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Effect of Cu^{2+}-complexation on the scavenging ability of chrysin towards photogenerated singlet molecular oxygen ($O_2(^1\Delta_g)$). Possible biological implications

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ABSTRACT

Visible-light irradiation of aqueous-ethanolic solutions of Riboflavin (Rf) in the individual presence of the flavone chrysin (Chr) and its complex with Cu^{2+} ([Chr_2Cu]; 2:1 L:M) generates singlet molecular oxygen $O_2(^1\Delta_g)$, that concomitantly interact with both flavone derivatives. Overall (k_t) and reactive (k_r) rate constants in the order of 10^7 M^{-1} s^{-1} were determined for the process. Metal chelation greatly enhances the scavenging ability of [Chr_2Cu] towards $O_2(^1\Delta_g)$ through a mechanism dominated, in >80%, by the physical component. In this way, practically all $O_2(^1\Delta_g)$ is deactivated by the complex without significant loss of the quencher. The isolated flavone quenches $O_2(^1\Delta_g)$ in a prevailing reactive fashion.

The very low value exhibited by [Chr_2Cu] for the k_r/k_t ratio constitutes a positive quality for antioxidative protectors in biological media, where elevated local concentration and high reactivity of significant molecules make them initial targets for $O_2(^1\Delta_g)$ aggression.

Finally, two interesting properties in the field of free radicals scavenging by [Chr_2Cu] must be mentioned. In first place metal chelation itself, in the obvious sense of free metal ion withdrawal from the oxidizable medium, prevents the initiation of a free radical-mediated oxidation processes through mechanisms of Fenton or lipid peroxidation. In addition, the incorporation of Cu adds to [Chr_2Cu] the ability of a free radical scavenger, already described for similar Cu-chelate compounds.

This collection of beneficial properties positions the complex as a remarkably promising bioprotector towards ROS-mediated oxidation.

A quantification of the efficiency on the initial anti-oxidative effect exerted by Chr and [Chr_2Cu] towards tryptophan was carried out. The amino acid is an archetypal molecular model, commonly employed to monitor oxidative degradation of proteinaceous media. It was efficiently photoprotected against $O_2(^1\Delta_g)$-mediated photooxidation by [Chr_2Cu].

1. Introduction

Flavonoids constitute an extensive family of natural compounds, with a number of beneficial effects in relation to human health [1,2]. Antitumoral, antioxidant, anti-HIV, antibiotic and antiinflammatory activities are only some of these well known qualities [3,4]. Nevertheless up to the present time, the main and more general recognized effect of flavonoids is the protection of cells against oxidative damaging provoked by reactive oxygen species (ROS) and free radicals. In particular ROS, such as singlet molecular oxygen ($O_2(^1\Delta_g)$), hydroxy radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and superoxide radical anion ($O_2^{\bullet-}$) [5–8], generated by the so-called oxidative stress, have been proven to produce serious deleterious effects, such as the induction of neurodegenerative disorders [9,10].

The effectiveness of flavonoids as ROS quenchers is well documented and depends on structural factors, as reported by other authors and by ourselves. A particularly important aspect is the number and position of the OH groups in the flavonoid skeleton [11–13]. Besides, the current research has clearly established that transition-metal chelation could affect the antioxidative ability of flavonoids as ROS scavengers [14–17].

Several works indicate that flavonoids posses a relatively high metal-ion complexation capacity [18–20]. We recently reported on the stability and thermodynamic parameters associated with the formation of the complex 2:1 L:M Chrysin-Cu (II) ([Chr_2Cu]) with a primary purpose centered in analytical applications [21]. The high stability of the complex, with apparent formation constant $K_f = 2.6 \times 10^8$, sparked our interest on its potential antioxidative activity compared to that of

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isolated Chrysin (Chr), in order to gain insight on the developing and evaluation of novel natural-biologically compatible systems. This topic constitutes the main objective of the present contribution.

A satisfactory approach for this kind of studies is the evaluation of the inhibitory ability of a potential antioxidative agent towards ROS produced by photosensitization. This system models, in some extent, a natural scenery in living environments through the study of photoprocesses occurring in the presence of visible light and a native ROS-generator dye-sensitizer. In the present case the chosen dye was Riboflavin (Rf, vitamin B2). The vitamin is an endogenous visible-light absorber in mammalians. It has been extensively postulated as a possible sensitizer for the in vivo photooxidative degradation of relevant biomolecules [22,23]. The ROS singlet molecular oxygen ($O_2(^1\Delta_g)$) and superoxide radical anion ($O_2^{\bullet-}$) are formed through reaction between Rf electronically triplet excited state ($^3Rf^*$) and ground state oxygen ($O_2(^3\Sigma_g^-)$) with quantum yields of 0.49 and 0.009 respectively [22,24,25].

In summary, we present in this paper a comparative study of the efficiency of Chr and [Chr_2Cu] as antioxidants. The piece of work constitutes a systematic kinetic and mechanistic investigation on possible reactions initiated through visible-light-induced excited Rf in the individual presence of the FLs. Besides, a quantification of the initial photo-protective effect exerted by Chr and by the complex towards tryptophan (trp) was carried out. The amino acid (AA) is an archetypal molecular model, frequently employed to monitor oxidative degradation of proteinaceous media [26,27].

