THE AEROBIC RIBOFLAVIN-SENSITIZED PHOTODECOMPOSITION OF TRYPTOPHAN, TRYPTAMINE AND INDOLE ACETIC ACID. GROUND-STATE AND PHOTOPROMOTED FLAVIN–INDOLE INTERACTIONS

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Received November 18, 1991
Accepted July 21, 1992

The interaction between riboflavin (RF) and the indole derivatives tryptophan, tryptamine and indole-3-acetic acid at visible light irradiation was studied in water and water/ethanol mixed solvent. Besides a dark indole–RF association, important only at indole concentration higher than 10^{-3} mol dm^{-3}, a complex picture of competitive reactions operate. Triplet excited RF is quenched by the indoles in concentrations ca 10^{-4} mol dm^{-3} (rate constants of the order of 10^{9} mol^{-1} dm^{3} s^{-1}) and by dissolved oxygen. Both, singlet molecular oxygen and superoxide anion (in a further reaction step) are produced. As a consequence the rate of indole photodecomposition decreases in order: indole-3-acetic acid > tryptophan >> tryptamine, and the rate of RF autofading suffers an important delay compared with that in the absence of the indoles.

Riboflavin (RF), in its ground and electronically excited states have been implicated in a wide range of photobiological processes[1 – 5]. This dye can also sensitize many reactions of important biomolecules[4 – 6]. Such interactions, occurring both in the dark and under the stimulus of visible light, would have important practical implications under environmental conditions.

Indole-containing substrates play important roles in the mentioned context. On this basis, we considered of a great interests to increase the knowledge about the relative weight of the so-called photodynamic effect and related aerobic mechanisms in the whole RF–indoles interaction.

There exists a great number of reports about dark-complexation[7,8] and photopromoted processes of flavins[9 – 11] in the presence of several indole derivatives, including flavin-sensitized photooxidations[5,14]. Those papers are focused on particular aspects of the complicated picture represented by the flavin–indole interaction. The aim of the present investigation is to obtain an integral idea on the aerobic sensitizing effect of RF

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and its interaction with some biologically relevant indole derivatives (indole-3-acetic acid (I_3Ac), tryptophan (Trp) and tryptamine (Trypta)). The conditions under which a given reaction mechanism operates, and the influence of the molecular structure of the indoles on the kinetics of different reaction steps, were evaluated.

EXPERIMENTAL

The indole derivatives I_3Ac, Trp and Trypta were purchased from Sigma. Riboflavin (Schuchart) was purified according to Silber et al.[15]. Furfuryl alcohol (FFA), and rose bengal (RB) (Aldrich) were employed as received. Ethanol was HPLC quality. Water was triply distilled. Phosphate buffer (for pH 7) was employed. Ground state absorption measurements were carried out using Hewlett–Packard 8452A diode array spectrophotometer. For absorbance determinations at fixed wavelength a Shimadzu UV-140-02 spectrophotometer was employed. An Aminco SPF 125 spectrophotofluorometer was used in all the stationary fluorescence measurements. The excitation and emission wavelengths were about 285 and 365 nm respectively for the indole derivatives. Fluorescence lifetime determinations were carried out using nitrogen laser (5 ns FMHW) as the excitation source and TRW filterfluorometer with output into a computer on which non linear least squares (time shift) analyses and deconvolution[16] were done. The irradiation device for static photolysis (cut off filter bellow 400 nm) including the specific oxygen electrode was described elsewhere[17].

RESULTS

The experiments were planned starting from the following reaction scheme:

$$\mathrm{RF} + \mathrm{Q} \xrightleftharpoons{K_{\mathrm{AS}}} [\mathrm{RF{-}Q}] \qquad (1)$$

$$\mathrm{RF} \xrightarrow{h\nu} {}^1\mathrm{RF}^* \qquad (2)$$

$${}^1\mathrm{RF}^* \xrightarrow{k_{\mathrm{di}}} \mathrm{RF} + h\nu' \qquad (3)$$

$${}^1\mathrm{RF}^* \xrightarrow{k_{\mathrm{IC}}} \mathrm{RF} + \mathrm{heat} \qquad (4)$$

