Journal of Photochemistry and Photobiology B: Biology 124 (2013) 42–49

Contents lists available at SciVerse ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

A comparative photochemical study on the behavior of 3,3′-dihydroxyflavone and its complex with La(III) as generators and quenchers of reactive oxygen species

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ARTICLE INFO

Article history:
Received 9 January 2013
Received in revised form 19 March 2013
Accepted 27 March 2013
Available online 6 April 2013

Keywords:
3,3′-Dihydroxyflavone–La(III) complex
Photodegradation
Photosenitization
Reactive oxygen species
Riboflavin

ABSTRACT

A 1:1 complex between 3,3′-dihydroxyflavone (DHF) and La(III) (DHF–La(III)) is formed in methanolic solution with the relatively high apparent stability constant value of 2.3×10^6 and a calculated standard entropy change of 88.2 J mol^{-1} K^{-1}, both at 25 °C.

The photophysical properties of the complex and the free flavonoid are discussed in comparison to the well known related compound 3-hydroxyflavone. The ligand photogenerates $O_2(^1\Delta_g)$ by energy transfer from its excited triplet state (^{3}DHF*) to dissolved ground state oxygen, with a quantum yield of 0.13. ^{3}DHF* is quenched by La(III) with a rate constant close to the diffusion-controlled value.

The respective abilities of the free flavonoid and DHF–La(III) as quenchers of the riboflavin-photogenerated reactive oxygen species singlet molecular oxygen ($O_2(^1\Delta_g)$) and superoxide radical anion ($O_2^{\cdot-}$) have been investigated. Both individual compounds were photoirradiated with visible light in the presence of the flavin as the only light-absorbing compound. A detailed kinetics and mechanistic study employing polarographic monitoring of oxygen uptake and time resolved detection of $O_2(^1\Delta_g)$ phosphorescence indicates that DHF and the complex react with $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$ by a non simple mechanism. The former deactivates $O_2(^1\Delta_g)$ in a predominant physical fashion, a fact that constitutes a desirable property for antioxidants. It was found that metal chelation greatly enhances the ability of DHF as an overall $O_2(^1\Delta_g)$ quencher.

1. Introduction

Flavonoids constitute an extensive family of compounds, widely distributed in the vegetable kingdom [1]. A number of beneficial effects in relation to human health have been described for these polyhydroxylated pigments, being antioxidant, antitumoral, anti-HIV and antiinflammatory activities only some of them [2,3].

The main antioxidant effect of flavonoids is the protection of cells against the damaging effects of reactive oxygen species (ROS), such as singlet molecular oxygen ($O_2(^1\Delta_g)$), hydroxy radicals, hydrogen peroxyde and superoxide radical anion ($O_2^{\cdot-}$) [4,5]. ROS are formed in many living systems, and themselves or subsequent reaction products, have been proven to produce serious deleterious effects, through the so called oxidative stress [6].

Regarding the involvement of flavonoids in the generation and quenching of ROS, a satisfactory approach to the actual situation in living environments is the study of photoprocesses occurring in the presence of visible light and eventually, in the presence of natural dye-sensitizers. Results from other authors and from ourselves indicate that the effectiveness of flavonoids as ROS quenchers mainly depends on structural effects, and particularly on the number and position of the OH groups in the flavonoid skeleton [5,7–9]. Also metallic-ion complexes of flavonoids have been reported as preventers of free radical and ROS formation [10].

Many flavonoids have a relatively high metal–ion complexation capacity [11,12]. Specially flavones, within the extensive family of poly-hydroxy compounds, have been widely examined in relation to complexation [13–16]. These studies are particularly important since it has been found that the biological properties of the ligands can be enhanced upon complexation [17].

DHF was recently synthesized in our group by a novel and simple procedure [18] being unknown its photophysical and photochemical properties. It is a derivative of 3-hyfroxyflavone (3HF), a particularly interesting compound whose non simple photochemistry still being profusely studied [19–22].

Several interesting biological properties of La-complexes have been described, including antitumoral, cytotoxic and antibacterial activities. Furthermore, it has been found that some ligands, when complexed with La(III), greatly enhance their biological action [16,23–25].

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http://dx.doi.org/10.1016/j.jphotobiol.2013.03.011

In synthesis, and in the context of the described properties of flavonoids and its complexes with metallic ions, this paper contributes to gain insight on (a) stoichiometry, apparent stability constant and thermodynamic parameters associated with the formation of flavonoid-metal complexes, (b) photophysical and photochemical processes of DHF, and the effect of its complexation with La(III) and (c) the properties of DHF and its complex DHF–La(III) in relation to generation and quenching of ROS upon visible-photoirradiation, in the absence and in the presence of known natural photosensitizer Riboflavin (vitamin B2). The last point constitutes the main part of the study.

2. Experimental

2.1. Materials

3,3′-Dihydroxyflavone was synthesized, purified, and identified according to previously reported methods [17]. The following reactants were used as received: $LaCl_3{\cdot}7H_2O$ (Tetrahedron); perinaphthenone (PN), furfurylacetate (FFAc), deuterated methanol (CH_3-OD) (all from Aldrich); furfuryl alcohol (FFA) (Riedel de Häen), quinine sulfate (Parafarm), riboflavin (Rf), rose bengal (RB), superoxide dismutase (SOD), sodium azide (NaN_3), vitamin C (VitC, ascorbic acid) (all from Sigma), potassium hydroxide (Cicarelli) and metanol HPLC grade (Sintorgan). Water was triply-distilled.