2. Experimental

2.1. Materials

Riboflavin (Rf), deuterium oxide 99.9% D (D_2O), Chrysin (Chr), the proteins superoxide dismutase (SOD; from bovine erythrocytes) and catalase (CAT, from bovine liver) were purchased from Sigma Chem. Co. The salt $CuSO_4 \cdot 5H_2O$ and sodium azide (NaN_3) were provided by Merck. Rose Bengal (RB), was from Aldrich and Furfuryl alcohol (FFA) was purchased from Riedel de Haën. All these compounds were used as received. Ethanol (EtOH) HPLC quality, was from Sintorgan. D_2O was employed in time-resolved phosphorescence detection (TRPD) in order to enlarge the $O_2(^1\Delta_g)$ phosphorescence lifetime [28]. The experiments were made at room temperature and with freshly prepared solutions. H_2O was triply distilled. The pH-pD values determined for the final aqueous solutions in the photochemical experiments were in the range 7.0 ± 0.2. Buffered aqueous solutions were prepared employing 0.02 M KH_2PO_4/0.02 M Na_2HPO_4 [29].

2.2. Methods

2.2.1. Preparation of the Complex

As already published, the complex solution was prepared adding 1 ml of 8.2 mM aqueous Cu^{2+} solution into 4.35 ml of 3.8 mM ethanolic Chr solution. [21] The complex was instantaneously formed, and this was evident by the intensification of yellow color of the solution.

2.2.2. Stationary Photolysis

Stationary aerobic photolysis of Chr and [Chr_2Cu], hereinafter called FLs solutions, employing RB or Rf as dye sensitizers were carried out in a home-made photolyzer with the filtered light from a 150-W quartz-halogen lamp. In the Rf- or RB-sensitized photoirradiations, cut-off at 480 nm. Under these conditions the FLs employed did not absorb any incident light.

Ground state absorption spectra were registered in an Agilent 8454 diode array spectrophotometer.

The anaerobic Rf degradation rates were determined from the decrease of the 445-nm absorption band of the vitamin, under N_2 saturation, as a function of photoirradiation time.

The rate constant k_r for the reaction $O_2(^1\Delta_g)$ + FLs (process (15), Scheme 1) was determined employing a described actinometric method [30]. Briefly, the expression slope / $slope_R = k_r$ [FLs] / k_{rR} [R] was used, where slope and $slope_R$ are the respective experimentally determined slopes of the first-order plots of FLs and R consumption upon photosensitized irradiation. The reference compound employed (R) was FFA, with a reported value for k_{rR} of 1.2×10^8 M^{-1} s^{-1} [30]. In the present case rates of oxygen uptake (ROU) were determined instead of rates of FLs consumption. The ROU were determined instead of rates of FLs consumption. The ROU were evaluated employing a specific oxygen electrode Orion 97–08, immersed in a 150 ml hermetically sealed pyrex cell. The cell contained the sample solution constituted by the mixture sensitizer + FLs. The system was irradiated with the above described photolysis device.

2.2.3. Quenching of Rf Electronically Excited States

Fluorescence quenching of Rf was determined employing a Fluoromax-4 Horiba Jobin Yvon spectrofluorimeter. The excitation and emission wavelengths employed were 445 and 525 nm respectively. The rate constant ^1k_q accounting for the fluorescence quenching of the electronically excited singlet state of Rf ($^1Rf^*$) by FLs (process (2)) was determined by a classical Stern-Volmer treatment employing the expression $I_0 / I = 1 + K_{sv}$ [Chr]. I and I_0 are the respective stationary fluorescence intensities in the presence and in the absence of FLs. K_{sv} is the Stern-Volmer constant ($K_{sv} = {}^1k_q \cdot {}^1\tau_0$) being $^1\tau_0 = 5$ ns the Rf fluorescence lifetime [31].

The conventional method of Laser Flash Photolysis (LFP) could not be used for the determination of the rate constants ^3k_q (process (5)), referred to the quenching of electronically excited Rf ($^3Rf^*$) by the FLs. This was so due to the superimposition of the ground state absorption bands of Rf and FLs at 337 nm, the available excitation wavelength in our LFP apparatus. Nevertheless, an alternative method was employed to solve the point. It is known that the anaerobic visible light-mediated degradation of Rf in solution, mainly occurs from $^3Rf^*$, for which a lifetime ($^3\tau_0$) of 15 μs has been reported [22]. The rate of the degradation process can be estimated through the time evolution of the absorbance decrease in the Rf absorption band at 445 nm. The decomposition rate of the vitamin was determined in the absence ($Rate_{Rf}$) and in the presence ($Rate_{Rf+FLs}$) of several FLs concentrations, in N_2 saturated atmosphere. Employing the Stern-Volmer treatment ($Rate_{Rf} / Rate_{Rf+FLs} = 1 + {}^3k_q{}_{app}\,{}^3\tau_0$ [FLs]) the apparent rate constant, accounting for the quenching of $^3Rf^*$ by FLs was roughly evaluated.

2.2.4. Time Resolved $O_2(^1\Delta_g)$ Phosphorescence Detection (TRPD)

The rate constant k_t for overall quenching of $O_2(^1\Delta_g)$ by the FLs, was determined employing time resolved phosphorescence detection (TRPD), as previously described [32]. The $O_2(^1\Delta_g)$ emission at 1270 nm was generated by excitation of RB solutions at 532 nm with a Nd:YAG laser (Spectron), after filtering with a 1270-nm interference and a wratten filter. The phosphorescence signal was detected at right angle using an amplified Judson J16/8Sp Germanium detector. Aerated solutions were employed in all cases. $O_2(^1\Delta_g)$ phosphorescence lifetimes were evaluated in the absence (τ_0) and in the presence (τ) of the quencher (FLs). The data were plotted according to a simple Stern-Volmer treatment: $\tau_0 / \tau = 1 + k_t\,\tau_0$ [FLs].