$${}^1\mathrm{RF}^* + \mathrm{Q} \xrightarrow{k_{\mathrm{qi}}} (\mathrm{RF} + \mathrm{Q}) \text{ or } \mathrm{P}_1 \qquad (5)$$

$${}^1\mathrm{RF}^* \xrightarrow{k_{\mathrm{ISC}}} {}^3\mathrm{RF}^* \qquad (6)$$

$${}^3\mathrm{RF}^* \xrightarrow{k_{\mathrm{d3}}} \mathrm{RF} \qquad (7)$$

$${}^3\mathrm{RF}^* \xrightarrow{k_{\mathrm{P2}}} \mathrm{P}_2 \qquad (8)$$

$$^3RF^* + Q \xrightarrow{k_{q3}} (RF + Q) \text{ or } P_3 \tag{9}$$

$$^3RF^* + O_2(^3\Sigma_g) \xrightarrow{k_{ET}} RF + O_2(^1\Delta_g) \tag{10}$$

$$O_2(^1\Delta_g) \xrightarrow{k_d} O_2(^3\Sigma_g) \tag{11}$$

$$RF + O_2(^1\Delta_g) \xrightarrow{k_{qRF}} RF + O_2(^3\Sigma_g) \tag{12}$$

$$RF + O_2(^1\Delta_g) \xrightarrow{k_{rRF}} P_4 \tag{13}$$

$$Q + O_2(^1\Delta_g) \xrightarrow{k_q} Q + O_2(^3\Sigma_g) \tag{14}$$

$$Q + O_2(^1\Delta_g) \xrightarrow{k_r} P_5\,, \tag{15}$$

where the meaning of the different steps is as follows: (*1*) dark association between RF and Q (the indole derivative); (*2*) absorption of light by RF; (*3*) fluorescence; (*4*) internal conversion; (*5*) quenching of RF singlet excited state by Q; (*6*) intersystem crossing; (*7*) deactivation of RF triplet excited state; (*8*) RF decomposition; (*9*) quenching of RF triplet excited state by Q; (*10*) energy transfer to ground state molecular oxygen, ($O_2(^3\Sigma_g)$), generating singlet molecular oxygen; (*11*) solvent deactivation of $O_2(^1\Delta_g)$; (*12*) physical and (*13*) chemical quenching of $O_2(^1\Delta_g)$ by RF; (*14*) physical and (*15*) chemical quenching of $O_2(^1\Delta_g)$ by Q. The symbols P_1 to P_5 are different photoproducts. The sum $k_q + k_r$ will be named k_t (the overall rate constant for $O_2(^1\Delta_g)$ quenching.

Dark Interaction RF–Indoles

The addition of either, Trp, Trypta or I_3A to aqueous or aqueous–ethanolic solution of RF causes typical changes in the absorption spectrum of the dye. They were similar to those recently published[3] for a series of indole-3-carboxylic acids, including I_3A. The spectral changes can be better visualized or magnified by taking the difference spectra of the RF–indole system against RF under otherwise identical conditions, as shown in Fig. 1*a* for Trypta, as a typical example. The presence of isosbestic points is an indication that two species are in equilibrium. If the positive band absorbance is plotted against the indole derivative concentration, a saturation curve is obtained (curve *6* in Fig. 1*b*) in the both solvents employed.

The values of the equilibrium constants, K_{as} determined by the method of Rose and Drago[18] are presented in Table I. The straight line in Fig. 1*b* shows the best fit obtained by this procedure for RF–Trypta. Similar graphs were obtained for RF–Trp and RF–I_3A, both in water and ethanol–water (1 : 1, v/v).

Fluorescence

RF exhibits an intense fluorescence emission, at 515 nm, with the reported quantum yield of 0.25 in water[19]. In our experiments, the stationary fluorescence quenching of RF by the indole derivatives was detectable at indole concentrations higher than 1.0 mmol dm^{-3} only. The only observed effect of indoles on RF fluorescence spectrum was a decrease in the intensity of the emission maxima. Through the simple procedure of fluorescence quenching (i.e. monitoring the RF fluorescence intensity in the both absence and presence of known indoles concentration) the values of Stern–Volmer constants (K_{SV}) listed in Table I were obtained (see Fig. 1*b*). If any interaction with the fluorophore singlet excited state is absent, these constants are often straightforwardly taken as a measure of the dark complexation product stability[1].

