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Vitamin B_2-sensitized Photo-oxidation of Dopamine

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ABSTRACT

Kinetics and mechanism of the photo-oxidation of the natural catecholamine-type neurotransmitter dopamine (DA) has been studied in aqueous solution, under aerobic conditions, in the presence of riboflavin (Rf, vitamin B_2) as a photosensitizer. Results indicate the formation of a weak dark complex Rf–DA, with a mean apparent association constant $K_{ass} = 30\ M^{-1}$, only detectable at DA concentrations much higher than those employed in photochemical experiments. An intricate mechanism of competitive reactions operates upon photoirradiation. DA quenches excited singlet and triplet states of Rf, with rate constants of 4.2×10^9 and $2.2 \times 10^9\ M^{-1}\ s^{-1}$, respectively. With the catecholamine in a concentration similar to that of dissolved molecular oxygen in air-saturated water, DA and oxygen competitively quench the triplet excited state of Rf, generating superoxide radical anion ($O_2^{\bullet -}$) and singlet molecular oxygen ($O_2(^1\Delta_g)$) by processes initiated by electron and energy-transfer mechanisms, respectively. Rate constants values of 1.9×10^8 and $6.6 \times 10^6\ M^{-1}\ s^{-1}$ have been obtained for the overall and reactive (chemical) interaction of DA with $O_2(^1\Delta_g)$. The presence of superoxide dismutase increases both the observed rates of aerobic DA photo-oxidation and oxygen uptake, due to its known catalytic scavenging of $O_2^{\bullet -}$, a species that could revert the overall photo-oxidation effect, according to the proposed reaction mechanism. As in most of the catecholamine oxidative processes described in the literature, aminochrome is the DA oxidation product upon visible light irradiation in the presence of Rf. It is generated with a quantum yield of 0.05.

INTRODUCTION

Visible light-induced photoprocess on bioactive molecules and pharmaceutical drugs has received particular attention in the last decades because the photoreaction can deeply alter their biologic activity of giving rise to products with different, null or even undesirable therapeutic response (1–3). As most of the biologically relevant molecules are transparent to visible light, the mechanisms governing these photoprocesses are strongly dependent on the characteristics of the photosensitizer (4–6).

A particularly interesting visible light-absorber sensitizer is riboflavin (Rf, see Scheme 1), a member of the vitamin B_2 complex, which is a natural compound present in most living organisms (7). Rf can potentially act as a natural endogenous photosensitizer toward natural or externally added compounds (8). The usual mechanism of action of this sensitizer is rather complex. Rf photodecomposes upon aerobic UV or visible illumination, in most of the cases with the concurrent involvement of reactive oxygen species (ROS) such as singlet molecular oxygen [$O_2(^1\Delta_g)$] and superoxide radical anion ($O_2^{\bullet -}$) (4,6,7,9,10).

In previous works we reported on the Rf-sensitized photo-oxidation of sympathomimetic drugs (SD) (11–13). SD consist of a series of compounds with properties resembling the neurotransmitters epinephrine, norepinephrine and dopamine (4-(2-amino-ethyl)-benzene-1,2-diol, DA) (14). The SD studied were phenylephrine (Phen) and isoproterenol (Iso), with phenolamine- and catecholamine-like structures, respectively. In these cases, the photodegradation of the vitamin, a known process taking place from its excited triplet state, was retarded. The photo-oxidation mechanism of both compounds is quite different. Whereas Phen acts as a typical antioxidant, fairly resistant toward the action of ROS, Iso oxidizes, in a process mainly mediated by superoxide radical anion ($O_2^{\bullet -}$), producing *N*-isopropylaminochrome (IAC) as a main photoproduct (13,15).

In this study we developed a kinetic and mechanistic study on the Rf-sensitized photo-oxidation of the natural catecholamine-type neurotransmitter DA. Thermal oxidative processes on catecholamines play an important role in human physiology and more precisely in the nervous system, and lead to the generation of neuromelanin (16,17). Nevertheless, in spite of the enormous relevance of the biologic processes in which catecholamines are involved, and as recently emphasized by Li *et al.*, its oxidation mechanism is uncertain (18). These compounds were found in the brain and probably might play a role in schizophrenia and in Alzheimer's disease (17,19,20). It is well known that DA is quite sensitive to air and light due to the oxidation of the catechol group to a quinonic derivative (19,21–24). On this basis, we considered a potentially interesting work—the systematic study of possible photodamages—leading to photo-oxidation of DA under vitamin B_2-photosensitizing conditions. The elucidation of kinetics, mechanism and photoproducts can contribute to a substantial understanding of unexpected biochemical transformations which could take place as decomposition pathways of the catecholamines due to photochemical stability of either 4-methylcatechol

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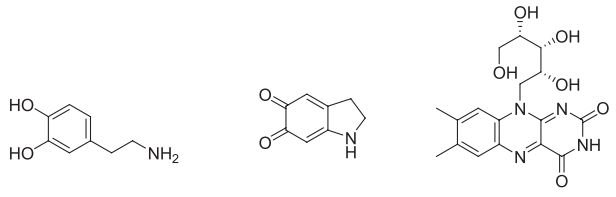


Dopamine (DA) Aminochrome (DPA) Riboflavin (Rf)

Scheme 1. Chemical structures of dopamine (DA), aminochrome (DPA) and riboflavin (Rf).

