pH, Arg, Lys and His amino acids.[1] In this regard, an interest is granted to the nature of the interactions between the moieties of the aminoacids mentioned above and RF, interactions which depend on the electric charge of these moieties and their hydrophobic character.

Using a molar ratio RF:Aminoacid of 1:1, the antioxidative activity of RF is strongly influenced. In Fig. 1 the antioxidative effect of RF is observed, this behaviour resulted from the decrease of chemilluminescence intensity, compared to that of the reference system. At the same time, one can notice that in the presence of His, the antioxidant activity of RF increases, while the presence of Arg and Lys aminoacids attenuates its antioxidative activity. It can be noticed that function of reaction time, especially at the beginning of the

reaction (first minute range) the RF antioxidative activity is decreased, while after this period its antioxidative activity increases. The increasing of chemilluminescence intensity for about 1 min from the beginning of the reaction could be due to the effect of oxygen reactive species, generated in the luminol-hydrogen peroxide system, which react more quickly with Arg and Lys amino acids and oxidise them, thus generating new free radicals that contribute to the increase of the chemilluminescent signal. The decrease of the chemilluminescence intensity in time, under the RF signal, corresponds to the neutralization of these free radicals formed in the oxidation process of these aminoacids by RF. The Cys aminoacid contributes to the increase of the antioxidative activity of RF.

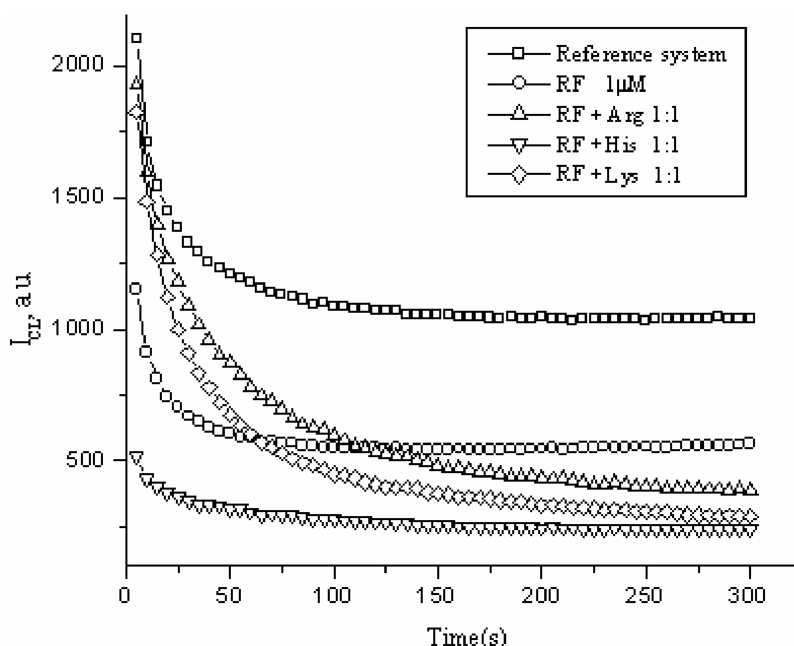


Fig. 1 – The CL emission kinetics produced by LH_2 (2.5×10^{-5} M) - H_2O_2 (30 mM) reaction, in Tris - HCl buffer 0.2 M, pH = 8.5 (reference system) in the presence of riboflavin and riboflavin - amino acid mixture.

Using the same RF:Cys molar ratio, the efficiency of the signal chemiluminesce extinction (S%) by Cys is about 100% (Table 1). The result proves the antioxidative activity of RF in the presence of Cys aminoacid is improved. This behaviour is due to the Cys antioxidant effect. As a result, in the luminol-hydrogen peroxide/RF, Cys has a positive influence on the redox process. The same behaviour was observed in the case of His and Lys amino acids (Fig. 2 A,B). The increase of Arg concentration has an effect on antioxidative activity of RF (Fig. 3a). 10 μM Arg concentration significantly increases the chemiluminescence intensity, completely attenuating the RF antioxidative activity. This could be explained by the RF catalytic effect on the reaction that produces the $NO^{\bullet}$ free radical, in the luminol-hydrogen peroxide/Arg/RF system, in Tris-HCl buffer, pH = 8.5, fact which could mean amino acid degradation, as a subsequent source for $NO^{\bullet}$ free radical.