In order to determine a nature of the fluorescence quenching by the indoles (wether it was due to an interaction with RF ground state (association), RF singlet excited state (Eq. (*4*)) or a combination of both mechanisms), the time resolved fluorescence measurements were carried out. The fluorescence lifetime of RF ($\tau_o = 1/k_{d1}$, Eq. (*3*)) was

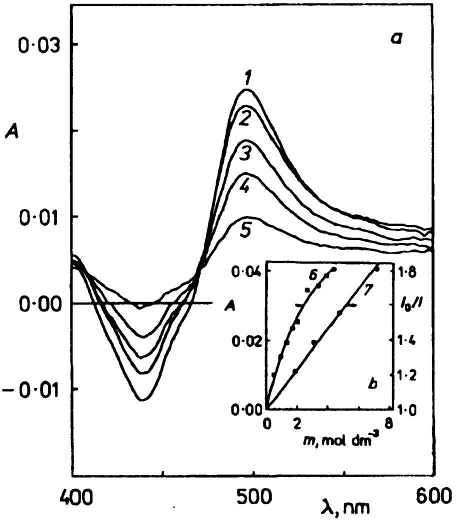

FIG. 1

Difference spectra of RF + Trypta vs RF (0.05 mmol dm^{-3}) in water at pH 7. Trypta concentration (mmol dm^{-3}): *1* 2.0; *2* 1.67; *3* 1.3; *4* 0.91; *5* 0.50. Inset: *6* corresponding saturation curve: absorbance vs Trypta concentration; *7* Stern–Volmer plot for quenching of RF fluorescence by Trypta (I_0 and I are the RF fluorescence intensities in the absence and in the presence of Trypta) ascertained to 4.1 + 0.3 ns, in excellent agreement with published data[20]. No changes in τ_0 exceeding the experimental error were detected in the presence of the indole derivatives in concentrations up to 5 mmol dm^3.

RF-Sensitized Photodegradation of the Indoles

The airated 0.25 mM indole solutions (in water) containing RF (A_{445} = 0.7) were irradiated by visible light. As the indoles do not absorb a light in this spectral region, the decrease in their fluorescence intensity, (usually up to 10%) with the irradiation time could be taken as the substrate consumption measure (see dependences shown in Fig. 2*a*). The experiments were done under the conditions in which, according to data in

TABLE I
Association constants (K_{as}, mol^{-1} dm^{-3}) of RF with several indole derivatives in H_2O and ethanol–H_2O (1 : 1, v/v) and Stern–Volmer constant (K_{SV}, mol dm^{-3}) for the stationary quenching of RF fluorescence by the indoles

Indole	K_{as} ± 10% H_2O	K_{as} ± 20% EtOH–H_2O	K_{SV} ± 5% H_2O
Trp	402	53	152
Tryp	289	150	110
I_3Ac	252	90	93[a]

[a] From ref.[3].

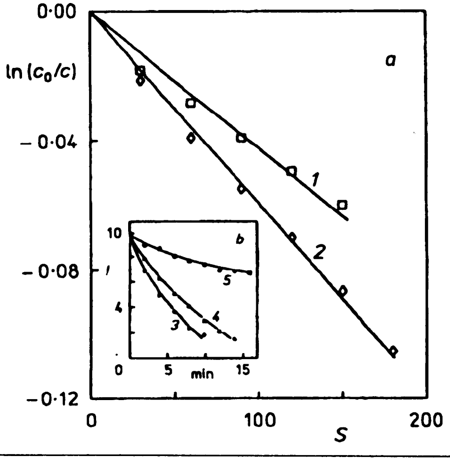

FIG. 2
First order plots for oxygen consumption upon RB-sensitized aerobic irradiation of Trypta (*1*) and FFA (*2*) in water, pH 7, both 0.2 mmol dm^{-3}. A_{560} for RB = 0.6. Inset: Fluorimetric monitoring (*I*) of indoles (0.1 mmol dm^{-3}) consumption as a function of irradiation time. Sensitizer RF (A_{445} = 0.5). *3*

Table I practically no dark association RF–indoles exists. The relative rates of Trp, Trypta and I_3A consumption (initial slopes of the curves of Fig. 2) are summarized in Table II.