(4-MC) *in vivo* or under laboratory conditions used in this study. The parent compound was used as a model, for comparative purposes.

MATERIALS AND METHODS

Materials. Riboflavin, deuterium oxide 99.9% (D_2O), superoxide dismutase (SOD) from bovine erythrocyte and dopamine hydrochloride (DA), were purchased from Sigma Chem. Co. Rose bengal (RB), perinaphtenone (PN) and furfuryl alcohol (FFA) were from Aldrich and 4-MC from Fluka. Triethylamine 99% was purchased from Sintorgan. All chemicals were used as received. Zinc tetraphenylporphyrine (ZnTPP) was prepared as previously published (25). Water was triply distilled. Methanol (MeOH) and acetonitrile (MeCN), both high-performance liquid chromatography (HPLC) quality, were provided by Sintorgan. The pH of the final aqueous solutions (Rf + DA, in water) for all photochemical experiments was in the range 6.0 ± 0.1.

Absorption measurements. Ground state absorption spectra were registered employing a Hewlett Packard 8452A diode array spectrophotometer.

Time-resolved $O_2(^1\Delta_g)$ phosphorescence detection (TRPD). The total quenching rate constant (k_t) for $O_2(^1\Delta_g)$ deactivation by DA and 4-MC was determined by near-IR time-resolved phosphorescence. Briefly, the third harmonic (λ = 355 nm) from a Nd:Yag laser (Spectron) was used as the excitation source. The emitted ($O_2(^1\Delta_g)$) phosphorescence at 1270 nm was detected at right angles using an Edinburgh EI-P Germanium detector, after having passed through 1270 nm interference and two Wratten filters. The output of the detector was coupled to a 400 MHz digital oscilloscope (HP 54504A) and to a personal computer to carry out the signal processing. Usually, 10 shots were needed for averaging so as to achieve a good signal-to-noise ratio, from which the decay curve was obtained. Air-equilibrated solutions were employed in all cases.

Stationary photolysis. Stationary aerobic photolysis of aqueous solutions containing DA or 4-MC and Rf was carried out in a home-made photolyzer which uses a blue light-emitting diode (LED) as photoirradiation source. A commercially available blue LED with emission maximum at 467 nm and 30 mA of polarization current was employed.

Determination of k_r and k_t. The reactive rate constant (k_r, see process [9]) for chemical reaction of $O_2(^1\Delta_g)$ with DA or 4-MC was determined using the method described by Scully and Hoigné (26) (Eq. 1), for which the knowledge of the reactive rate constant for the photooxidation of a reference compound R is required:

$$\text{slope}/\text{slope}_R = k_r/k_{r_R} \quad (1)$$

where slope and slope_R are the respective slopes of the first-order plots of DA or 4-MC and reference consumption, or oxygen consumption, under sensitized irradiation. Oxygen uptake in water was monitored with a 97-08 Orion electrode. Assuming that the reaction of $O_2(^1\Delta_g)$ with the quencher is the only means of oxygen consumption, the ratio of the first-order slopes of oxygen uptake by the substrate and the reference compound yields k_r/k_{rR}. The reference was FFA, with a reported pH-independent k_r value of 1.2 10^8 M^{-1} s^{-1} (27).

$O_2(^1\Delta_g)$ lifetimes were evaluated in the presence (τ) and absence (τ_0) of the quencher and the data were plotted as a function of quencher concentration, according to a simple Stern-Volmer treatment (Eq. 2).

$$1/\tau = 1/\tau_0 + k_t[Q] \quad (2)$$

where Q is DA or 4-MC.

The mixture D_2O/H_2O (60:40%, vol/vol) was employed as a solvent in the TRPD experiments in order to enlarge the phosphorescence lifetime of $O_2(^1\Delta_g)$ within temporal range attainable by our photodetector (27).

Fluorescence measurements. Steady-state fluorescence was measured with a Spex Fluoromax spectrofluorometer at 25 ± 1°C in air-equilibrated solutions. Synchronous fluorescence spectroscopy, in which the excitation and emission wavelengths are scanned simultaneously at a fixed offset wavelength ($\Delta\lambda$), generally yields spectra with more features than conventional spectroscopy, because of its high selectivity and fluorimetric sensitivity. For the synchronous fluorescence mode, the following instrumental arrangement was used: 2.1 nm for the excitation and emission slits and $\Delta\lambda$ = 20 nm. The evolution of Rf and DA or 4-MC upon visible light photoirradiation was determined simultaneously.

Fluorescence lifetimes were determined with a time-correlated single photon counting technique (SPC) on an Edinburgh FL-900CD instrument, equipped with a blue LED (PicoQuant PLS-8-2-208). Excitation and emission wavelengths for Rf were 450 and 515 nm, respectively.

For the determination of the rate constants for the interactions of ^{1}Rf* with DA or 4-MC (reaction [4]) a classical Stern-Volmer treatment of the data was applied through the following equations:

$$I_0/I = 1 + K_{SV}[Q] \quad (3)$$

$$1_{\tau 0}/1_\tau = 1 + {}^1k_{q\tau 0}^1[Q] \quad (4)$$

where I, I_0, $^1\tau$ and $^1\tau_0$ are the respective stationary intensities and lifetimes for Rf fluorescence in the presence and absence of the quenchers (DA and 4-MC), being $K_{SV} = {}^1k_q\,{}^1\tau_0$.