Table 1

The CL changes in the LH_2 - H_2O_2 system, in Tris - HCl buffer, pH = 8.5, in the presence of RF and Cys

System	I_{CL}, a.u.	S, %
LH_2/Tris – HCl pH 8.5/H_2O_2	2099	-
LH_2/Tris – HCl pH 8.5/H_2O_2 / 1 μM RF	1153	45. 06
LH_2/Tris – HCl pH 8.5/H_2O_2 /1 μM RF/ 2.5 μM Cys	34. 30	98. 36
LH_2/Tris – HCl pH 8.5/H_2O_2 / 1 μM RF/ 5 μM Cys	2. 23	99. 89
LH_2/Tris – HCl pH 8.5/H_2O_2 / 1 μM RF/ 10 μM Cys	1. 63	99. 92

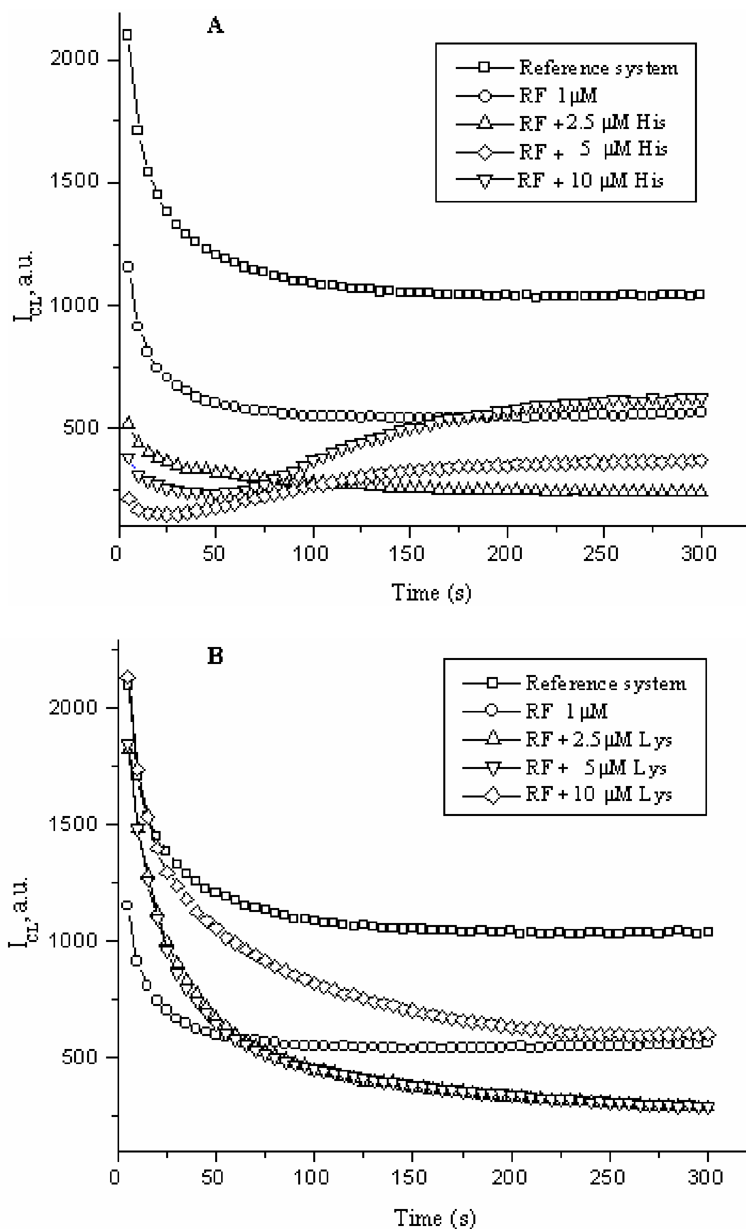
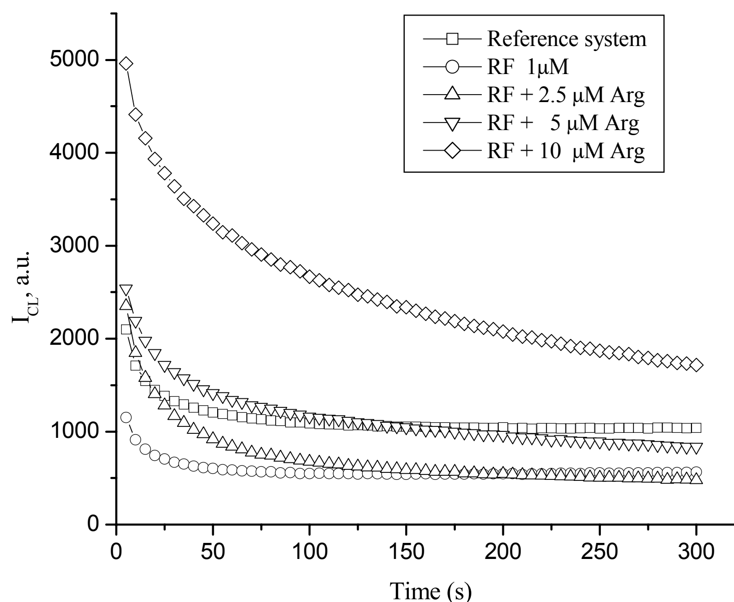