Photoprotection of trp by Chr and [Chr_2Cu]: Relative rates for Rf- and RB-sensitized photooxidation of the systems, trp, Chr and [Chr_2Cu] and their mixtures were evaluated through the initial slope of oxygen consumption as a function of photoirradiation time, employing the specific oxygen electrode already described. Normalized rates for each sensitizer family were obtained as the quotient between the respective rate of oxygen uptake for a given sample and that for the faster oxygen-consumer sample of the family.

3. Results

3.1. Preliminary Assays

When individual 0.14 mM Chr and 0.07 mM [Chr_2Cu] solutions, employing aqueous buffer pH 7/EtOH 70:30 (v/v) as a solvent and in the presence of Rf ($A_{445} = 0.4$) were photoirradiated, spectral modifications very similar to each other were observed as shown in Fig. 1A and B. The respective absorption spectra of 0.08 mM Chr (Fig. 1A), 0.028 mM [Chr_2Cu] (Fig. 1B) and 0.06 mM Rf (in Fig. 1A and B) were included for comparative purposes.

In parallel experiments on similar solutions, oxygen consumption upon sensitized photoirradiation was detected. Relative values for this process, evaluated as ROU, are shown in Table 1.

Both families of experiments indicate photoconsumption of all three species the individual FLs, oxygen and the very sensitizer, Rf. This could be attributed to reactions initiated by Rf electronic excited states and/or reactive oxygenated species produced through these states, or even to both processes operating simultaneously [22,24,25].

Rf is highly reactive under visible light irradiation in aqueous solutions due to the generation of radical and/or ROS species such as $O_2^{\bullet-}$ and $O_2(^1\Delta_g)$. On this basis, we have carried out a systematic kinetic study in order to evaluate and characterize the nature, mechanism and extent of the possible processes involved in the Rf-sensitized degradation of Chr and [Chr_2Cu].

3.2. Sensitized Photooxidation Processes

Scheme 1 has been employed for interpretation and discussion of the results. This self-defined reaction sequence include photoprocesses

$Rf + h\nu \rightarrow {}^1Rf^*$	(1)
${}^1Rf^* + FLs \longrightarrow Rf + FLs \text{ or } P$	(2), rate constant ${}^1k_q$
${}^1Rf^* \longrightarrow {}^3Rf$	(3)
${}^3Rf^* + O_2({}^3\Sigma_g^-) \longrightarrow Rf^{\bullet+} + O_2$	(4)
${}^3Rf^* + FLs \longrightarrow Rf^{\bullet-} + FLs^{\bullet+}$	(5) rate constant ${}^3k_q$
$Rf^{\bullet-} + H^+ \rightleftharpoons RfH^{\bullet}$	(6)
$2\ RfH^{\bullet} \longrightarrow Rf + RfH_2$	(7)
$RfH_2 + O_2({}^3\Sigma_g^-) \longrightarrow RfH_2^{\bullet+} + O_2^{\bullet-}$	(8) rate constant k_8
$RfH_2^{\bullet+} + O_2^{\bullet-} \longrightarrow Rf + H_2O_2$	(9)
$O_2^{\bullet-} + FLs \longrightarrow P(10)$	(10) rate constant k_{10}
$H_2O_2 + FLs \longrightarrow P(11)$	(11)
${}^3Rf^* + O_2({}^3\Sigma_g^-) \longrightarrow Rf + O_2({}^1\Delta_g)$	(12) rate constant k_{ET}
$O_2({}^1\Delta_g) \longrightarrow O_2({}^3\Sigma_g^-)$	(13)
$O_2({}^1\Delta_g) + FLs \longrightarrow O_2({}^3\Sigma_g^-) + FLs$	(14) rate constant k_q
$O_2({}^1\Delta_g) + FLs \longrightarrow P(15)$	(15) rate constant k_r
Being $k_t = k_r + k_q$	

Scheme 1. Possible pathways in a Riboflavin-photosensitized process in the presence of an electron donor transparent to the incident light (FLs).