Laser flash photolysis experiments. Transient absorption spectra were determined in Argon-saturated 0.04 mM Rf aqueous solutions using a flash photolysis apparatus. The above described Nd:YAG laser was employed to generate ^{3}Rf* and a 150 W xenon lamp as a source for the analyzing light. The detection system comprised a Photon Technologies International (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* a HPIB parallel interface, where it was analyzed and stored. ^{3}Rf* disappearance was monitored from the first-order decay of the absorbance at 670 nm, a zone where the interference from other possible species is negligible. To avoid self-quenching and triplet-triplet annihilation, the triplet decay was measured at low Rf concentration (typical 0.05 mM) and at low enough laser energy.

For the determination of the rate constant for interaction of ^{3}Rf*-DA or 4-MC, the Stern-Volmer expression (Eq. 5) was employed

$$1/{}^3\tau = (1/{}^3_{\tau 0}) + 3k_q[\text{DA}] \text{ or } [\text{4-MC}] \quad (5)$$

where $^3\tau$ and $^3\tau_0$ are the experimentally determined lifetimes of ^{3}Rf* in the presence and absence of DA or 4-MC.

Electron transfer quantum yield ($\Phi_{RfH\bullet}$) was determined relative to the triplet yield of zinc tetraphenylporphyrin (Φ_T) in benzene, employing Eq. 6 (28,29)

$$\Phi_{RfH\bullet} = \Phi_T\varepsilon_T OD_{RfH\bullet}/OD_T\varepsilon_{RfH\bullet} \quad (6)$$

where OD_T is the triplet absorbance at 470 nm immediately after the laser pulse, ϵ_T is the molar absorption coefficient of ZnTPP triplet, and $OD_{RfH\bullet}$ and $\epsilon_{RfH\bullet}$ are the absorbance and the molar absorption coefficients of the Rf radical anion at 570 nm. The molar absorption coefficient of 5.1 10^3 M^{-1} cm^{-1} for Rf neutral radical ($\varepsilon_{RfH\bullet}$) was used (30) and values of 7.3 10^4 M^{-1} cm^{-1} and 0.83 were used for ϵ_T and Φ_T, respectively (29).

Determination of the quantum yield of aminochrome production through Rf-sensitized process. The quantum yield of DPA production (Φ_{DPA}), photosensitized by Rf in water, was determined employing the production of IAC by Iso photosensitized by Rf in water (13) as a reference reaction, and by means of Eq. 7.

$$\Phi_{DPA} = R_{DPA} \times \Phi_{IAC}/R_{IAC} \quad (7)$$

R_{IAC} is the rate of IAC generation, spectrophotometrically monitored by the absorbance increase at 495 nm as a function of irradiation time. Φ_{IAC} is the reported quantum yield of IAC generation, in water ($\Phi_{IAC} = 0.15$) (13) and R_{DPA} is the rate of DPA generation, spectrophotometrically monitored by the increase in the absorption band of DPA at 494 nm as a function of the irradiation time. The molar absorption coefficients employed for IAC and DAC were 4400 and 2907 M^{-1} cm^{-1} respectively (23,31).

The photolyzed independent aqueous solutions contained Rf (Abs $Rf_{446} = 0.4$) and Iso or DA 0.4 mM. Photoirradiation was carried out in 1 cm path quartz cells, magnetically stirred, containing 3 mL solution, employing the blue LED described above.

HPLC experiments. The aminochrome (DPA) employed as a standard was obtained by steady-state photolysis at 280 nm of aqueous solutions carried out in a PTI unit, composed by a high pass monochromator and a 150 W Xe lamp as described in the literature (23). The formation of aminochrome was determined by HPLC analysis, employing a Varian 5000 coupled to a UV–Vis detector (Varian 2550). A stainless steel analytical column (Varian SP-C8-IP-5 5μm 4 × 250 mm) was used for the chromatographic analysis. The mobile phase consisted of H_2O:MeOH (60:40) or H_2O:MeCN (90:10), in both cases at pH = 5 and in the presence of 1 mM triethylamine. The flow rate was adjusted to 1 mL min^{-1}. The detection wavelength was 490 nm.

RESULTS

The usual photochemical mechanism of Rf sensitization is rather complex. Results were interpreted and discussed on the basis of the following reaction scheme (Scheme 2) and other reaction steps included elsewhere in the next pages.

Upon light absorption, Rf gives rise to its excited singlet state (^{1}Rf*) and, through intersystem crossing, to the excited triplet state (^{3}Rf*) (process [2], Scheme 2). ^{3}Rf* transfers energy and one electron to ground state oxygen, $O_2(^3\Sigma_g^-)$ dissolved in the solution. Consequently, $O_2(^1\Delta_g)$ (process [5]) and ($O_2^{\bullet-}$) (process [6]) are produced with quantum yields of 0.49 and 0.009, respectively (10). Moreover, Rf can associate in dark with different compounds, including SD (process [1]).

Dark complexation Rf–DA and interaction ^{1}Rf*-DA

The fluorescence quenching method was employed for evaluation of the apparent association constant (K_{ass}, Scheme 2) for the systems Rf–DA and Rf–4-MC (32), in aqueous solution.

Rf presents an intense fluorescence emission band, centered at 515 nm, with a reported fluorescence quantum yield (Φ_F) of 0.25 (33). In the presence of DA or 4-MC, the fluorescence quenching of excited singlet Rf (^{1}Rf*, Scheme 2) produces a decrease in the stationary emission intensity, but the shape of the emission spectrum does not change. In parallel, the fluorescence decay of Rf in the absence and presence of DA or 4-MC, as determined by the SPC technique, was monoexponential. Figure 1 shows the respective Stern-Volmer plots obtained from static and time-resolved methods.