Fig. 2 – The effect of the His (A) and Lys (B) on riboflavin antioxidative activity; the CL generating system (reference system) is the same as previously.

In order to prove the generation of $NO^{\bullet}$ free radical in the luminol-hydrogen peroxide/Arg/RF system, in Tris-HCl buffer, pH = 8.5, electronic spin resonance was undertaken, using the EPR spin trapping technique. For this purpose 5, 5-dimethyl-1-pyrolin-N-oxide (DMPO) was used as a spin trapper, a less toxic nitrone and more easily convertible in nitroxide-more stable free radicals adduct types.[22] Fig. 3b shows the presence of the nitroxide free radicals in the luminol-hydrogen peroxide/Arg/RF system.

The spectrum could be simulated as a sum of two existent species: an DMPO-$HO^{\bullet}$ adduct with hyperfine splitting constants $a_N = 14.98$ G, $a_H = 14.57$ G and another one corresponding to a degradation product, a nitroxid type with $a_N = 14.69$ G. The degradation of DMPO-$HO^{\bullet}$ giving the nitroxid is fast, the percentage of degradation product found from simulated spectra being 83%, the difference corresponds to DMPO-$HO^{\bullet}$ adduct, 17% respectively. We supposed the generation of $NO^{\bullet}$ in the oxidation process of Arg amino acid. The fast degradation of DMPO adduct could be attributed to a faster oxidation of Arg amino acid. Probable, this process does not permit the trapping of $NO^{\bullet}$ radical, even when nitroxid-nitrone was used.

Fig. 3a – The variation of Arg concentration on riboflavin antioxidative activity in the CL generating system.