Oxygen Uptake Experiments

The ability of RF to generate $O_2(^1\Delta_g)$ upon an adequate irradiation is sufficiently documented. A quantum yield Φ of 0.46 – 0.50 has been reported in water for that process[6,21]. On the other hand, the indole derivatives are known as relatively efficient $O_2(^1\Delta_g)$ quenchers[3,22,31]. The participation of the $O_2(^1\Delta_g)$-mediated mechanism in the overall photooxidation was studied in the following way: in parallel runs, under identical conditions as those described in the preceding paragraph, the relative rates of oxygen consumption were measured by means of an specific oxygen electrode (see Table II).

The k_r value (Eq. (*15*)) for Trypta was determined employing RB as a highly effective $O_2(^1\Delta_g)$ photosensitizer ($\Phi = 0.8$ in water[23]). Absolute rates of oxygen uptake were measured for the indole derivatives and FFA. The latter was used as a reference sacrificial substrate, from which the rate constant k_{rFFA} of the $O_2(^1\Delta_g)$-mediated photooxidation is well known[24] ($1.2 \cdot 10^8$ mol^{-1} dm^3 s^{-1}). The ratio of the first order plot slopes of oxygen uptake by the reference and the substrate (see e.g. Fig. 2) yields the ratio k_{rFFA}/k_r, from which a value of constant k_r can be calculated (see Table II). This method is a modification of that one employed by Foote and Ching[25]. The k_r values for Trp and I_3Ac determined by ourselves, and already published[3,26] were also included in Table II. On the basis of this data, I_3Ac is about three times a more efficient chemical quencher of $O_2(^1\Delta_g)$ than Trp, Trypta is of a mediate reactivity.

The oxygen uptake experiments with RF used as a sensitizer were also performed. Sodium azide (5 mmol dm^{-3}) was applied as the physical quencher of $O_2(^1\Delta_g)$ ($k_q = 5.1 \cdot 10^8$ mol^{-1} dm^3 s^{-1}, process (*14*), with Q = sodium azide). Under these experimental

TABLE II

Relative rates for: (i) indole consumption, v_{ind}; and (ii) oxygen uptake sensitized by RF, v_{Ox}; rate constants for reactive interaction indole–$O_2(^1\Delta_g)$ (k_r, mol^{-1} dm^3 s^{-1}) and rate constants for the quenching of $^3RF^*$ by the indoles (k_{q3}, mol^{-1} dm^3 s^{-1})

Compound	v_{ind}	v_{Ox}	$k_r \cdot 10^{-9}$	$k_{q3} \cdot 10^{-9}$
Trp	0.75	0.81	3.2[a]	8
Tryp	0.25	0.70	7.8	10
I_3Ac	1	1	9.2[b]	3[b]

[a] From ref.[26]; [b] from ref.[3].

conditions, the lifetime of $O_2(^1\Delta_g)$ in water was reduced by a factor of ca 10 in the case of I_3Ac but by a factor about 3 only, in the case of Trp and Trypta. Thus, it is clear that the oxygen uptake upon RF-sensitized irradiation does not obey the pure singlet oxygen mechanism.