The first one presents a neat positive curvature, whereas the plot for the time-resolved data is linear. This behavior corresponds to the typical case in which a fluorescent probe is simultaneously quenched by dark association with its ground state and by collisional interaction with its excited singlet state. It is also well known that these systems can be treated using the modified Stern-Volmer equation (32):

$$\mathrm{Rf} + \mathrm{DA} \underset{K_{ass}}{\rightleftharpoons} [\mathrm{Rf}\cdots\mathrm{DA}] \quad (1)$$

$$\mathrm{Rf} \xrightarrow{h\nu} {}^1\mathrm{Rf}^* \longrightarrow {}^3\mathrm{Rf}^* \quad (2)$$

$${}^1\mathrm{Rf}^* \xrightarrow[{}^1k_d]{} \mathrm{Rf} \quad (3)$$

$${}^1\mathrm{Rf}^* + \mathrm{DA} \xrightarrow[{}^1k_q]{} \mathrm{Rf} + \mathrm{DA} \quad (4)$$

$${}^3\mathrm{Rf}^* + O_2({}^3\Sigma_g^-) \xrightarrow[k_{ET}]{} \mathrm{Rf} + O_2({}^1\Delta_g) \quad (5)$$

$${}^3\mathrm{Rf}^* + O_2({}^3\Sigma_g^-) \xrightarrow[k_{eT}]{} \mathrm{Rf}^{\bullet+} + O_2^{\bullet-} \quad (6)$$

Scheme 2. Possible reactions in the visible light irradiation of riboflavin (Rf) in the presence of dopamine or 4-methylcatechol (Q).

$$I_0/I = (1 + K_D[Q])(1 + K_{ass}[Q]) \quad (8)$$

where the constant $K_{D,}$ being $K_D = {}^1k_q\ {}^1\tau_0$, accounts for the dynamic component of the fluorescence quenching and was determined independently of the time-resolved measurements. From Fig. 1, K_D values of 20 ± 1 and 25 ± 1 M^{-1} were found for DA and 4-MC, respectively. Employing the obtained $^1\tau_0$ value of 4.8 ns, which is in excellent agreement with literature reports (7), ${}^1k_q = 4.2 \times 10^9$ M^{-1} s^{-1} and ${}^1k_q = 5.2 \times 10^9$ M^{-1} s^{-1} were calculated for DA and 4-MC, respectively. Hence, values of 30 ± 1 and 43 ± 1 M^{-1} were obtained, respectively, for K_{ass} by nonlinear least square fitting of Eq. 8, using the previously mentioned values.

Stationary photolysis

As shown in Fig. 2, visible light photoirradiation of the mixture Rf (0.030 mM)–DA (0.60 mM) in water produces spectral changes in the UV–Vis spectra that can be attributed to transformations in both components of the mixture. Synchronous fluorescence allows the monitoring of Rf (483 nm) and DA (289 nm) evolutions, simultaneously. These changes can be neatly observed in the synchronic fluorescence spectra shown in Fig. 2 (inset).

These results strongly suggest the intermediacy of electronically excited states of Rf, with or without the concomitant participation of active oxygenated species such as $O_2(^1\Delta_g)$ and/or $O_2^{\bullet-}$, as depicted in Scheme 2.

Interaction $O_2(^1\Delta_g)$-DA. Determination of the rate constants k_t and k_r

$O_2(^1\Delta_g)$ can be physically quenched either by the medium (process [9]) or by DA (process [10]), or can react with DA (process [11]). Reaction (11), with the so-called reactive or chemical rate constant k_r, is the main pathway of disappearance of DA in $O_2(^1\Delta_g)$-mediated processes.

$$O_2({}^1\Delta_g) \xrightarrow[k_d]{} O_2\left({}^3\sum\nolimits_g^-\right) \quad (9)$$

$$O_2({}^1\Delta_g) + \mathrm{DA} \xrightarrow[k_q]{} O_2\left({}^3\sum\nolimits_g^-\right) + \mathrm{DA} \quad (10)$$

$$O_2({}^1\Delta_g) + \mathrm{DA} \xrightarrow[k_r]{} P_1 \quad (11)$$

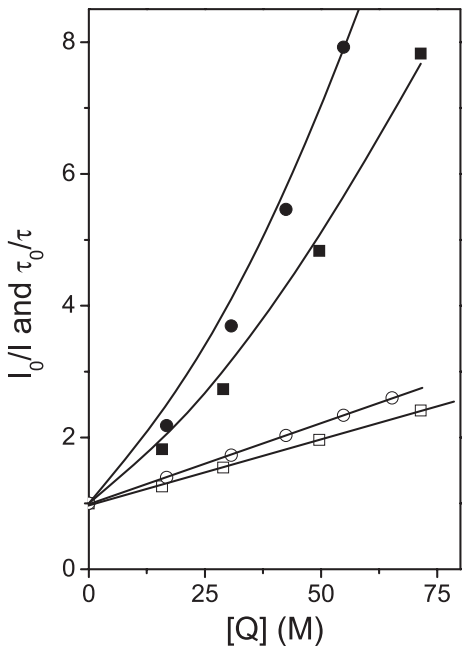


Figure 1. Stern-Volmer plots for the stationary (filled symbols) and time-resolved fluorescence (open symbols) quenching of $^1Rf^*$ by DA (■) and 4-MC (●).