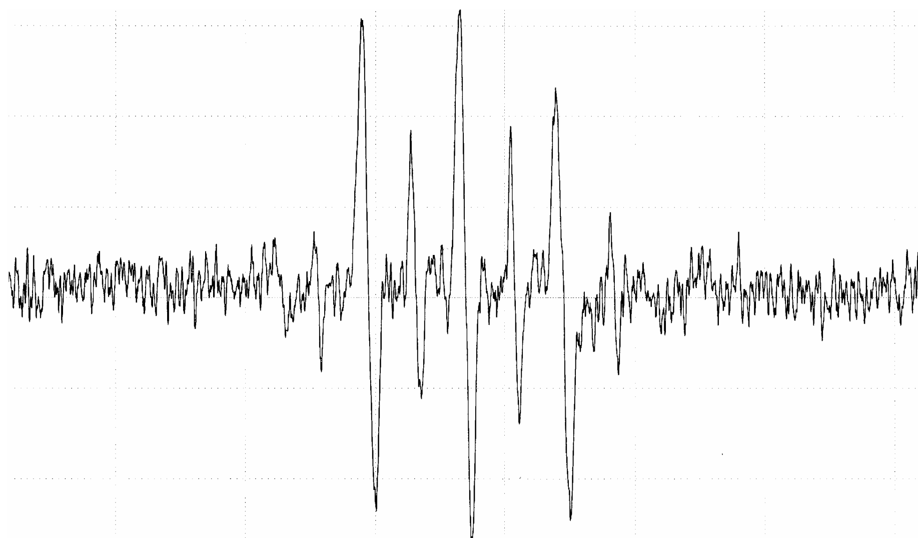


Fig. 3b – The ESR spectrum of DMPO - LH_2 - H_2O_2 - Arg - RF mixture, in Tris - HCl buffer, pH = 8.5.

At the same time, by chemilluminescence study, it was found that although the peptides His-Arg and Lys-Arg, contain the Arg in their structure, RF antioxidative activity is improved. It was also found that the His-Lys peptide leads to an increased antioxidant activity of RF. As about the interference of these amino acids with luminol in the absence of RF, a strong antioxidant effect was observed, for all studied amino acids, with a percentage inhibition of about 100%.

Therefore, the order of improving the RF antioxidative activity is the following: Cys > His > Lys > Arg. In the studied luminol-hydrogen peroxide-RF-Arg system, RF has a relevance regarding $NO^{\bullet}$ free radical generation (Fig. 3b), the major role of this free radical is in neurotransmission processes as well as in the immune answer against bacteria, viruses and tumor cells.

Fluorescence studies

Studies regarding RF photosensitising action are known,[23] especially the case of photooxidation processes when energy transfer occurs to molecular oxygen, which is transformed into singlet oxygen, an oxidizing species. RF structure confers to these processes, the property of a photosensitizing.[24, 25]

The previous results based on chemiluminescence measurements were completed with RF fluorescence measurements in the presence of Cys, Lys, Arg, His aminoacids.

It was found that in the presence of Lys and Arg aminoacids, the RF fluorescence intensity significantely decreases (the results are not shown), this decrease being correlated with the increase of aminoacid concentration. The RF fluorescence quenching by Lys and Arg can be attributed to a dynamic process, analyzed by the Stern-Volmer equation ($I_0/I=1+\tau_0 k_q$; $[Q]=1+K_{SV}[Q]$). For very small quencher concentrations (up to 5×10^{-4} M), the variation of the ratio I_0/I versus molar quencher concentration [Q] was linear, and from the slope, Stern-Volmer constant, K_{SV}, was obtained. Then the bimolecular extinction rate constant, k_q, of the process was calculated, considering excited-state lifetime of the RF fluorescence, τ_0, being 4.64 ns.[19] Thus the values of K_{SV} =11.3 M^{-1} and k_q=$2.43 \times 10^{+9}$ $M^{-1}s^{-1}$ were obtained. The k_q value is in the range of a diffusion - controlled rate constant. In the case of Cys amino acid no more changes were evidenced in the RF fluorescence spectrum, indifferently of the Cys concentration used.

It was found that at lower concentration of His amino acid, the RF fluorescence intensity increases, while at higher concentration of His, a decrease takes place (Fig. 4a). We assumed the possibility of His oxidation as a result of a photosensitizing reaction involving the singlet oxygen, as a function of this amino acid concentration. A reaction mechanism was proposed (fig. 5). One can observe the reaction through which peroxide II can be obtained (electron transfer *vs* hydrogen abstraction), responsible for an increase of fluorescent intensity at low concentration of His. In this case riboflavin speeds its oxidation by singlet oxygen.