2.2. Methods

The stoichiometry of the complex was determined using Yoe-Jones method [26], which consists in preparing a set of solutions keeping constant the metallic ion concentration and varying the ligand concentration. The absorbance of these solutions is measured, at a wavelength where only the complex absorbs, and used to plot a graphic of absorbance vs. ligand/metal concentration ratio, L/M. The intersection points between straight lines of experimental data indicates the ligand/metal molar ratio. This method was also employed for the calculations of apparent stability constants according to the expression:

$$K = \frac{(A/A_{ext})C}{[M-(A/A_{ext})C][L-(A/A_{ext})C]} = \frac{[ML]}{[M][L]} \quad (1)$$

where M and L are the total analytical concentrations of metal and ligand respectively, C is the total analytical concentration of the ligand or the metal, whichever is the limiting concentration at the equivalence point, A_{ext} is the extrapolated value of the absorbance, A is the actual value of absorbance and K is the apparent stability constant.

The standard enthalpy associated to the process, $\Delta_r H°$, was determined using VanťHoff expression: $lnK = \Delta_r H°/RT + c$, while the Gibbs free energy, $\Delta_r G°$, was determined using the equation: $\Delta_r G° = -RT\ ln\ K$, and the change in entropy, $\Delta_r S°$, was calculated using the expression: $\Delta_r G° = \Delta_r H° - T\,\Delta_r S°$, where, R is the universal gases constant, T is the absolute temperature and c, an integration constant.

The ground state absorption spectra were registered with a Hewlett Packard 8452A or an Agilent 8453 diode-array spectrophotometer. Steady-state fluorescence measurements were made employing a Shimadzu RF5301-PC or a Fluoromax-4 Horiba Jobin Yvon spectrofluorimeter at 25 ± 1 °C. Fluorescence lifetimes were measured by the time-correlated single photon counting technique on an Edinburgh FL-9000CD instrument. The quantum yields were obtained by comparison of the wavelength-integrated intensity of the unknown, I, to that of a standard, I_R, both with the same absorbance at the excitation wavelength, and using the expression: $\Phi/\Phi_R = I/I_R$ [27]. As quantum yield standard a 0.5 M H_2SO_4 quinine sulfate solution was used ($\Phi = 0.55$) [28].

Transient absorption spectra were detected using a Laser Flash Photolysis apparatus. A nanosecond Nd:YAG laser system (Spectron) at 355 nm was the excitation source employing a 150 W Xenon lamp as the analyzing light. The detection system comprised a PTI monochromator and a red-extended photomultiplier (Hamamatsu R666). The signal, acquired and averaged by a digital oscilloscope (Hewlett–Packard 54504A), was transferred to a PC via an HPIB parallel interface, where it was analyzed and stored.

The quantum yield for $O_2(^1\Delta_g)$ generation, Φ_Δ, was determined from the slopes of the first order plots of an oxidizable reference consumptions, furfurylacetate (FFAc), in the presence of the unknown and the reference applying the expression: $\Phi_\Delta/\Phi_{\Delta R} = \text{slope/slope}_R$, where the subscript R refers to the reference of known $O_2(^1\Delta_g)$ production quantum yield. The well-known photosensitizer perynaphthenone ($\Phi_\Delta = 1$) was used as reference [29]. Reference and unknown compound must be matched at the irradiation wavelength in order to ensure both absorb the same portion of light ($A_{350} = 0.5$).

The overall quenching rate constant of deactivation of $O_2(^1\Delta_g)$ by the flavonoid and the complex (k_t, the sum of k_q plus k_r, (processes (11) and (12) respectively, Scheme 1) was determined using a previously reported system [30]. Briefly, a Nd:YAG laser (Spectron) was used for the excitation (532 nm) of the sensitizer RB ($A_{532} = 0.4$), and the emitted radiation ($O_2(^1\Delta_g)$ phosphorescence at 1270 nm) was detected at right angles using an amplified Judson J16/8Sp germanium detector, after passing through two Wratten filters. The output of the detector was coupled to a digital oscilloscope and to a personal computer for the signal processing. Usually, 16 shots were needed for averaging, so as to achieve a good signal to noise ratio, from which the decay curve was obtained. Air-saturated solutions were employed in all the cases. In the dynamic determinations, MeOD, instead of MeOH, was used as a solvent in order to enlarge the lifetime of $O_2(^1\Delta_g)$ [31]. The $O_2(^1\Delta_g)$ lifetimes were evaluated in the presence (τ) and in the absence

$$Rf + h\nu \rightarrow {}^1Rf^* \rightarrow {}^3Rf \quad (1)$$

$${}^1Rf^* + Q \rightarrow Rf + Q \text{ or } P(2) \quad (2), \text{ rate constant } {}^1k_q$$

$${}^3Rf^* + O_2({}^3\Sigma_g^-) \rightarrow Rf^{\bullet+} + O_2^{\bullet-} \quad (3)$$

$${}^3Rf^* + Q \rightarrow Rf^{\bullet-} + Q^{\bullet+} \quad (4), \text{ rate constant } {}^3k_q$$

$$Rf^{\bullet-} + H^+ \leftrightarrow RfH^\bullet \quad (5)$$

$$2\ RfH^\bullet \rightarrow Rf + RfH_2 \quad (6)$$

$$RfH_2 + O_2({}^3\Sigma_g^-) \rightarrow RfH_2^{\bullet+} + O_2^{\bullet-} \quad (7)$$

$$O_2^{\bullet-} + Q \rightarrow P(8) \quad (8)$$

$${}^3Rf^* + O_2({}^3\Sigma_g^-) \rightarrow Rf + O_2({}^1\Delta_g) \quad (9), \text{ rate constant } k_{ET}$$

$$O_2({}^1\Delta_g) \rightarrow O_2({}^3\Sigma_g^-) \quad (10)$$

$$O_2({}^1\Delta_g) + Q \rightarrow O_2({}^3\Sigma_g^-) + Q \quad (11), \text{ rate constant } k_q$$

$$O_2({}^1\Delta_g) + Q \rightarrow P(12) \quad (12), \text{ rate constant } k_r$$

Being $k_t = k_r + k_q$

Scheme 1.