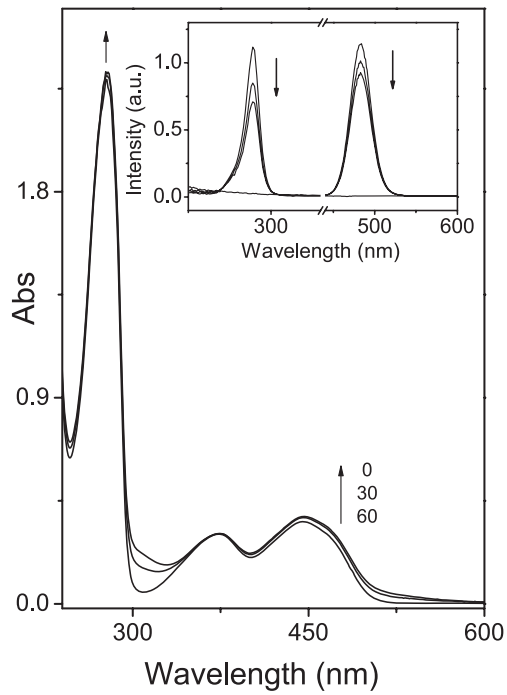


Figure 2. Absorption spectra of Rf (30 μM) + DA (0.60 mM) upon irradiation at 467 nm under air-saturated conditions. Numbers on the spectra represent the irradiation time in minutes. Main: changes in the UV–Vis absorption spectra. Inset: changes in the synchronous fluorescence signal.

In all experiments involving possible $O_2(^1\Delta_g)$-mediated reactions, the sensitizers were exclusive $O_2(^1\Delta_g)$ generators, such as PN (quantum yield for $O_2(^1\Delta_g)$ generation, $(\Phi_\Delta) \sim 1$, in water [34]) and RB ($\Phi_\Delta = 0.75$, in water [35]). Rf was not employed as a sensitizer in order to avoid all possible interferences by other oxidative species potentially generable by the vitamin.

By means of the time-resolved quenching of $O_2(^1\Delta_g)$ phosphorescence the overall quenching constant ($k_t = k_q + k_r$) value of $(1.9 \pm 0.1)\ 10^8\ M^{-1}\ s^{-1}$ was obtained for DA and $(2.1 \pm 0.1) \times 10^8\ M^{-1}\ s^{-1}$ for 4-MC, employing PN as a sensitizer in a D_2O/H_2O (60:40%, vol/vol) solution (Fig. 3, inset). The sensitizer exhibits absorption of 0.22 at 355 nm.

As SOD was used in complementary photolysis experiments (see below), the possible quenching of $O_2(^1\Delta_g)$ by the enzyme was evaluated through TRPD. No quenching of the oxidative species was observed up to a concentration of 0.5 μM, higher than those employed in this study, under the same experimental conditions described for the determination of k_t for DA and 4-MC.

The k_r value was independently determined by means of oxygen uptake measurements (see Materials and Methods) employing 0.05 mM RB as a dye sensitizer (Fig. 3). This dye, possibly the most widely employed in $O_2(^1\Delta_g)$ research (15), strongly absorbs in the spectral region above 450 nm.

k_r values of $(8.8 \pm 0.3) \times 10^6$ and $(2.4 \pm 0.1) \times 10^6$ were obtained for DA and 4-MC, respectively, from the first-order plots shown in Fig. 3, employing FFA as a reference compound. Experimental data from stationary and time-resolved experiments unambiguously demonstrate the interaction between $O_2(^1\Delta_g)$ and both DA and 4-MC.

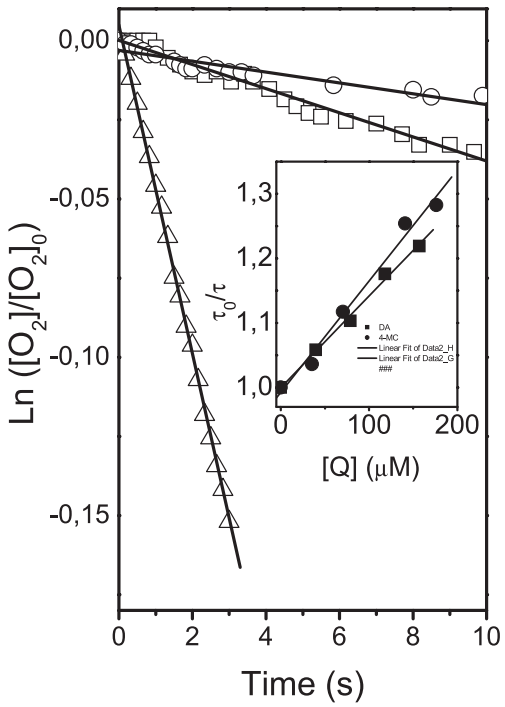


Figure 3. First-order plots for oxygen uptake upon visible light irradiation in aqueous solutions containing: (△) RB (Abs. at 549 nm = 0.442) + [FFA] = 0.69 mM; (□) RB (Abs. at 549 nm = 0. 442) + [DA] = 0.77 mM; (○) RB (Abs. at 549 nm = 0. 442) + [4-MC] = 0.82 mM. (–) Adjustment of the experimental data fitted by means of linear least square. Inset: Stern-Volmer plot for O_2 ($^1\Delta_g$) quenching by DA (■) and 4-MC (●).