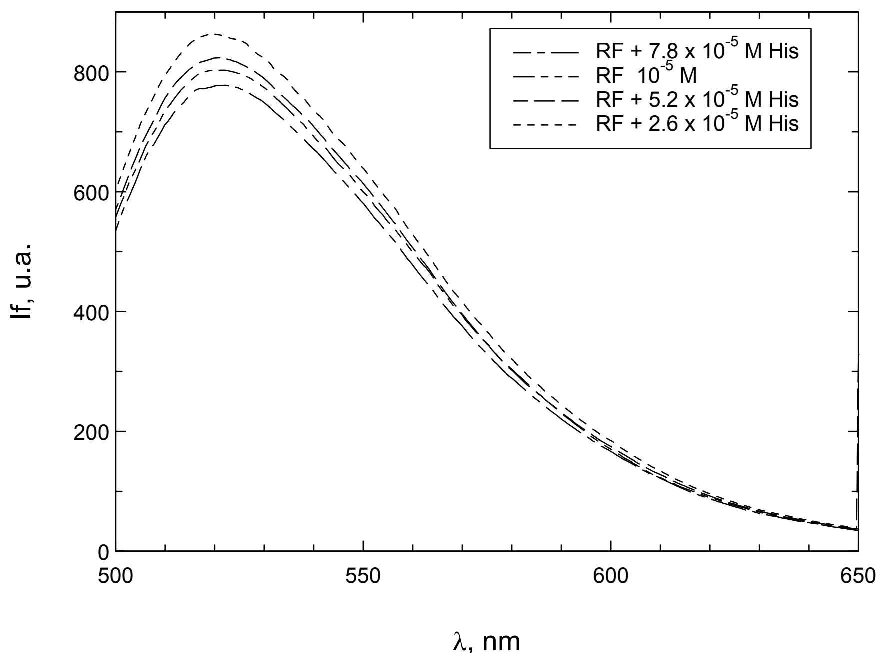


Fig. 4a – The variation of riboflavin fluorescence in the presence of His aminoacid; λ_{ex} = 365 nm.

Comparatively, in Fig. 4b, RF fluorescence spectra in the system luminol -hydrogen peroxide, in Tris-HCl buffer, pH = 8. 5, in the presence of the following amino acids Cys, Lys, Arg, His, are shown. The amino acid concentration was 2.5×10^{-5} M. In all these cases, the appearance of two emission bands was evidenced. The first band at 425 nm, specific for luminol emission, becomes more intense in the presence of Lys and His amino acids, without shifting the wavelength. This behaviour is in correlation with the previous results, these amino acids can be oxidised more quickly. Regarding the 520 nm band, characteristic for RF fluorescence emission, one can notice that the fluorescence is significantly quenched and the emission spectra is hypsocromicaly shifted in the presence of Lys and His. These are arguments supporting the mechanism proposed in Fig. 5, when RF is consumed in such a process.