(τ_0) of the quencher (Q), and the data were plotted as a function of concentration, according to a simple Stern–Volmer treatment, $\tau_0/\tau = 1 + k_t\tau_0[Q]$.

The rate constant k_r, for chemical reaction of $O_2(^1\Delta_g)$ with the substrates was determined by a relative method involving oxygen consumption upon photosensitized irradiation, according to a described method [32]. The knowledge of the reactive constant k_{rR} for the photooxidation of a reference compound R is required. Assuming that the reaction of $O_2(^1\Delta_g)$ with the quencher is the only pathway of oxygen consumption the expression: slope/slope$_R$ = k_t/k_{rR} allows to determine k_r. In this method the slope of the first order plot of oxygen consumption by DHF or DHF–La(III) (slope) and by a reference compound R (slope$_R$) are experimentally determined in the same experimental conditions. The reference compound used here was furfuryl alcohol (FFA), with a reported pH-independent k_{rR} value of 3×10^7 M^{-1} s^{-1} in methanolic media [30]. Photolysis were performed in a home-made photolyzer with a 300-W-quartz halogen lamp, using cut-off filters or a monochromator whenever was necessary and oxygen consumption was measured with Orion 810A+ specific oxygen electrode.

3. Results

3.1. About the complex DHF–La(III)

Although the main aim of this work is the study of DHF and its complex with La(III) as eventual photogenerators and deactivators of ROS, necessary first steps are the physico-chemical characterization of the complex and the photophysical and photochemical characterization of both species, the free flavone and the complex.

The formation of a complex between DHF and La(III) was suggested by the intensification of yellow color when La(III) solution was added into the ligand solution. The bathochromic shift produced when the complex is formed was confirmed by recording the spectra of the ligand solution and that of the same solution after La(III) addition. Several additions of La(III) were made in order to study the spectral behavior of the mixture, as shown in Fig. 2.

Compared to DHF, that exhibits a main band centered at 347 nm and a shoulder at 306 nm, the electronic absorption spectra of the complex shows single new band centered at 410 nm, assigned at the $L \rightarrow M$ (ligand → metal) charge transfer. The presence of a unique isosbestic point indicates that only one new species is formed, without regarding the decrease in the L/M ratio. No significant spectral changes were observed at L/M ratios lower than one.

A 1:1 L:M stoichiometry was graphically determined for the complex by means of the Yoe-Jones method [25], as shown in Fig. 3.

The apparent formation constant (K) of the complex was determined at several temperatures and the experimental data is shown in Table 1.The enthalpy change, being −10 kJ mol^{-1}, was determined from ln K vs. T^{-1} plot (Fig. 3, inset). The calculated standard entropy change was 88.2 J mol^{-1} K^{-1} at 25 °C.

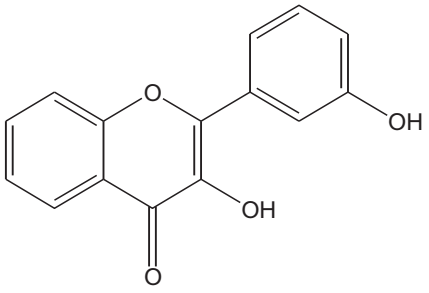

Fig. 1. Chemical structure of 3,3′-dihidroxiflavone (DHF).

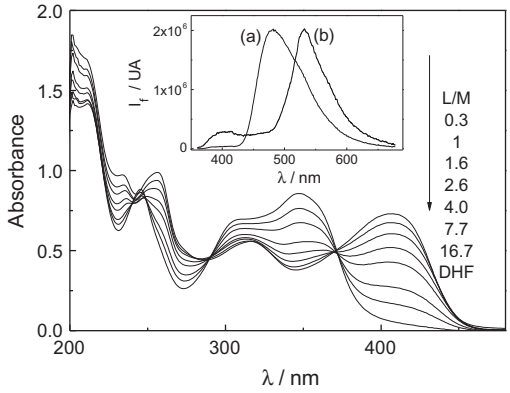

Fig. 2. Main: spectra of a methanolic 0.37 mM DHF solution upon addition of La(III). L/M indicates the ratio ligand to metal, and DHF indentifies the spectrum of the flavonoid in the absence of La(III). Inset: fluorescence spectra of MeOH DHF–La(III) (a) and DHF (b) normalized at their respective maxima. λ_{exc} = 350 nm.

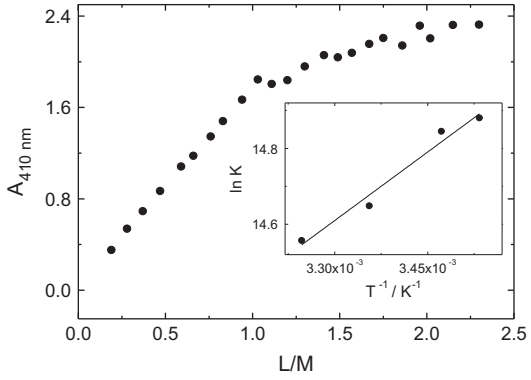

Fig. 3. Stoichiometry and apparent constant determination for the complex DHF–La(III) at 10 °C (see experimental section). Inset: graphical representation of Vaṅt Hoff equation.

Table 1
Concentration of ligand (L) and metal (M), extrapolated (A_{ext}) and actual (A) values of the absorbance, apparent equilibrium constants (K) and $\Delta_rG°$ values obtained at several temperatures.