Interaction ^{3}Rf*-DA. Laser flash photolysis experiments and kinetic data

^{3}Rf* lifetime was neatly reduced by the individual presence of DA or 4-MC, demonstrating the occurrence of an interaction between these substrates and the electronically excited triplet pigment. Bimolecular rate constants ^3k_q (process [12]) of (2.2 ± 0.2) 10^9 м$^{-1}$ s^{-1} and (3.6 ± 0.2) 10^9 м$^{-1}$ s^{-1} were graphically obtained (Fig. 4, inset) for DA and 4-MC, respectively.

$$^3\mathrm{Rf}^* + \mathrm{DA} \underset{^3k_q}{\longrightarrow} \mathrm{Rf}^{\bullet -} + \mathrm{DA}^{\bullet +} \quad (12)$$

$$\mathrm{Rf}^{\bullet -} + \mathrm{H}^+ \longrightarrow \mathrm{RfH}^{\bullet} \quad (13)$$

$$2\mathrm{RfH}^{\bullet} \longrightarrow \mathrm{RfH_2} + \mathrm{Rf} \quad (14)$$

$$\mathrm{RfH_2} + \mathrm{O_2}(^3\Sigma_g^-) \rightarrow [\mathrm{RfH_2^{\bullet +}} + \mathrm{O_2^{\bullet -}}] \rightarrow \mathrm{Rf} + \mathrm{H_2O_2} \quad (15)$$

The transient absorption spectrum of the Rf solution immediately after the laser pulse (Fig. 4) is coincident with that attributed to ^{3}Rf*, whereas the shape of the spectrum obtained in the presence of 0.71 mм DA is similar to that reported to account for the species RfH$^{\bullet}$ (11,36) (process [12] followed by process [13]). It is well documented (11,15,36) that this species is produced by electron transfer from different electron-donor substrates, some of them of biologic importance, to ^{3}Rf* (Fig. 4).

Quantum yields of 0.34 ± 0.03 and 0.10 ± 0.01 for semireduced neutral Rf generation ($\Phi_{\mathrm{RfH}^{\bullet}}$) by DA and 4-MC was determined using Eq. 6. It is known that the species RfH$^{\bullet}$, with a pK value of 8.3 disproportionates through process (14), and the byproduct RH_2 generates $O_2^{\bullet -}$ through step (15).

Oxygen consumption upon Rf sensitization

Photoirradiation of aqueous solutions of Rf (34 μм) + DA (0.43 mм) gave rise to oxygen consumption (Fig. 5) compared to a solution of Rf (34 μм) in the absence of DA. Results on $\Phi_{\mathrm{RfH}^{\bullet}}$, in the preceding paragraph, show that Rf can produce a considerable amount of the species $O_2^{\bullet -}$ (reaction [15]). In order to evaluate the participation of this species in the reaction mechanism, the photolysis experiments, for DA and 4-MC, were performed in the presence of SOD in the range 0–0.38 μм. The enzyme is a specific $O_2^{\bullet -}$ quencher and has been frequently employed, in similar concentrations, to confirm/discard the participation of the oxygenated species in a given reaction step (15,16,21,37–39). Results (Fig. 5, main) indicate an increase in the rate of oxygen uptake in the presence of SOD.

Besides, photoirradiation of aerobic solutions of Rf (34 μм) and DA (0.43 mм) results in a progressive oxidation of DA to a product whose accumulation could be followed at 494 nm. The presence of SOD, explored in the concentration range 0–0.057 μм, increased the rate of product formation (Fig. 5, inset). Light, DA, oxygen and Rf were all essential components, in that no reaction was observed in the absence of any of them.

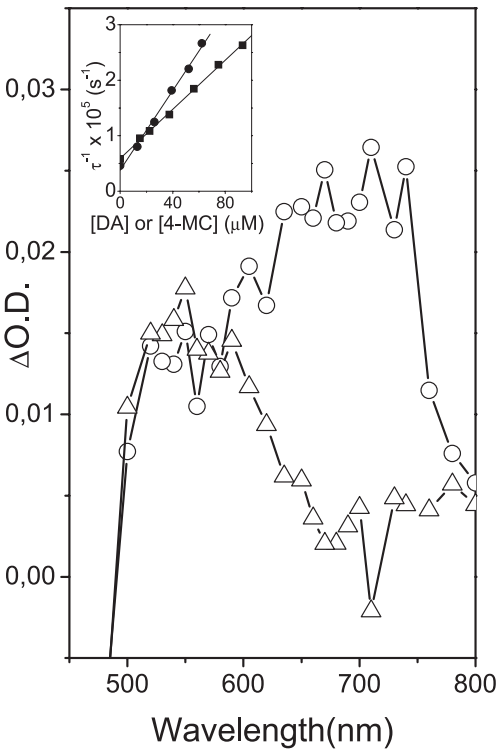


Figure 4. Transient absorption spectra of Rf (Abs 0.318 at 355 nm) in argon-saturated aqueous solution, in the absence (○) and presence (□) of DA (0.71 mм) taken at 1 and 20 μs after the laser pulse, respectively. Inset: Stern-Volmer plot for the ^{3}Rf* quenching by DA (■) and 4-MC (●).