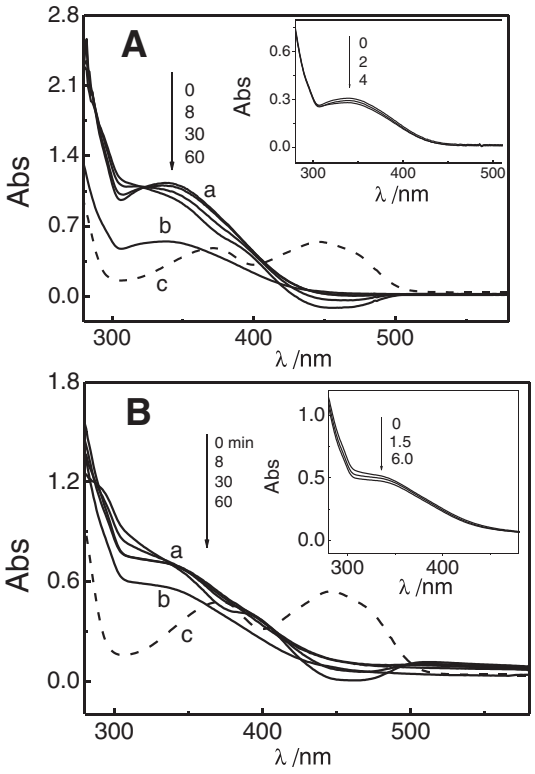

Fig. 1. Panel A. Set a: Changes in the UV–Vis absorption spectrum of an aerated solution of 0.06 mM Rf plus 0.14 mM Chr, taken vs. 0.06 mM Rf, upon photoirradiation; b) absorption spectrum of 0.08 mM Chr vs. solvent. c) Absorption spectrum of 0.06 mM Rf vs. solvent. Inset A: changes in the UV–Vis absorption spectrum of an aerated solution of RB ($A_{549} = 0.5$) plus 0.029 mM Chr, taken vs. RB ($A_{549} = 0.5$) upon photoirradiation. Panel B. Set a: Changes in the UV–Vis absorption spectrum of an aerated solution of 0.04 mM Rf plus 0.07 mM [Chr_2Cu], taken vs. 0.04 mM Rf, upon photoirradiation. b) Absorption spectrum of 0.028 mM [Chr_2Cu] vs. solvent. c) Absorption spectrum of 0.06 mM Rf vs. solvent. Inset A: changes in the UV–Vis absorption spectrum of an aerated solution of RB ($A_{549} = 0.5$) plus 0.057 mM [Chr_2Cu], taken vs. RB ($A_{549} = 0.5$) upon photoirradiation. Numbers on the spectra represent irradiation time in minutes. In all cases the solvent was pH 7/EtOH 70:30 (v/v) and photoirradiation λ > 480 nm.

in the presence and in the absence of an electron donor transparent to the incident light. P(n) are eventual products for each corresponding (n) reaction and $O_2({}^3\Sigma_g^-)$ represents ground state molecular oxygen.

The prevalence of a given photoprocess lies on experimental conditions and nature of the involved compounds. A similar reaction sequence has been previously employed and discussed in relation to other biomolecules [35,36].rate constant k_r

Being $k_t = k_r + k_q$.

3.3. Interaction of Rf Electronically Excited States with Chr and [Chr_2Cu]

Rf presents an intense fluorescence band centered at 515 nm, with a reported emission quantum yield of 0.25 [22]. In the presence of the Chr, the quenching of ${}^1Rf^*$ (Scheme 1, process (2)) produces a decrease in the stationary emission intensity (data not shown). The fluorescence quenching rate constant, ${}^1k_q$, determined as described in the Experimental section, is included in Table 1, as well as the corresponding ${}^1k_q$ value for Cu^{2+}, taken from the literature [33]. Due to solubility limitations ${}^1k_q$ was not determined for [Chr_2Cu].

The rate constant ${}^3k_{q\ app}$, accounting for the quenching of ${}^3Rf^*$ by the FLs (process (5)) was evaluated through the already described Stern-Volmer treatment. The photodecomposition rate of the vitamin, monitored through the absorbance decrease in the 445 nm absorption band, diminished with the increase of FLs concentration (see Fig. 2A for a typical case) and remained constant in the presence of Cu^{2+}, in all cases as compared to the Rf degradation rate in the absence of the

Table 1
Rate constant values for the fluorescence quenching of Riboflavin (k_{q1}); apparent rate constant for the quenching of triplet excited state of Riboflavin ($^3k_{q\text{-}app}$); rate constants for the reactive and overall quenching of $O_2(^1\Delta_g)$ (k_r and k_t), ratio k_r/k_t and relative rates of Chr and [Chr_2Cu] consumption upon Rf (RR_{Rf}) and RB (RR_{RB}) photosensitization. Solvent: aqueous buffer pH 7/EtOH 70:30 v/v.

Compound	^1k_q 10^9 M^{-1} s^{-1} ±10%	$^3k_{q\ app}$ 10^{10} M^1 s^1 ±20%	k_r 10^7 M^{-1} s^{-1} ±5%	k_t 10^7 M^{-1} s^{-1} ±5%	k_r/k_t	RR_{Rf}	RR_{RB}
Chrysin (Chr)	7.0	1.2	1.0 (5.6) (a)	1.1 (b)	~1	1	1
Chrysin-Cu ([Chr_2Cu])	Nd (c)	1.9	0.8 (1.45) (a)	4.8 (b)	0.17	0.5	0.8
Cu^{++}	1.2 (d)	Not observed (e)		6.4 (f)			

(a) Determined in aqueous buffer pH 10/EtOH 70:30 v/v. (b) Determined in aqueous buffer pD 10/EtOD 70:30 v/v. (c) Not determined due to the low solubility of the complex (see text). (d) From ref. [33]. (e) Not observed quenching of $^3Rf^*$ up to $[Cu^{2+}] = 0.01$ mM. (f) From ref. [34].

individual FLs and Cu^{2+}. The obtained $^3k_{q\ app}$ values (see Fig. 2B for a typical case) are shown in Table 1. Even when ±20% error must be attributed to $^3k_{q\ app}$ values, due to the experimental procedure employed, results are in line with the participation of a long-lived intermediary in the photolysis, represented by $^3Rf^*$, which is efficiently intercepted by a relatively low FLs concentration.