Quenching of RF Photodestruction

It is known that RF photodegradation in water solutions proceeds predominantly from the triplet exited state[27] (process (*8*)). Nitrogen saturated aqueous solutions containing RF (A_{460} = 0.7) were photolyzed by visible light, and the rates of dye consumption (v_o) were determined from the rate of a decrease absorption at 460 nm. Similar experiments were carried out in the presence of different indole ([Q]), in concentrations of the order of 10^{-4} mol dm^{-3}, (the rate v). As a result, the rate of RF decomposition was drastically diminished as it is shown in the inset of Fig. 3. Within the mentioned range of concentration, the indoles interact neither with ground state nor with the singlet excited state of RF. On this basis, the reduction in the rate of RF photodegradation must be considered as a result of the long-lived RF triplet state quenching by very low concentrations of the indoles. From a simple Stern–Volmer plots (Fig. 3) the rate constant k_{q3} (process (*8*)) can be evaluated (Eq. (*16*)). The k_{q3} values are listed in Table II.

$$v_0 / v = 1 + k_{q3}\,\tau_{0,T}\,[Q]\,, \qquad (16)$$

where $t_{0,T}$ (the reciprocal of k_{d3}, Eq. (*7*)) represents the RF triplet lifetime in water, reported as 14 μs by Edmonson et al.[28].

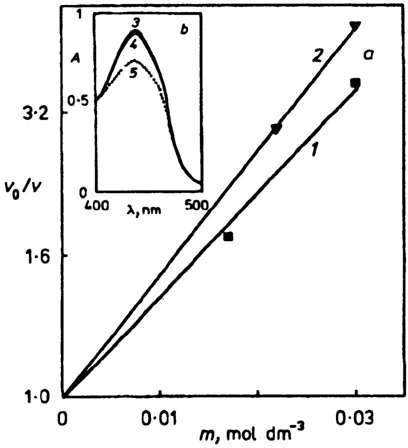

FIG. 3
Stern–Volmer plots for the quenching of anaerobic RF autobleaching (monitored by the decrease in absorbance at 445 nm) by *1* Trypta; *2* Trp. v_0 and v are the velocities of RF bleaching (see text) in the absence and in the presence of the indoles. Inset: Absorption spectra of anaerobic RF, *3* non photolized; *4* 3 min photolysis, in the presence of Trypta 0.05 mmol dm^{-3}; *5* 3 min photolysis in the absence of Trypta

DISCUSSION

Dark Complexation

In our previous paper[3], we reported about the complexation of RF with a series of indole-3-carboxylic acids that, likewise Trp, Trypta and I_3Ac, have been found as association partners of RF (refs[1,29]). In all cases, the charge-transfer mechanism was proposed as the driving force of the interaction, in which RF acts as an electron acceptor. Equimolar stoichiometry of all complexes was ascertained. However, the contribution of an hydrophobic interaction, playing an additional associative role, has been also postulated[3]. In the present case the decrease in values of K_{as}, when changing the solvent from pure water to ethanol–water mixture as well as the relatively high value of K_{as} for Trp (possessing the more hydrophobic side chain) in water observed in present study are in agreement with both, charge-transfer and hydrophobic components of interactions.

A pronounced discrepancy can be observed (Table I) between the K_{as} values (obtained by absorption spectrometry) and the K_{SV} values (determined fluorometrically). In the first case (Rose–Drago method[18]) an iterative routine was employed for the fitting of the saturation curve (Fig. 1*b*) with a simple assumption of 1 : 1 association. On the other hand, in order to consider the the Stern–Volmer constants as the actual association constants for the system RF–indoles, the strong requirement of zero fluorescence in the spectral region of the free fluorophore must be fulfilled by the complex. In this context, the K_{SV} values should be taken as a lower limit for the association constants. In our opinion the K_{as} values should be considered as more a representative measure of the complex stability. However, at least two important points arise from the data in Table I: *a*) a moderate dark association RF–indoles takes place; *b*) the complexing is only important at indole concentration substantially higher than 1 mmol dm^{-3}.

Light-Promoted Interactions

As stated in Results, RF efficiently sensitizes the photodecomposition of the analyzed indoles. On the other hand, these indoles are inhibitors of RF decomposition (by means of the competitive quenching of $^3RF^*$). It follows from Table II that the rates of indoles degradation behave in a reciprocal relationship with the rate constants for the quenching of $^3RF^*$. This fact indicates (in view of the inefficiency of the indoles as $^1RF^*$ quenchers at concentrations lower than 1 mmol dm^{-3}) that they react with an intermediate generated by $^3RF^*$.