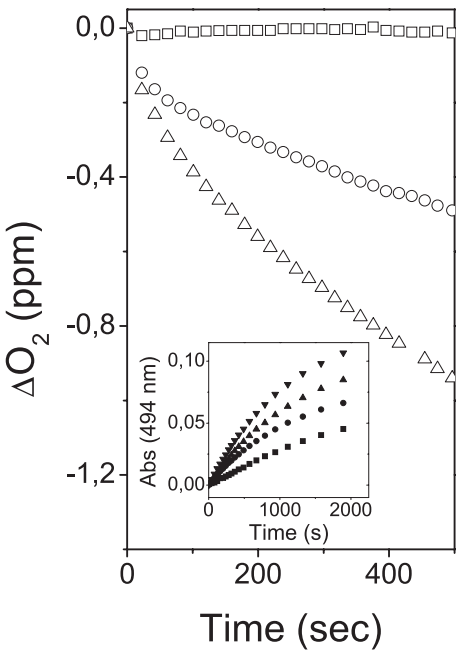


Figure 5. Effect of SOD on the photosensitized oxidation of dopamine. Oxygen uptake upon visible light irradiation (cutoff 300 nm) of aqueous solutions containing: (□) Rf (0.033 mм); (○) Rf (0.033 mм) + DA (0.4 mм); (△) Rf (0.033 mм) + DA (0.4 mм) + SOD (0.38 μм). Inset: Effect of SOD in the rate of dopaminochrome by photolysis of the Rf (32.6 μм) + DA (430 μм) system; (■) plus [SOD] = 0 μм, (●) plus [SOD] = 0.014 μм, (▲) plus [SOD] = 0.029 μм, (▼) plus [SOD] = 0.057 μм.

Elucidation of the main photoreaction product

High-performance liquid chromatography analysis on the photolyzed Rf–DA aqueous solution showed the presence of only two peaks, belonging to the photoproduct and to remaining Rf, respectively. The first peak exhibits the same retention time as the photoproduct of DA, obtained by direct UV photolysis of DA (Table 1). To assign the chemical identity of the eluting band, the matching of the capacity factors k' (Eq. 16) obtained in two different media was used as criteria for the identification of photoproducts (13,40).

$$k' = \frac{(t_r - t_0)}{t_0} \tag{16}$$

In the above equation, t_r is the retention time of the analyte and t_0 is the retention time of the injection peak. Using the injection peak as t_0, the respective capacity factors of DPA and the photoproduct obtained in the Rf-sensitized photolysis were 0.16 and 0.14, respectively, for both derivatives with MeOH-water (60:40, vol/vol) as a mobile phase and 0.68 in MeCN-water (10:90, vol/vol) as a mobile phase, in the HPLC analysis. These results strongly suggest that the identity of both photoproducts is the same (Table 1).

Quantum yield of aminochrome generation

An overall quantum yield of 0.05 was graphically obtained for the production of DPA from DA, photosensitized by Rf in water.

DISCUSSION

Flavins are one of the most extensively studied families of biomolecules, in relation to their ability to form dark association complexes (33,41,42). The K_{ass} values of 30 and 43 M^{-1} for Rf–DA and Rf–4-MC complexation denote a weak interaction and indicates that the fraction of complexed pigment can be ignored when the work is developed under sensitizing conditions, *i.e.* Rf and DA or 4-MC in the sub-mM concentration range. Similar observations have been reported for the Rf–SD complexes (11,12,15).

From the stationary photolysis experiments on the Rf–DA system, the photodecomposition of DA was detected. According to the quenching rate constant values of ^{1}Rf* and ^{3}Rf*, both closer to the diffusion limit, a DA concentration of *ca* 0.6 mM produces *ca* 96% deactivation of ^{3}Rf* and only *ca* 1% deactivation of ^{1}Rf*. These values suggest that the main photodegradation pathways on DA and 4-MC should be focalized on those processes that involve ^{3}Rf* and the reactive oxygenated species produced from this electronic state.

Table 1. Retention times (R_t) and capacity factors (k') for aminochrome obtained through direct (A) and Rf-sensitized (B) photolysis of dopamine in two mobile phase systems. Row C shows values for the eluting band corresponding to Rf, employed in the Rf-sensitized runs.

	R_t (min)	k'	R_t (min)	k'
	MeOH-water (40:60 vol/vol)		MeCN-water (10:90 vol/vol)	
A	4.97	0.16	4.58	0.68
B	4.93	0.14	4.60	0.68
C	5.73	0.33	31.6	10.6

Under aerobic conditions, and within typical concentrations employed in this work for the stationary photolysis experiments, *i.e. ca* 0.6 mM for DA and approximately the same value for dissolved oxygen in water (35), employing the determined value of 2.2 10^9 M^{-1} s^{-1} for ^3k_q and assuming a value $k_{ET} = 7 \times 10^8$ M^{-1} s^{-1}, equivalent to 1/9 of the diffusion-controlled rate constant value in water (43), the rates of $O_2(^1\Delta_g)$ and $RfH^\bullet$ generation are quite similar. The interaction of ^{3}Rf* with oxygen should be totally ascribed to the $O_2(^1\Delta_g)$ generation pathway (step [5]) due to the reported extremely low quantum yield of direct $O_2^{\bullet-}$ production (10) (step [6]). Nevertheless, the upper limit of 0.34 calculated for $\Phi^\bullet_{RfH}$ strongly suggests that step (13) could be the dominant source of $O_2^{\bullet-}$ production and concomitantly, reaction (17) could represent an important photo-oxidative pathway.

$$O_2^{\bullet-} + DA \rightarrow P_2 \tag{17}$$

The ratio $k_r/k_t \sim 0.05$ and $k_r/k_t \sim 0.01$, for DA and 4-MC, respectively, clearly indicates that $O_2(^1\Delta_g)$-mediated events predominantly proceed by a physical pathway, and the effective photo-oxidation of the substrates by that way should constitute a minor contribution.