3.4. The Interaction of Chr and [Chr_2Cu] With Photogenerated ROS

Eventual oxidative processes due to photoinduced reactions of the FLs with ROS (see Introduction section) were tested through oxygen consumption experiments, using specific ROS interceptors. Similar experiments are currently employed to evaluate the participation of $O_2(^1\Delta_g)$, $O_2^{\bullet-}$ and H_2O_2 in a given oxidation event [37–39].

The rate of oxygen uptake (ROU) in photoirradiated solutions of ($A_{445} = 0.5$) Rf plus individual 0.053 mM Chr or 0.026 [Chr_2Cu] decreases ca. 45% and y 39% respectively in the presence of 1 mM NaN_3, as compared with the ROU for the same solutions in the absence of the salt. To the opposite, for similar experiments replacing NaN_3 by 1 μg/ml SOD or 1 μg/ml CAT, an increase in the ROU was observed for both FLs, always compared to the respective ROU for the solutions in the absence of the ROS interceptors. The effect of SOD is scarcely observed in the case of [Chr_2Cu]. Results are shown in Fig. 3. It is known that NaN_3 deactivates $O_2(^1\Delta_g)$, with a rate constant $k_q = 6.4 \times 10^8$ M^{-1} s^{-1} (reaction (14) with NaN_3 instead of FLS) [28]; SOD dismutates the species $O_2^{\bullet-}$ and CAT decomposes H_2O_2 (reactions (16) and (17) respectively).

$$2O_2^{\bullet-} + 2H^+ + SOD \rightarrow O_2(^3\Sigma_g^-) + H_2O_2 \quad (16)$$

$$2\ H_2O_2 + CAT \rightarrow 2\ H_2O + O_2(^3\Sigma_g^-) \quad (17)$$

3.5. Interaction of $O_2(^1\Delta_g)$ With Chr and [Chr_2Cu] and Determination of the Rate Constants k_t and k_r

In order to quantify the possible interaction FLs-$O_2(^1\Delta_g)$, Rose Bengal (RB) - a well known exclusive photosensitizer for the oxidative species - was utilized [40]. The xanthene dye was selected to focalize on the potential $O_2(^1\Delta_g)$-mediated process. In this way, all possible interferences due to interactions of the FLs with Rf electronically excited states and/or with other photogenerated ROS were eliminated.

The individual photoirradiation of 0.029 mM Chr and 0.028 mM [Chr_2Cu] in the presence of RB ($A_{560} = 0.5$), produces spectral changes shown in Fig. 1A and B, insets. The spectral modifications, quite similar in shape to those shown in Fig. 1 for the case of Rf-sensitization, strongly suggest some degree of interaction of $O_2(^1\Delta_g)$ with the substrates.

Results of TRPD of $O_2(^1\Delta_g)$ of RB ($A_{532} = 0.4$) in aqueous buffer pD 7/EtOD 70:30 v/v, in the absence and in the presence of Chr and [Chr_2Cu] exhibited a first order decay kinetics. The addition of the substrates to the RB solution led to a decrease of the $O_2(^1\Delta_g)$ lifetime. This fact constitutes unambiguous evidence for the interaction $O_2(^1\Delta_g)$-FLs. The respective k_t values were obtained as described in the Experimental section. Typical runs are shown in Fig. 4.

The reactive $O_2(^1\Delta_g)$-FLs interaction was quantified by determining the corresponding rate constants k_r (process (15)) through the actinometric method described in the Experimental section, measuring ROU. RB ($A_{532} = 0.4$) was employed as dye sensitizer. The concentrations of the substrates and the reference were all 0.05 mM in aqueous buffer pH 7/EtOH 70:30 v/v and aqueous buffer pH 10/EtOH 70:30 v/v (Fig. 4). The considerably increment observed in the k_r values constitutes an expected result, typical for an interaction $O_2(^1\Delta_g)$ - phenolic compounds, due to the increase in the electron donating ability produced by the ionization of the OH groups [41]. Results for the neutral aqueous-ethanolic solution are shown in Fig. 4, inset.

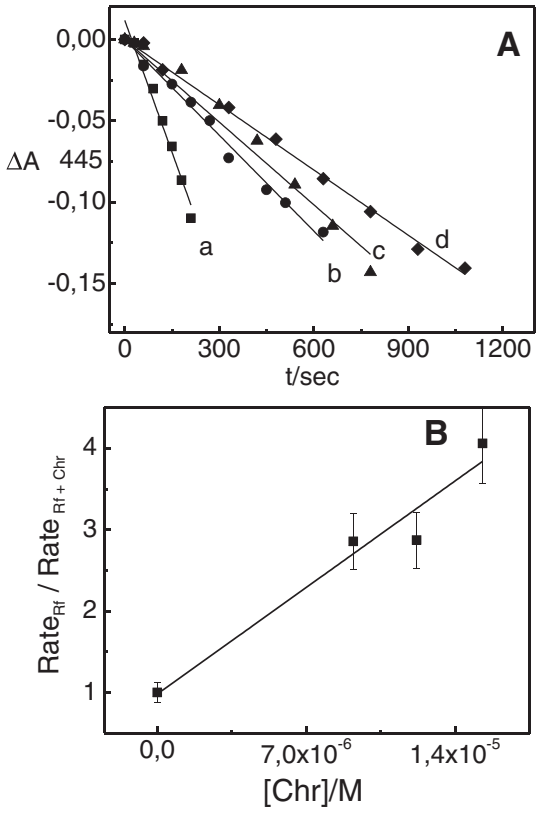

Fig. 2. A. Degradation of Rf as a function of photoirradiation time (cut-off 480 nm), monitored through the decrease in the 445 nm absorbance peak, in the absence (a) and in the presence of Chr: 9,22 μM (b); 12,2 μM (c) and 15.3 μM (d). B. Stern-Volmer treatment for the evaluation of the rate constant for the quenching of $^3Rf^*$ by Chr in buffered pH 7/EtOH 70:30 (v/v) deaerated solutions. $Rate_{Rf}$ and $Rate_{Rf+Chr}$ represent the respective rates of Rf degradation, upon photoirradiation (cut-off 480 nm), in the absence and in the presence of Chr.