T (°C)	A_{ext}	A	$M \times 10^4$	$L \times 10^4$	$K \times 10^{-6}$	$\Delta_rG°$ (kJ mol^{-1})
10	1.810	1.731	1.04	1.07	2.9	−35.0
15	1.787	1.708	1.03	1.07	2.8	−35.5
25	1.734	1.648	1.02	1.06	2.3	−36.3
35	1.676	1.589	1.01	1.04	2.1	−37.3

3.2. Characterization of electronic excited states of DHF and DHF–La(III)

Fluorescence spectra of MeOH solutions of DHF and DHF–La(III) (Fig. 2, inset) upon excitation at 350 and 410 nm respectively comprise a double band for the flavone, centerd at *ca.* 400 and 545 nm and a single band for the complex, centerd at 485 nm. The fluorescence quantum yields for DHF and for the complex, obtained as described in the experimental section, were 0.01 and 0.2 respectively.

The transient absorption spectrum of DHF in oxygen-free MeOH, obtained 2 μs after the laser pulse is shown in Fig. 4, main. It exhibits some similarities in shape with that reported for 3HF in methyl-pentane by Tokumura et al. [19]. Nevertheless, 3HF presents a shoulder in the region of 430–460 of the second order-decaying transient absorption which is absent in the corresponding one for DHF. The spectrum is dominated by triplet–triplet absorp-

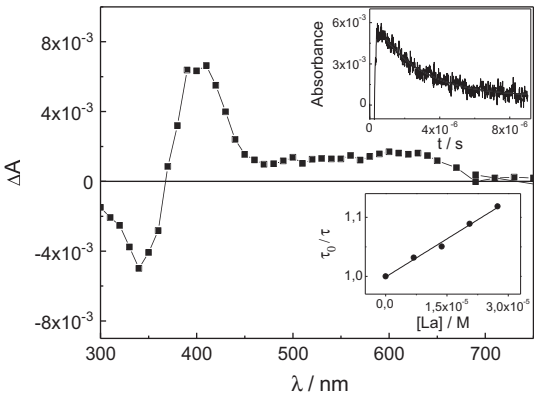

Fig. 4. Transient absorption spectrum of DHF in N_2 saturated methanolic solution obtained 2 μs after the laser pulse. Upper inset:transient decay at 400 nm. Lower inset: Stern–Volmer plot for the quenching of triplet excited DHF by La(III).

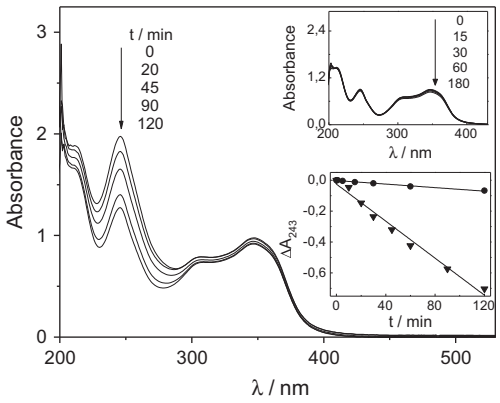

Fig. 6. Spectral evolution of a methanolic solution of 0.56 mM DHF + 1.1 mM VitC upon photoirradiation. Upper inset: spectral evolution of methanolic solution of 0.56 mM DHF upon photoirradiation. Lower inset: plot of ΔA_{245} vs. time for the described solutions of DHF (●) and DHF + VitC (▼) upon photoirradiation. λ_{irr} = 360 nm (cut-off filter).

tion and breaching of DHF. The transient absorption of DHF, remarkably decreased in intensity by dissolved molecular oxygen and exhibits a simple exponential decay (Fig. 4, upper inset) that appreciably increases in the presence of La(III). The signal totally disappears when the stoichiometric ratio La(III)-flavonoid is 1:1. No transient signal at all could be observed for the complex DHF–La(III). A rate constant value $^3k_q = 1.3 \times 10^9\ M^{-1}\ s^{-1}$ (reaction (4) of Scheme 1, with DHF instead of Rf, see below) was determined for the quenching of $^3DHF^*$ by La(III), as shown in Fig. 4, lower inset.

3.3. Evaluation of DHF and DHF–La(III) as $O_2(^1\Delta_g)$ generators

The quantum yields of $O_2(^1\Delta_g)$ generation ($\Phi\Delta$) by DHF and DHF–La(III) upon irradiation at 350 nm were evaluated employing FFAc as a photooxidizable target and PN as a reference. The Absorbance changes due to FFAc, attributed to a reaction with $O_2(^1\Delta_g)$, were monitored at 216 nm. A $\Phi\Delta = 0.13$ was obtained for DHF (Fig. 5) whereas no photoconsumption of the furfuryl derivative at all was observed for the complex as a photosensitizer ($\Phi\Delta \sim 0$), even at irradiation times up to fivefold higher than that employed for DHF.