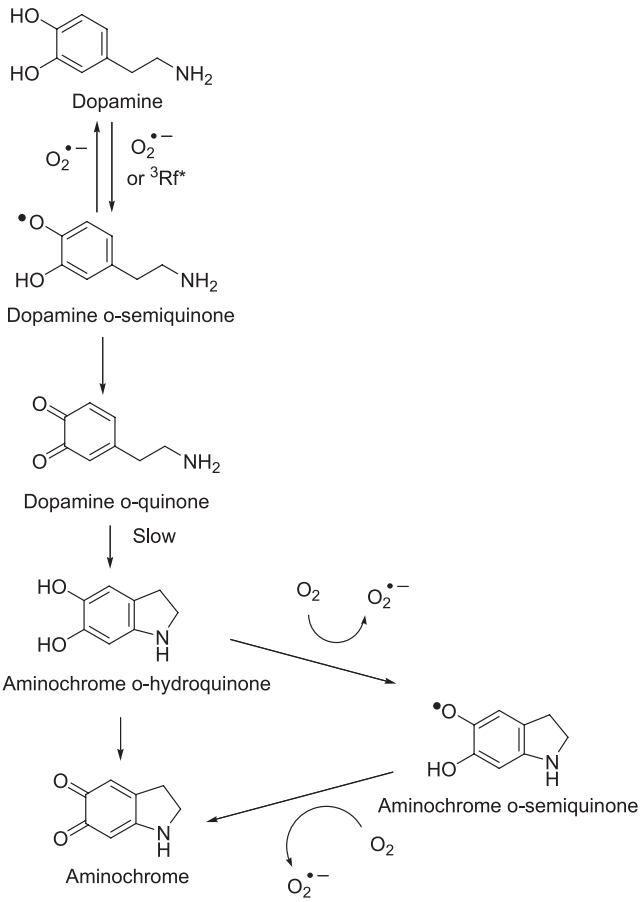
The observed increment in the rates of oxygen uptake and photoproduct generation upon Rf-sensitized irradiation of DA (Fig. 5), in the presence of SOD, confirm the involvement of $O_2^{\bullet-}$ in the overall oxidative photoprocess. It has been reported for other systems that the enzyme competes for the available $O_2^{\bullet-}$ with the oxidizable substrate by catalysis of reaction (15), inhibiting the oxidative pathway process (reaction [17]) (15,16,21,37–39). In our case, and on the basis that SOD is a specific $O_2^{\bullet-}$ scavenger, and does not quench the species $O_2(^1\Delta_g)$, within the SOD concentration employed, the initial photo-oxidation of DA generates both $O_2^{\bullet-}$ and the radical species $DA^{\bullet+}$ (reactions [14] and [12] respectively), which can react to reverse the overall effect of the photo-oxidation by reduction of the intermediate species $DA^{\bullet+}$ (reaction [19]), as already reported for the Rf-sensitized photo-oxidation of dianisidine (44).

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \tag{18}$$

$$O_2^{\bullet-} + DA^{\bullet+} \longrightarrow O_2 + DA \tag{19}$$

Regarding the photo-oxidation products, oxidation of DA to aminochrome by $O_2^{\bullet-}$ (45) or by one-electron transfer step (24,31,46) mechanisms have been described previously. In a similar fashion we propose that DA semiquinone could be produced by $O_2^{\bullet-}$ or by electron transfer from ^{3}Rf* (reaction [12]). As shown in Scheme 3, after disproportionation ortho-quinone is produced and this compound undergoes slow cyclization, forming aminochrome o-hydroquinone, which is oxidized to adenochrome or, upon reaction with ground state O_2, generates the respective aminochrome and $O_2^{\bullet-}$ (24,47–49).

Adenochrome and IAC have been described as final products in the photo-oxidation of the structurally related DA compounds adrenaline and Iso, photosensitized by



Scheme 3. Proposed pathways for the riboflavin-sensitized photooxidation of dopamine.

methylene blue and Rf, respectively, in both cases by $O_2^{\bullet-}$-mediated processes (13,21).

The value of 0.05 determined for the overall quantum yield of DPA production in the Rf-sensitized process appears relatively low, considering the relatively high Rf quantum yields of $O_2(^1\Delta_g)$ and $RfH^{\bullet}$ generation. Nevertheless, this result can be rationalized on the basis that the $O_2(^1\Delta_g)$-mediated interaction is dominated by the physical pathway and that the generation of $RfH^{\bullet}$—the $O_2^{\bullet-}$ precursory species—is greatly reduced under aerobic conditions, due to competitive reactions.

CONCLUSIONS

Dopamine is oxidized in a Rf-photosensitized process. Although $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$ are generated due to the sensitization and quenching of $^3Rf^*$, only $O_2^{\bullet-}$ and the interaction between $^3Rf^*$ and DA is involved in the effective photooxidation of DA.

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