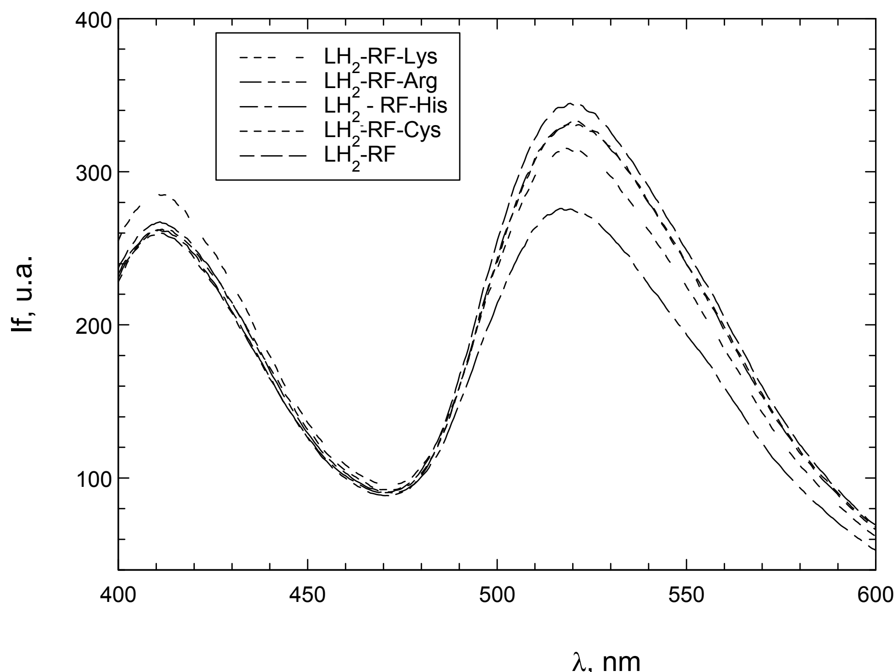


Fig. 4b – Fluorescence spectra of riboflavin in the LH_2 - H_2O_2 system, in Tris - HCl buffer pH = 8.5, in the presence of Cys, Lys, Arg and His; λ_{ex} = 365 nm.

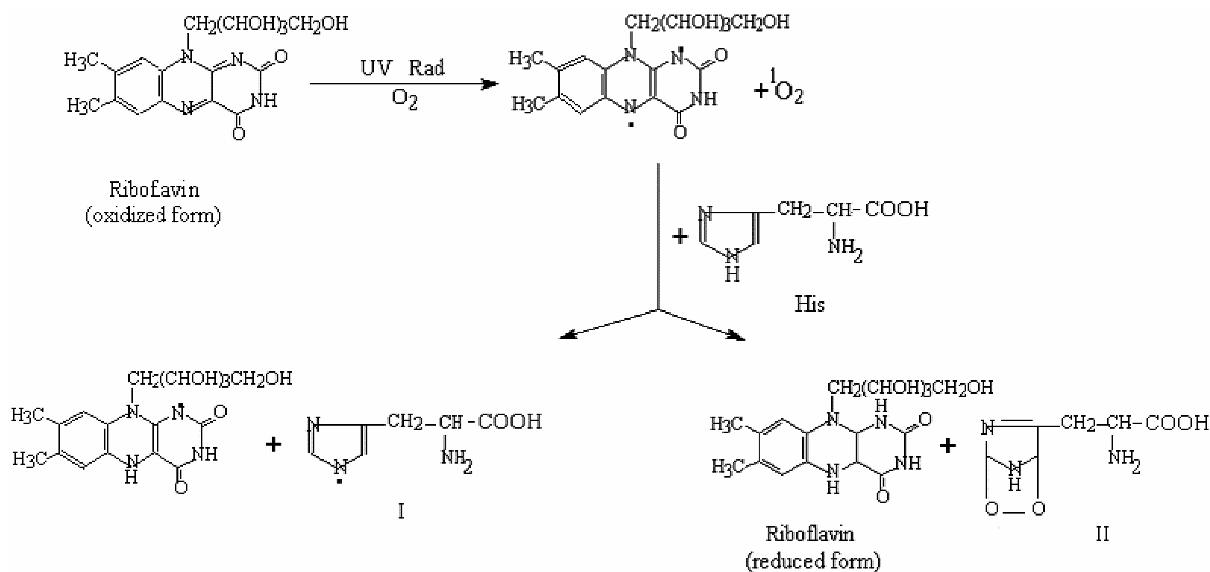


Fig. 5 – The mechanism of His photooxidation in the presence of riboflavin.

CONCLUSIONS

In this study, the oxidative activity of riboflavin *in vitro*, in the presence of Cys, Arg and His amino acids and generation of free radicals in this process were investigated by chemiluminescent, fluorescence and EPR techniques. In the system luminol –hydrogen peroxide as chemilluminescence generator system in alkaline medium (Tris-HCl buffer, pH 8.5), the antioxidative activity of riboflavin vary with the structure of amino acids in the following order: Cys > His > Lys > Arg. Generation of $NO^{\bullet}$ free radical in the presence of Arg was taken in account. The results of the present study can be relevant in understanding of some biochemical processes.

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