Given that Trp, Trypta and I_3Ac are efficient chemical quenchers of $O_2(^1\Delta_g)$ (Table II) a direct connection between this fact and the possibility of RF to generate the reactive intermediate can be established. Nevertheless, according to the relative values of rates for oxygen uptake by the indoles both, in the absence and in the presence of sodium azide, a pure $O_2(^1\Delta_g)$-mediated mechanism should be disregarded, at least for

Trp and Trypta. In addition, the lack of a certain parallelism between the v_{ind} and the k_r values supports the mentioned postulate. Even so, it is clear that oxygen is efficiently scavenged from the reaction system with a similar rate independently of the indole derivative. The data can be better rationalized if we correlate our results whit those reported for a series of chlorophenols[2] and 2,6-dimethyl phenol (P) (ref.[30]), where the occurrence of reaction (*17*) was confirmed by laser flash photolysis

$$^3RF^* + P \longrightarrow RF^{\bullet -} + P^+ . \qquad (17)$$

In the presence of oxygen, superoxide ion can be produced through reaction (*18*)

$$RF^{\bullet -} + {}^3O_2 \longrightarrow RF + O_2^{\bullet -} \qquad (18)$$

and

$$O_2^{\bullet -} + RF \text{ or } P \longrightarrow \text{products} . \qquad (19)$$

A similar global mechanism operates in the lumiflavin-sensitized photooxygenation of indole, investigated by means of steady light photolysis and flash photolysis by Yoshimura and Ohno[11]. Besides a $O_2(^1\Delta_g)$ path , the oxidized radical of indole (generated by reaction of triplet lumiflavin with indole) produces superoxide anion by reaction with molecular oxygen. A combination of Type I (radical-mediated) and Type II ($O_2(^1\Delta_g)$-mediated) mechanisms[12] was proposed by the authors to explain the experimental results. Miyoshi et al[13]. came to same conclusions on the base of analysis of the flavin mononucleotide-sensitized aerobic photooxidation of indoleacetic acid: a combination of the above mentioned mechanisms operates.

The assumed presence of superoxide ion in our system offers an explanation for both, the similarity within the v_{Ox} for all the indoles and their non-parallelism with the k_r values. On the other hand, the trend in the rates of indole consumption (v_{ind}) as compared with that of v_{Ox}, could indicate the participation of a reactive step $^3RF^*$–indole.

Additional factor must be considered within this complicated reaction scheme: depending on the relative concentrations of the reactants, the system can be switched over from one to another mechanism. Dark association is only important at indole concentration higher than 1 mmol dm^{-3}. In air saturated aqueous solution, RF decomposes by a combination of two mechanisms[6,11,31] originated in $^3RF^*$ (steps (*8*) and (*13*)). The excited RF is efficiently quenched by the indoles (especially by Trp and Trypta (see k_{q3} values in Table II). The competitive reactions (*9*) and (*10*) can take place in the presence of the indoles. Considering the value of 7 . 10^9 mol^{-1} dm^3 s^{-1} for k_{ET} in water[3], and the oxygen concentration of 0.26 mmol dm^{-3} (air saturated aqueous solution[32]), generation of $O_2(^1\Delta_g)$ (*10*) becomes significant at I_3Ac concetrations lower than 0.5 mmol dm^{-3} (under these conditions k_{q3} $[I_3AC]$ = k_{ET} $[O_2(^3\Sigma_g)]$. This relationship is satisfied only at indoles concentration lower than 0.1 mmol dm^{-3} for both Trp and Trypta. The last fact justifies the significant contribution of a Type II reaction in the case of I_3Ac (as arises from the oxygen uptake experiments). As a consequence, Trp and Trypta are decomposed by both, a reactive interaction with $^3RF^*$ and a reaction with $O_2^{\bullet -}$.

It can be concluded that the irradiated RF–indole systems react by a complex mechanism, the results of which is that the indoles are decomposed and RF suffers a delay in its rate of autofading.

Thanks are given to CONICOR (Pcia. de Córdoba) CONICET (República Argentina) an Universidad Nacional de Río Cuarto, for financial support. O. E. Z. thanks Prof. N. J. Demas for providing SPC program.

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