As an additional experiment the photosensitizing ability of DHF was tested employing VitC as a $O_2(^1\Delta_g)$-sensitive target. VitC efficiently reacts with the oxidative species with a reported rate constant k_t of $1.5 \times 10^8\ M^{-1}\ s^{-1}$ in methanolic solution [33]. The

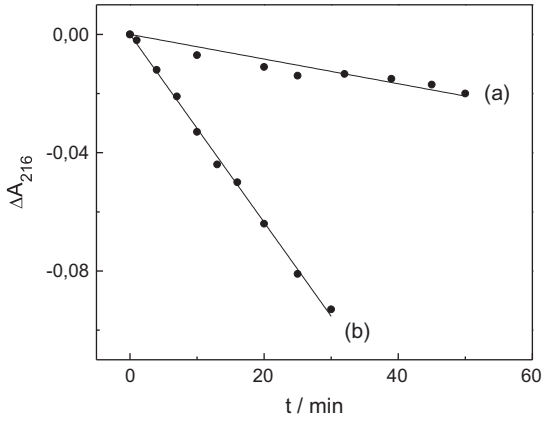

Fig. 5. Absorbance decrease of 0.1 mM furfuryl acetate at 216 nm upon photoirradiation in the presence of (a) DHF (A_{350} = 0,5) and (b) Perynaphthenone (A_{350} = 0,5). λ_{irr} = 350 nm.

absorbance decrease of the vitamin was monitored at 243 nm (Fig. 6). In the same figure are shown the slight spectral changes experimented by DHF upon photoirradiation of a methanolic solution in the absence of VitC. The rate of VitC photoconsumption was extremely low in the presence of 2 mM NaN_3, being the salt a recognized efficient physical quencher of $O_2(^1\Delta_g)$ [30]. The possibility that NaN_3, a recognized quencher of triplet state molecules, could deactivate $^3DHF^*$ should be also considered as an additional source of VitC photoprotection [34].

Employing the complex as a sensitizer, the graphical representation of VitC absorbance changes as a function of photorradiation time indicates a very low slope (data not shown), attributable to changes experimented by photodegradation of the very complex but not to the vitamin.

3.4. The interaction of DHF and DHF–La(III) with photoexcited Rf and photogenerated ROS

Riboflavin is a pigment of particular interest in the field of the photobiology. The vitamin has been postulated as a possible sensitizer for the photooxidative degradation of a number of relevant natural substrates present in different classes of foods and living organisms [35]. On this basis, we decided to investigate the specific interactions of DHF and the complex with singlet and triplet excited states of the vitamin, under aerobic and anaerobic conditions.

The reaction scheme shown below (Scheme 1), already employed to interpret the mechanism of interaction of electron donors with a photoexcited Rf [8], was utilized in the present case.

Rf is the sensitizer, i.e. the species that absorbs radiation in a wavelength range where Q is transparent. The absorption of incident light promotes Rf to electronically excited singlet and triplet states (reaction (1)). Both states can be quenched through reactions (2), (3) and (5) respectively. By means of the electron transfer reaction (4), the respective semireduced and semioxidized Rf and Q forms are produced. Reaction (7) represents the generation of the reactive species superoxide anion ($O_2^{\cdot-}$) which can react with Q (reaction (8)) and/or with the pigment (reaction not shown). P(2)–P(8) represent eventual photoproducts.

From the triplet state, an energy transfer reaction to the ground state-triplet molecular oxygen $O_2(^3\sum_g^-)$, dissolved in the medium, can take place, yielding the excited state oxygen species $O_2(^1\Delta_g)$ (reaction (9)). This can decay either by collision with surrounding solvent molecules (reaction (10)) or by interaction with Q and/or Rf through an exclusive physical (reaction (12)) or chemical (pho-

tooxidation, reaction (12)). An overall rate constant for $O_2(^1\Delta_g)$ quenching (k_t) is defined as the sum of the rate constants for processes (11) and (12).

Methanolic solutions containing 0.05 mM Rf and individual 8.8 mM DHF (λ_{irr} 400 ± 5 nm) or 4.2 mM DHF–La(III) (cut-off > 480 nm), in the presence of air, experimented modifications in their respective absorption spectra, attributed to photoreactions involving the substrates or the substrates plus the sensitizer. The case of the system Rf + DHF–La(III) is shown in Fig. 7. The slight negative absorbance observed 450–500 nm wavelength region corresponds, in this case, to Rf degradation. Similar qualitative results were obtained for DHF (not shown). From parallel experiments on the same photoirradiated solutions, oxygen consumption was observed. The solutions did not consume any oxygen in the dark.

The reported lifetime of $^1Rf^*$ in MeOH is *ca.* 5 ns [8]. A sub-mM concentration of an eventual $^1Rf^*$quencher, similar to those employed for DHF and DHF–La(III) in all experiments performed in this work, is not enough to intercept the excited species of the vitamin, even assuming a diffusion-controlled value for the rate constant ^1k_q of process (2) in Scheme 1. Hence, the deactivation of $^1Rf^*$ by the flavonoid and its complex must be disregarded under work conditions.

It is known that the photodegradation of Rf in solution, in the absence of oxygen and under visible light irradiation, predominantly proceeds through $^3Rf^*$ [36,37]. It is also known that the rate of the process can be evaluated by the absorbance decrease in the Rf absorption spectrum at 445 nm as a function of photoirradiation time. The rate of Rf decomposition was diminished in the individual presence of DHF and its complex, clearly indicating the occurrence of a quenching of $^3Rf^*$ by these substrates.

The described set of preliminary qualitative results strongly suggests that the electronically triplet state of Rf and/or ROS generated from this state react with DHF and DHF–La(III). On this basis we carried out a systematic kinetic study in order to establish the mechanism and parameters involved in the mentioned photopromoted interactions.

In Fig. 8 is shown the inhibition of Rf photodegradation in N_2 saturated methanolic solution by increasing concentrations of DHF–La(III). Qualitative similar results were obtained for DHF.

Under photostationary conditions, taking the slopes of $^3Rf^*$ degradation traces of Fig. 8 as the respective photodegradation rates in the absence and in the presence of the $^3Rf^*$quencher Q, and a value of 15 μs for the triplet lifetime of Rf in MeOH [34], $^3k_{qApp}$ values of 9.4×10^8 and 1.6×10^9 M^{-1} s^{-1} were obtained for DHF and DHF–La(III) respectively (process (4) of Scheme 1).