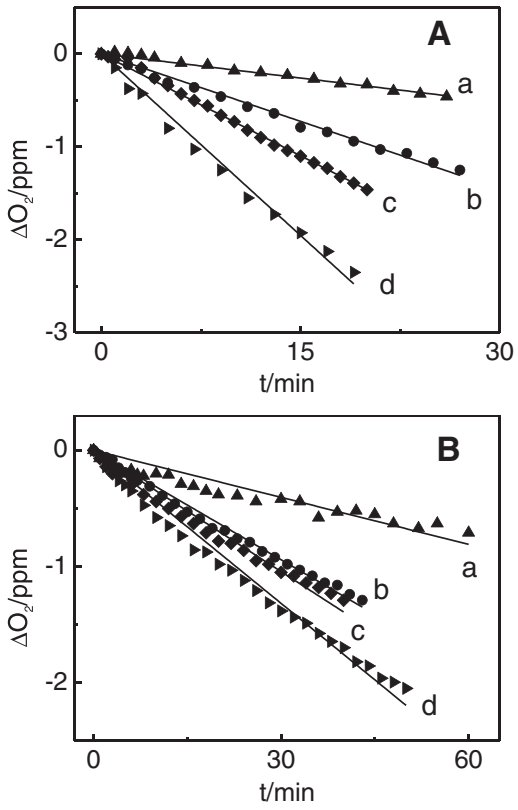

Fig. 3. Oxygen uptake as a function of photoirradiation time (cut-off 480 nm) in buffered pH 7/EtOH 70:30 (v/v) aerated solutions of 0.04 mM Rf plus: Panel A. 0.053 mM Chr in the absence (b) and in the presence of 1 μg/ml CAT (d); 1 μg/ml SOD (c) and 1 mM NaN_3 (a) and Panel B. 0.026 mM [Chr_2Cu] in the absence (b) and in the presence of 1 μg/ml CAT (d); 1 μg/ml SOD (c) and 1 mM NaN_3 (a).

The rate constants k_t and k_r and the k_r/k_t ratios are shown in Table 1. The later constitute a useful information on the substrate oxidability since indicates the fraction of overall quenching of $O_2(^1\Delta_g)$ that effectively leads to a chemical transformation. It can be seen that the interaction of the Chr-$O_2(^1\Delta_g)$ is exclusively reactive in nature, i.e., the oxidative species is entirely deactivated through Chr oxidation. To the contrary, the reactive fraction for the complex only reaches 17% of the overall interaction.

3.6. Potential Activity of Chr and [Chr_2Cu] as Photoprotectors of trp Against Photogenerated ROS

In order to check and compare possible protective effects of Chr and [Chr_2Cu] against oxidative damaging in biological environments, the Rf-photosensitized oxidation of trp was selected as a model system. The amino acid is a classical oxidizable site in photodynamic processes of proteins [28].

The ROU in photoirradiated solutions 0.2 mM of trp 40 μM Rf was determined in the individual presence and absence of 0.2 mM Chr and [Chr_2Cu], employing aqueous buffer pH 7/EtOH 70:30 v/v as a solvent. Again, in order to selectively search for an eventual $O_2(^1\Delta_g)$-mediated process, the same experiments were also performed replacing Rf by RB ($A_{560} = 0.5$) as a sensitizer, in the mentioned solvent. These rates, obtained by monitoring up to 10–15% of the starting dissolved oxygen, represent a measure of the overall photooxidability of each photoirradiated system. The photooxidation rate of trp + Chr, as estimated by the ROU upon Rf-photosensitization (Fig. 5), is close to the simple addition of the respective individual rates of the isolated amino acid plus the isolated flavone. In parallel, the rate for the system trp + [Chr_2Cu] suffers a decrease of ca. 33%, as compared to the addition of the individual ROS of the complex plus trp. Fig. 5 shows that the ROUs

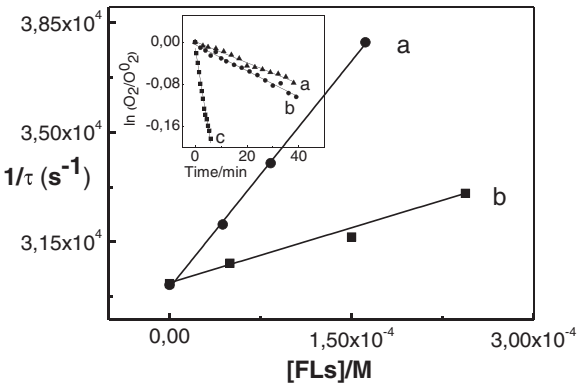

Fig. 4. Stern-Volmer plot for the quenching of $O_2(^1\Delta_g)$ phosphorescence by FLs in buffered pD 7 D_2O/EtOD 70:30 (v/v) aerated solution (a) [Chr_2Cu] and (b) Chr. Sensitizer RB ($A_{532} = 0.31$). Ϯ represents the $O_2(^1\Delta_g)$ phosphorescence lifetime. Inset: first order plots for oxygen uptake in pD 7 H_2O/EtOH 70:30 (v/v) aerated solution upon photoirradiation >480 nm by (a) 0.05 mM [Chr_2Cu]; (b) 0.05 mM Chr and (c) 0.05 mM FFA. $O^0{}_2$ and O_2 represent the dissolved oxygen concentration at photoirradiation time t = 0 and t = t, respectively. Sensitizer RB ($A_{548} = 0.41$).

