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PAPER

Mechanism of electron transfer processes photoinduced by lumazine

M. Paula Denofrio,^a M. Laura Dántola,^a Patricia Vicendo,^b Esther Oliveros,^b Andrés H. Thomas^a and Carolina Lorente^{*a}

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UV-A (320–400 nm) and UV-B (280–320 nm) radiation causes damage to DNA and other biomolecules through reactions induced by different endogenous or exogenous photosensitizers. Lumazines are heterocyclic compounds present in biological systems as biosynthetic precursors and/or products of metabolic degradation. The parent and unsubstituted compound called lumazine (pteridine-2,4(1,3*H*)-dione; Lum) is able to act as photosensitizer through electron transfer-initiated oxidations. To get further insight into the mechanisms involved, we have studied in detail the oxidation of 2'-deoxyadenosine 5'-monophosphate (dAMP) photosensitized by Lum in aqueous solution. After UV-A or UV-B excitation of Lum and formation of its triplet excited state (³Lum*), three reaction pathways compete for the deactivation of the latter: intersystem crossing to singlet ground state, energy transfer to O₂, and electron transfer between dAMP and ³Lum* yielding the corresponding pair of radical ions (Lum^{•−} and dAMP^{•+}). In the following step, the electron transfer from Lum^{•−} to O₂ regenerates Lum and forms the superoxide anion (O₂^{•−}), which undergoes disproportionation into H₂O₂ and O₂. Finally dAMP^{•+} participates in subsequent reactions to yield products.

Introduction

Within the pteridine family, pterins are those compounds derived from 2-aminopteridine-4(3*H*)-one (pterin; denoted Ptr), whereas lumazines are derived from pteridine-2,4(1,3*H*)-dione (lumazine; denoted Lum). Lumazines are present in living systems as the main products of the metabolic degradation of reduced pterins.^{1,2} In addition 6,7-dimethyl-8-ribityllumazine is the biosynthetic precursor of riboflavin (vitamin B2).³ Although Lum presents different acid–base equilibria in aqueous solutions, the only relevant equilibrium at physiological pH involves the neutral form and the monoanion (Fig. 1), with a p*K*_a value of 7.95.⁴ The absorption spectra of both acid and base forms, although quite different, have intense bands in the UV-B (280–320 nm) and UV-A (320–400 nm) spectral regions (Fig. 1).

Solar radiation induces modifications to genomic DNA and is implicated in the generation of human skin cancers.^{5,6} UV radiation at wavelengths lower than 300 nm damages DNA as a result of the direct excitation of the nucleobases.⁷ On the other hand, although nucleobases absorb very weakly above 300 nm, both UV-B and UV-A radiation can induce modifications to DNA through photosensitized reactions.^{7,8} This indirect action may be mediated

Fig. 1 Molecular structures of Lum and dAMP, and corresponding absorption spectra in air-equilibrated aqueous solutions; solid line: acid form of Lum (pH = 5.5); dashed line: basic form of Lum (pH = 10.5); dashed–dotted line: dAMP.

by endogenous or exogenous sensitizers. The chemical changes in DNA and its components resulting from photosensitized reactions can take place through different mechanisms. Energy transfer from the triplet excited state of the photosensitizer to pyrimidine bases leads to the formation of pyrimidine dimers.^{9,10} Photosensitized oxidations also contribute to DNA damage induced by UV-A and UV-B radiation. These processes involve the generation of radicals (type I mechanism), *e.g.*, via electron transfer or hydrogen

^aInstituto de Investigaciones Físicoquímicas Teóricas y Aplicadas (INIFTA), Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CCT La Plata-CONICET, Casilla de Correo 16, Sucursal 4, (1900), La Plata, Argentina. E-mail: clorente@inifta.unlp.edu.ar; Fax: +54 221 4254642; Tel: +54 221 4257430 int.153

^bLaboratoire des Interactions Moléculaires Réactivité Chimique et Photochimique (IMRCP), UMR 5623-CNRS/UPS, Université Paul Sabatier (Toulouse III), 118, route de Narbonne, F-31062, Toulouse cedex 9, France

abstraction, and/or the production of singlet oxygen ($^1\text{O}_2$) (type II mechanism).¹¹