3.5. Deactivation of reactive oxygen species by DHF and DHF–La(III)

In Fig. 9 are shown the results of oxygen uptake experiments obtained upon photoirradiation of 0.05 mM Rf and of the mixtures 0.05 mM Rf plus 0.5 mM DHF–La(III). The participation of ROS was evaluated through oxygen consumption experiments in the presence of specific ROS quenchers. Thus, the presence of both 5 mM NaN_3 and 1 μg/mL SOD produced a decrease in the rate of oxygen uptake by the mixtures. The salt and the enzyme are selective quenchers for the species $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$ respectively, and concentrations similar to those herein employed have been formerly used in order to confirm or discard the participation of any of said species in a given reaction mechanism [38,39].

On the basis that Rf generates $O_2(^1\Delta_g)$ and other ROS in the presence of electron-donating substrates as the flavonoids family [8], the well-known $O_2(^1\Delta_g)$ generator RB (A_{530} = 0.4) [40] was employed as a sensitizer, in order to exclusively study the $O_2(^1\Delta_g)$-interaction. The decay kinetics of $O_2(^1\Delta_g)$ phosphorescence was first order, and the lifetime agreed well with literature data [30]. The addition of DHF, La(III) and DHF–La(III) in the sub mM concentration range, lead to a decrease of the $O_2(^1\Delta_g)$ lifetime, unambiguously confirming the interaction of the flavone derivatives with this oxidative species. The k_t values, 8.6×10^5 M^{-1} s^{-1} for DHF, 1.0×10^5 M^{-1} s^{-1} for La(III) and 3.8×10^7 M^{-1} s^{-1} for the complex were graphically obtained in MeOD (Fig. 10).

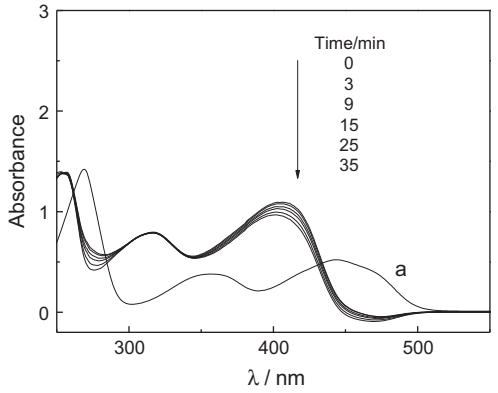

Fig. 7. Spectral evolution of the mixture 0.05 mM Rf + 4.2 mM DHF–La(III) vs. 0.05 mM Rf (λ_{irr} > 480 nm, cut off filter). The absorption spectrum of 0.05 mM Rf (a) was included for comparative purposes.

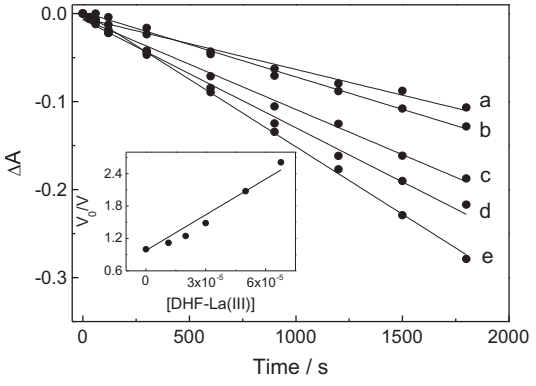

Fig. 8. Absorbance changes of a 0.04 mM Rf N_2-saturated methanolic solution of Rf, monitored a 445 nm, as a function of photoirradiation times in the absence (V_0) (e) and in the presence (V) of different concentrations of DHF–La(III): [DHF–La(III)] = 11 μM (d); [DHF–La(III)] = 20 μM (c); [DHF–La(III)] = 30 μM (b); [DHF–La(III)] = 50 μM (a). Inset: Stern–Volmer for the decrease in the rate of the absorbance changes of Rf by the presence of DHF–La(III).

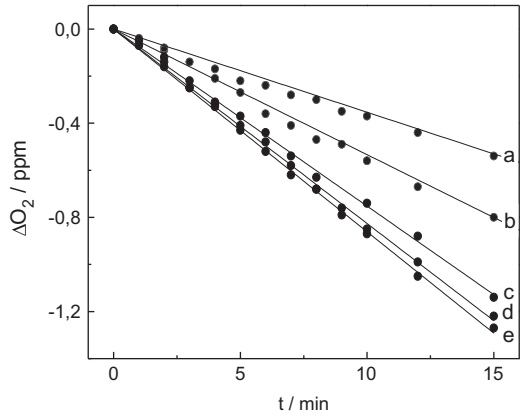

Fig. 9. Oxygen uptake by a solution of 0.05 mM Rf in MeOH–H_2O1:1 as a function of photoirradiaton time (v/v) in the absence (a) and in the presence of 0.5 mM DHF–La(III) +5 mM NaN_3 (b); 0.5 mM DHF–La(III) +1 μg/ml SOD (c); 0.5 mM DHF–La(III) (d).

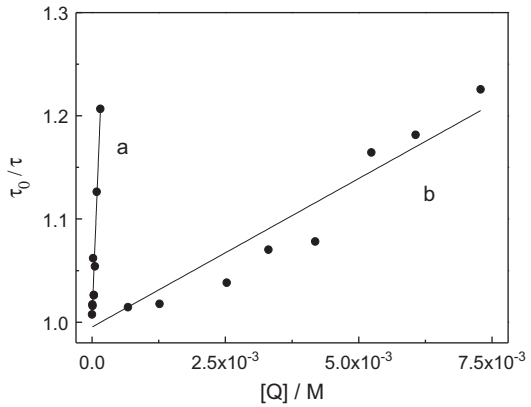

Fig. 10. Stern–Volmer plot for the quenching of $O_2(^1\Delta_g)$ phosphorescence by DHF–La(III) (a) and DHF (b) in MeOD. Sensitizer: RB, A_{560} = 0.5.