qualitatively behaved in a similar fashion when RB instead of Rf was employed as a sensitizer, with ca. 27% decrease for the mixture trp + [Chr_2Cu] as compared to addition of the respective individual ROUs. Again the ROU for the mixture AA + Chr is close to the addition of the respective individual ROU values.

4. Discussion

4.1. Interaction of FLs With Rf Electronically Excited States

The observed spectral perturbations in Chr and [Chr_2Cu] and the decrease in the ROU in the presence of NaN_3, all upon Rf-sensitized photoirradiation, strongly support the occurrence of an interaction $O_2(^1\Delta_g)$-FLs. Ulterior experiments, such as those performed employing RB as a dye sensitizer, and very especially those on TRPD, confirm the feasibility of this reaction pathway.

Chr interacts with $^1Rf^*$ with a rate constant value in the order of $10^9\ M^{-1}\ s^{-1}$. Nevertheless, this pathway should not affect the photosensitization results: quencher concentrations much higher than those employed in these experiments are necessary to significantly intercept the electronically excited state of the vitamin, due to its reduced lifetime. Hence photodegradation effects due to reaction (2) should be disregarded. The same can be said for the fraction of free Cu^{2+} present in solution, especially when a maximal concentration of 0.6 μM can be evaluated for the free ion according to already mentioned apparent formation constant and a mean value of 0.2 mM for bulk complex concentration employed in this work.

In parallel, the possible involvement of the interaction of the FLs with ^3Rf, obtained employing concentrations similar to those of the Rf-photosensitization should be discussed.

The operation of reaction (5), under aerobic conditions, depends on whether it is kinetically competitive with reaction (11). The k_{ET} value of reaction (12) in H_2O-EtOH is ca. 1/9 of the diffusional value i.e. $7 \times 10^8\ M^{-1}\ s^{-1}$ [42], while the values obtained for the rate constant ^3k_q in the case of both FLs have a mean value of ca. $1.6 \times 10^{10}\ M^{-1}\ s^{-1}$ (Table 1). For typical photosensitization runs, dissolved oxygen in H_2O-EtOH 70:30 v/v and FLs concentration were 0.85 mM and ca. 0.1 mM respectively [43]. By employing these data a value ca. 2 for the quotient *rate for reaction (5)/rate for reaction (11)* indicates that both processes may simultaneously operate. In other words, this simple kinetic analysis supports the viability of $O_2(^1\Delta_g)$ production under work conditions.

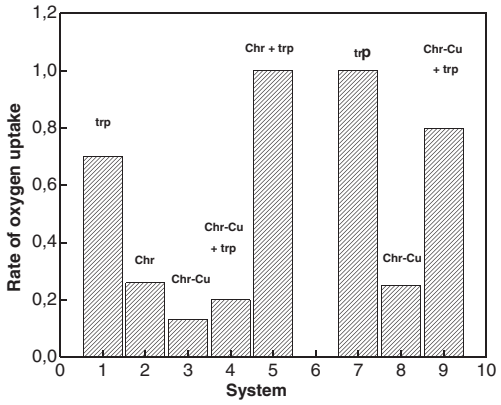

Fig. 5. Rates of oxygen consumption by buffered pH 7/EtOH 70:30 (v/v) aerated solutions employing 0.04 mM Rf as a sensitizer in the presence of 0.05 mM trp (1); Chr (2); [Chr_2Cu] (3); [Chr_2Cu] + trp (4) and Chr + trp (5). The same employing RB ($A_{549} = 0.5$) as a sensitizer in the presence of 0.05 mM (7) trp; [Chr_2Cu] (8) and [Chr_2Cu] + trp (9).

4.2. Interaction of Chr and [Chr_2Cu] With ROS

It is well known that the proteins CAT and SOD are $O_2(^1\Delta_g)$ quenchers [27]. However, the probability that in the stationary photoirradiation runs the ROU were incremented due to possible $O_2(^1\Delta_g)$-mediated oxidation of the proteins, should be disregarded: Results on TRPD show that the $O_2(^1\Delta_g)$ emission lifetime in D_2O-EtOD 70:30 (v/v) remains unchanged in the individual presence and in the absence of 1 μg/ml of the proteins.