Ptr and Lum are able to act as sensitizers in photoinduced oxidations through both type I and type II mechanisms.^{12–15} Although Lum is a relatively efficient $^1\text{O}_2$ photosensitizer, studies performed using 2'-deoxyadenosine 5'-monophosphate (dAMP) and 2'-deoxyguanosine 5'-monophosphate (dGMP) as substrates and Lum as photosensitizer showed that the predominant mechanism of the photosensitized oxidations involves an electron transfer process.^{14,15} In these studies, carried out in aqueous solutions under UV steady-state irradiation, consumption of a given nucleotide and O_2 , production of H_2O_2 , and constant concentration of Lum during irradiation were observed. Interestingly, it was also found that the rate of nucleotide disappearance was lower in O_2 -saturated solutions than in air-equilibrated solutions. Since quenching of the triplet state of Lum by O_2 has already been reported,¹⁶ this result was attributed to a competition between two pathways involving the Lum triplet state: energy transfer to O_2 and electron transfer from the nucleotide. However, to the best of our knowledge, the interaction of Lum triplet state with nucleotides and the kinetics of its quenching by O_2 have not been studied so far. Another interesting finding reported in the mentioned studies consists in the effect of superoxide dismutase (SOD), an enzyme that catalyzes the disproportionation of the superoxide radical anion ($\text{O}_2^{\cdot-}$) and its conjugated acid (the perhydroxyl radical, $\text{HO}_2^{\cdot}$) into H_2O_2 and O_2 .¹⁷ The photosensitized oxidations were faster in the presence of SOD than in its absence, thus suggesting the participation of $\text{O}_2^{\cdot-}$ in the mechanism, that still remains to be demonstrated.

In this work, we present a study aimed at establishing a general mechanism for the electron transfer-initiated oxidations of biomolecules photosensitized by Lum. dAMP was chosen as a model substrate for several reasons: (i) if a given photosensitizer produces $^1\text{O}_2$, its presence in the medium does not interfere with the analysis of the electron transfer process because adenine is not oxidized by this reactive oxygen species;^{18,12} (ii) dAMP is highly soluble in H_2O , (iii) it is easily quantified by chromatographic methods and (iv) some general features of the Lum-dAMP system are already known (*vide supra*). In this work, we have investigated the formation of $\text{O}_2^{\cdot-}$ as a product of the photosensitized reaction, studied the quenching of the Lum triplet state by dAMP and dissolved O_2 in time-resolved experiments and carried out analysis of photoproducts.

Experimental

Chemicals

Lumazine (Lum) was purchased from Schircks Laboratories (Jona, Switzerland) and used without further purification. Superoxide dismutase (SOD, bovine erythrocytes), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), tris(hydroxymethyl)aminomethane (TRIS), 2'-deoxyadenosine 5'-monophosphate (dAMP), and ammonium acetate (NH_4OAc) were provided by Sigma. DMPO was purified by activated carbon and stored at $-20\text{ }^\circ\text{C}$ before use.

The pH of the aqueous solutions was adjusted by adding drops of HCl or NaOH solutions from a micropipette. The concentrations of the acid and base used for this purpose ranged from 0.1 M to 2 M. The ionic strength was approximately 10^{-3} M in all the experiments.

Electron paramagnetic resonance: spin trapping experiments

Electron paramagnetic resonance (EPR) set-up. EPR spectra were collected on a Bruker ESP 500E spectrometer. Samples were irradiated directly inside the microwave cavity of the spectrometer using a 150 W Oriel Hg lamp (Palaiseau, France). The light was delivered *via* an optical fiber to the grid of the cavity. Under these conditions, the samples were irradiated with polychromatic light. The following instrumental settings were employed for the measurements: microwave power: 20 mW; field modulation amplitude: 0.1 mT; field modulation frequency: 100 kHz; microwave frequency: 9.77 GHz.

EPR-spin trapping detection of superoxide anion ($\text{O}_2^{\cdot-}$). Nitrones are common reagents for the detection and identification of transient radicals due to their ability to form persistent radical adducts that are detectable and fingerprintable by EPR spectroscopy.^{19,20} In our experiments, DMPO was used as a spin trap.²¹

The samples used (500 μL) contained Lum (10^{-4} M) and DMPO (5×10^{-2} M) in an aqueous buffer of 1 mM TRIS/HCl and 0.5 mM NaCl (pH 7.2). Samples were transferred in quartz flat cells and irradiated in the EPR cavity at room temperature. EPR spectra were recorded at different times since the beginning of the irradiation and until a plateau was observed in the EPR signal intensity (after 3 to 4 min under our experimental conditions). To confirm the formation of $\text{O}_2^{\cdot-}$ upon irradiation of the samples, similar EPR-spin trapping experiments were performed in the presence of SOD (22 U mL^{-1}).