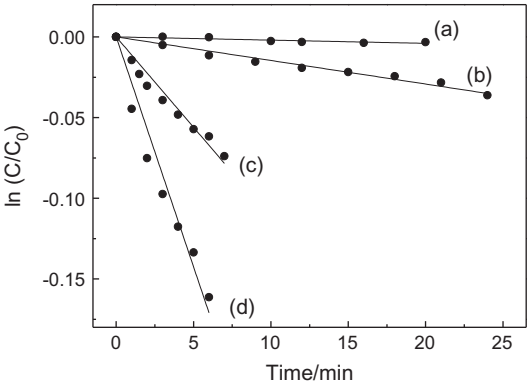

Fig. 11. First order representation for oxygen uptake by photoirradiated methanolic solutions of (a) RB + DHF; (b) RB + DHF–La(III); (c) RB + FFA; and (d) RB + DHF + KOH (0.01 M). In all cases RB (A_{558} = 0.56), [DHF] = [DHF–La(III)] = [FFA] = 1.1 × 10^{-4} M.

The rate constant k_r (process (12)) was determined by the above mentioned actinometric method, monitoring oxygen photoconsumption (Fig. 11). The obtained k_r value for DHF–La(III) was 4.0×10^6 M^{-1} s^{-1}. No oxygen consumption was observed for DHF and for La(III), even employing relatively prolonged irradiation times. On the other hand a k_r value of 6.4×10^7 M^{-1}s^{-1} was obtained for the flavone in the presence of 0.01 M KOH, which is within the typical values of reactive rate constants of flavonoids in alkaline media [7]. In these cases the electron-donating ability of the flavones is highly increased due to the ionization of the respective hydroxyl-groups [41,42].

The $O_2(^1\Delta_g)$-mediated photooxidation quantum efficiency Φ_r ($\Phi_r = k_r[Q]/(k_d + k_t[Q])$ is not easy to evaluate, particularly in biological environments, because its determination includes the knowledge of the actual concentration of the photooxidizable substrates, represented by the Q in this case. A simpler and useful approach is the evaluation of the k_r/k_t ratio, which indicates the fraction of overall quenching of $O_2(^1\Delta_g)$ by the substrate that effectively leads to a chemical transformation. The calculated k_r/k_t ratios in neutral MeOH for DHF and for the complex were respectively ~0 and 0.1.

4. Discussion

4.1. About the complex DHF–La(III)

The stoichiometry and the relatively high apparent equilibrium constant value for DHF–La(III) indicate that this ligand could be considered a possible candidate as an analytical reagent for La(III).This is an important point in the context of the extraction and recovery of the metal. Other metal complexes of flavonoids with similar or higher stability constants have been reported as potential analytical reagents: 2′-hydroxychalcone was proposed as a new specific reagent for the extraction of beryllium in the presence of elements such as aluminium and iron. The ratio of metal to ligand in the complex is 1:2 and the stability constant is 4.59×10^5 [12]. It is also been reported the use of morin as spectrophotometric reagent of aluminium in micellar media [43]. The stoichiometry of the complex in 0.16% w/v Triton X-100 is 1:3 *L*:*M* and the stability constant 2.63×10^{11} [44].

The thermodynamic data calculated for the formation of the complex show that it is a spontaneous exothermic process. The thermal energy emitted by the system is absorbed by the surroundings increasing the entropy. These changes indicate that the driving force of the complexation is entropic: initially the reactants are solvated, limiting solvent mobility. When complexation occurs solvent molecules are released, increasing disorder.

4.2. Photophysical studies and generation of $O_2(^1\Delta_g)$ by DHF and DHF–La(III)

The structural similitude between the novel DHF and the profusely studied parent compound 3HF, and their mutual close parallelism in spectroscopic data, allows the possibility to characterize the former through a simple comparison of the obtained results with reported information for 3HF and related compounds.

Regarding the absorption spectrum of DHF, the two broad bands centered at 260 and 350 nm (Fig. 1) respectively correspond to the cynnamolic and benzylic molecular moieties, whereas the important shoulder at 305 nm arises from the pyronic ring, as already described for 3HF [45].

The fluorescence spectrum of DHF in MeOH (Fig. 2, inset) is quite similar to that published by Sytnik et al. [46] for 3HF and Fisetin (3,3′,4′,7-tetrahydroxyflavone) in EtOH. All three compounds possess a 3-hydroxy group able to interact with an *ortho*-carbonyl group (Fig. 1). The photochemistry of 3HF and Fisetin in solution has been described in terms of an excited state proton transfer involving the mentioned structure [42–44]. On this basis, the 400 nm-centered emission band of DHF should be assigned to the normal tautomer and the 532 nm-centered band to the proton transferred species, that is formed in the subpicosecond range after light absorption [43,47]. The fluorescence quantum yield for DHF in MeOH is relatively low and fairly similar to the reported value of 3HF in MeOH ($\Phi_{F\ 3HF}$ = 0.02) [48]. The increase in Φ_F of the complex as compared to the free flavonoid can be attributed to an increase in the molecular rigidity due to the presence of the ligand structure, by reducing the probability of competitive energy-dissipative processes. One of these dissipative pathways can be the above mentioned intramolecular proton transfer process in DHF. These arguments has been already employed by de Souza et al. to explain an increment in the relative fluorescence intensities of the flavonoids quercetin and galantine upon Al (III) and Zn(II) complexation [49].