As mentioned in the Results section, neatly arises from the ROU traces in the presence of SOD (Fig. 2A and B) a lower increase for [Chr_2Cu] than the increase observed for Chr. Being quite similar the respective ^3k_q values, accounting for the initial step responsible for $O_2^{\bullet-}$ generation (see Scheme 1), it should be expected, in principle, a similar increase in the ROU for both FLs in the presence of SOD. A possible reason for the observed results could be due to a competition SOD-[Chr_2Cu] for the species $O_2^{\bullet-}$. This mechanistic pathway should decrease the amount of $O_2^{\bullet-}$ recycled to $O_2(^3\Sigma_g^-)$ through step (15). This observation is in coincidence with reported results on several metal-chelate compounds in the sense that the presence of Cu adds to the complex the ability of a free radical scavenger [44,45]. Nevertheless, this possibility seems to represent as a minor contribution to the observed overall ROS-FLs interaction. In fact, the reaction FLs-$O_2(^1\Delta_g)$ appears as the dominant mechanistic pathway in a Rf-photosensitized scenery. A quantitative analysis of the results on ROU inhibition by NaN_3 could provide additional insight into the problem: Considering the Stern-Volmer expression $V_o/V_{Az} = 1 + k_{tAz}\tau_{\Delta o}[NaN_3]$, where V_{Az} and V_o are the ROU in a $O_2(^1\Delta_g)$–mediated process in the presence and in the absence of 1 mM NaN_3, where $\tau_{\Delta o} = 4$ μs is the mean $O_2(^1\Delta_g)$ lifetime in water and $k_{tAz} = 6.4 \times 10^8\ M^{-1}\ s^{-1}$ is the rate constant value for overall quenching of $O_2(^1\Delta_g)$ by azide in water [28], the obtained V_o/V_{Az} value is 2.5. It constitutes an intermediate value between 2.7 and 2.1 experimentally determined (Fig. 2A and B) for the respective ROU ratios in the presence and in the absence of Chr and [Chr_2Cu] respectively. Since NaN_3 constitutes a recognized exclusive $O_2(^1\Delta_g)$ quencher, this acceptable agreement between expected and experimental results constitutes an additional argument supporting the occurrence of a preponderant $O_2(^1\Delta_g)$-mediated process in the Rf-sensitized oxygen uptake determinations for the FLs.

4.3. Photoprotective Effect of [Chr_2Cu] Towards trp Oxidation

The effect of the presence of an added oxidizable/electron-donating substrate such as FLs on the photooxidation effectiveness of a given oxidizable target like trp, is not straightforwardly predictable. The effect - if any - depends on several concurrent elements, given by the respective abilities of target and substrate as quenchers of both the long-lived Rf triplet excited state and $O_2(^1\Delta_g)$, the main photogenerated ROS in the present case. The interaction trp-$O_2(^1\Delta_g)$ is well typified, with rate constant values $k_t = 7.2 \times 10^7\ M^{-1}\ s^{-1}$ and $k_r = 4.7 \times 10^7\ M^{-1}\ s^{-1}$ [41].

The photoprotective effect exerted by the complex towards trp-photooxidation is evident by the clear decrease observed in the ROU of the photosensitized system trp – [Chr_2Cu] as compared to the rate in the absence of the complex. The considerably low values for the quotient k_r/k_t exhibited by the complex arises as the main reason for this behaviour. In the case of Chr, the absence of protective effect is evident, i.e.: the ROU for the mixture Chr + trp is practically the same that the addition of individual rates. In this case every quenching event produces the degradation of the scavenger ($k_r/k_t \sim 1$, Table 1). The close agreement in the photoprotective results between Rf- and RB-sensitization observed for [Chr_2Cu], already pointed out in the Results section, adds another argument in favour of a dominant $O_2(^1\Delta_g)$ process in the Rf-sensitization.

Another possible reason for the protective effect could be attributable to the quenching of $^3Rf^*$ by the FLs. These compounds exhibit high ^3k_q values, and could decrease by this way the stationary concentration of $O_2(^1\Delta_g)$ with the concomitant reduction of the overall ROU by the mixture FLs-trp. Nevertheless, a second reactive pathway in the photooxidation of trp is given by reaction (10), with trp instead of FLs. The AA is a known quencher of $^3Rf^*$ with a reported rate constant value $^3k_q = 2.5 \times 10^9\ M^{-1}\ s^{-1}$, ca. one order of magnitude lower than those estimated for FLs [23]. The initial described products of reaction (10) for trp are radicals from the reduced flavin whereas the overall reaction consists in the $O_2^{\bullet-}$-mediated oxidation of the AA [46]. In this context, results shown in Fig. 4, strongly suggest that the fraction of $O_2^{\bullet-}$ produced by the FLs through reactions (5)–(8) is not highly significant to considerably increase the ROU in the mixtures FLs – trp.

5. Conclusions

Photoirradiation of Rf with visible light, in the presence of the FLs Chr and [Chr_2Cu], generates ROS. Direct and indirect evidence demonstrates that mainly $O_2(^1\Delta_g)$ significantly interacts with the FLs. In the case of [Chr_2Cu], this interaction is largely dominated by the physical component. In this way, practically all $O_2(^1\Delta_g)$ is deactivated without significant loss of the quencher. The remarkably low values for the k_r/k_t ratio shown by [Chr_2Cu], constitutes a positive quality for antioxidative protectors in a bio-environment, where high local concentration and high reactivity of relevant biomolecules make them primary targets for the $O_2(^1\Delta_g)$ attack.

Besides, two interesting properties in the field of free radicals scavenging by [Chr_2Cu] must be mentioned. In first place metal chelation itself, in the obvious sense of free metal ion withdrawal from the oxidizable medium, prevents the initiation of a free radical-mediated oxidation processes through mechanisms of Fenton or lipid peroxidation [47,48]. In addition and as already described for similar Cu-chelate compounds, the incorporation of Cu adds to [Chr_2Cu] the ability of a free radical scavenger [44,45].

This set of qualities positions the complex as a promising biocompatible protector towards $O_2(^1\Delta_g)$-mediated oxidation.

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