Regarding the laser flash photolysis studies, after a detailed analysis on 3HF, Tokumura et al. [19] attributed the transient species to two oxygen sensitive triplet species represented by the lowest energy triplet of the normal form of DHF absorbing at 395 nm and the lowest energy triplet from the tautomer, represented by the shoulder of 440 nm. As mentioned in the results section, the behavior of DHF is some different to that reported by Tokumura et al. [19] for 3HF, especially by the single-exponential decay observed for the transient species. The lowest triplet DHF (^{3}DHF*) is thus suggested for the mentioned oxygen-sensitive transient.

4.3. Generation of $O_2(^1\Delta_g)$ by DHF

According to the results employing FFAc and VitC as reference photooxidizable susbtrates, the visible light photoirradiation of DHF in MeOH solution, produces $O_2(^1\Delta_g)$. It is presumably generated through step (9) of Scheme 1 with DHF instead of Rf. The flavonoid seems to be unreactive towards the oxidative species as demonstrated by the constancy of the absorption spectra upon prolonged photolysis.The value of $\Phi\Delta = 0.13$ for DHF, that represents a lower limit for the quantum yield of triplet population, is in the range of triplet quantum yields of a series of flavones in solution [50].

In parallel, the experimental results did not show any evidence for the photogeneration of the oxidative species by the complex.

4.4. The interaction of DHF and DHF–La(III) with Rf electronically excited triplet

The spectral changes observed upon aerobic Rf-photosensitization clearly show chemical transformations in both DHF and DHF–La(III), whereas oxygen uptake experiments strongly suggest the occurrence of photoprocesses in which ROS, generated by Rf-electronically excited states take part.

Both $^1Rf^*$ and $^3Rf^*$ are quenched by the flavone and the complex with rate constant values ^1k_q and $^3k_{qApp}$, respectively, close to the diffusion limit. Nevertheless, under the experimental conditions employed, with the quenchers in the sub-mM range, the $^1Rf^*$ quenching process (process (2)) is undetectable.

It is known that $^3Rf^*$ in solution generates both $O_2(^1\Delta_g)$ (process (9)) and $O_2^{\cdot-}$ (process (3) upon visible light irradiation with reported quantum yields of 0.48 and 0.009, respectively [51].

Several authors, including ourselves, demonstrated that flavonoids are fairly to good quenchers of $O_2(^1\Delta_g)$ (k_t in the range 10^7–$10^8\ M^{-1}\ s^{-1}$), being the phenolic moiety mainly responsible for the interaction [30,52].

The experiments employing RB as a dye-sensitizer indicate that both DHF and the complex are moderate to good quenchers of $O_2(^1\Delta_g)$, being mostly physical in character the interaction with the oxidative species. This is an important characteristic in the context of these compounds as eventual protectors against photogenerated ROS.

The predominance of a given process (oxidation of Q *via* either $O_2^{\cdot-}$ or $O_2(^1\Delta_g)$) will depend, in principle, on the competition between $O_2(\sum_g^-)$ and Q for the quenching of $^3Rf^*$. It is currently accepted that the quenching of $^3Rf^*$ by $O_2(\sum_g^-)$ occurs with an approximate rate constant k_{ET} of 1/9 of the difussional value [53]. Considering a value of $1.2 \times 10^9\ M^{-1}\ s^{-1}$ in MeOH for 1/9 k_{ET} [54], and a mean value of $1.27 \times 10^9\ M^{-1}\ s^{-1}$ for $^3k_{qApp}$ of DHF and DHF–La(III), it arises that at equal concentration of dissolved $O_2\left(\sum_g^-\right)$ and Q the rates of reactions (4) and (10) are practically the same with DHF and DHF–La(III) instead of Q in reaction (4). This result, in principle, indicates that $O_2^{\cdot-}$ and $O_2(^1\Delta_g)$ can be formed upon selective photoirradiation of Rf in the presence of DHF and DHF–La(III), under aerobic conditions.

According to the oxygen consumption experiments, the decrease in the rate of oxygen uptake due to the presence of SOD (Fig. 9) suggests the participation of $O_2^{\cdot-}$ in the photooxidative process of DHF and DHF–La(III). Although in the direct generation of $O_2^{\cdot-}$ by electron transfer from $^3Rf^*$ to $O_2\left(\sum_g^-\right)$ must be considered negligible due to the extremely low values for the quantum yield of process (3), in the presence of any the flavone or the complex, process (4) operates, and subsequently the species $O_2^{\cdot-}$ could be formed through electron transfer (processes (5–7)). This sequence, with high efficiency of $Rf^{\cdot-}$ production, has been described already for other phenolic derivatives [55,56]. The high apparent rate constant values obtained for step (4) and the inhibitory effect of SOD in oxygen uptake experiments, indicate that $O_2^{\cdot-}$, generated by steps (4)–(7), is chemically deactivated by DHF and DHF–La(III) through process (8)).

5. Conclusions

DHF and La(III) form a 1:1 complex in methanolic solution with relatively high stability constant.

The flavonoid photogenerates $O_2(^1\Delta_g)$ with a quantum yield of 0.13, by energy transfer from $^3DHF^*$ to dissolved ground state oxygen. No evidence for the production of the oxidative species by the complex could be detected.

The Rf-photogenerated ROS $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$ are quenched by both the flavonoid and the complex. The former deactivates $O_2(^1\Delta_g)$ in a predominant physical fashion, a fact that constitutes a desirable property for antioxidants. It was found that metal chelation enhances the ability of DHF as an overall $O_2(^1\Delta_g)$ quencher.

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