Laser flash photolysis

Time-resolved absorption experiments. Time-resolved absorption experiments were performed as described previously.²² Briefly, the frequency-tripled output (355 nm) of a Quanta-Ray GCR 230 Nd:YAG laser operating at the repetition rate of 10 Hz was used as the excitation source (pulse fwhm ~ 5 ns). Transient species thus produced were monitored using the spectrally-resolved output of a steady-state Xe lamp. To increase the signal-to-noise ratio, data from ~ 250 independent laser pulses were typically averaged.

Singlet oxygen experiments. Samples were irradiated with the laser described in the previous paragraph. $^1\text{O}_2$ was monitored in time-resolved experiments *via* its phosphorescence at 1270 nm using a 77 K Ge detector (EOL-817-P North Coast, Santa Clara, CA). The detector output was monitored using a digital oscilloscope (Lecroy 9410; Chestnut Ridge, NY), and acquired by a computer for storage and analysis. To improve signal-to-noise ratios, data recorded from 10 to 100 independent laser pulses were generally averaged.

Steady-state irradiation

UV irradiation. Solutions containing Lum and dAMP were irradiated in 1 cm path length quartz cells at room temperature with Rayonet RPR lamps (Southern N.E. Ultraviolet Co.) with emission centered at 300 or 350 nm (bandwidth (fwhm) ~ 20 nm). These differences in irradiation wavelength allow a better overlap between lamp emission and pH-dependent absorption spectra of Lum (Fig. 1). To avoid irradiation below 300 nm, where the nucleotide absorbs (Fig. 1), a cutoff filter was placed between the

lamp emitting at 300 nm and the cell. The experiments were performed in open cells with air-equilibrated aqueous solutions under stirring. The measurements were carried out under conditions of reduced environmental light.

High performance liquid chromatography (HPLC). An HPLC chromatograph Prominence from Shimadzu was employed for monitoring the reaction. A Synergi Polar-RP column (150 × 4.6 mm, 4 μm, Phenomenex) was used for product separation. 10 mM NH₄OAc aqueous solution (pH 6.8) was used as the mobile phase. HPLC runs were monitored by UV/visible spectroscopy at different wavelengths.

Detection and quantification of H₂O₂. H₂O₂ was determined by its reaction with 4-aminophenazone and phenol catalyzed by the enzyme peroxidase to yield 4-(*p*-benzoquinone-monoimino)-phenazone, which is detected by its absorbance in the visible region.^{23,24} This assay has high sensitivity and specificity due to the intense absorbance of the product at 505 nm and the enzymatic catalysis, respectively. The reactants were purchased from Wiener Laboratorios S.A.I.C. (Cholesterol Kit). Briefly, 500 μL of irradiated solution were added to 600 μL of reagent. The absorbance at 505 nm of the resulting mixture was measured after 30 min at room temperature, using the reagent as a blank. Aqueous H₂O₂ solutions prepared from commercial standards were employed for obtaining the corresponding calibration curves.

Mass spectrometry analysis

An LC/MS system, equipped with an HPLC chromatograph (Agilent 1100) and a triple quadrupole mass spectrometer (Q TRAP Applied Biosystems), were employed. HPLC analyses were performed by using an X-bridge column (150 mm, Waters), and isocratic elution with 100% formic acid (0.1%) at a flow rate of 0.3 mL min⁻¹. The mass spectrometer was equipped with an electrospray ion (ESI) source (Turbo Ion Spray (TIS)) and was operated in both positive and negative ion modes. Nitrogen served as auxiliary, collision gas and nebulizer gas. The nitrogen temperature of the TIS source was 450 °C and the declustering potential (DP) 30 V. The detection was scan mode with a step size of 0.1 atomic mass units (amu) and a scan range of 150–500 amu. Mass chromatograms, *i.e.* representations of mass spectrometry data as chromatograms (the *x*-axis represents time and the *y*-axis represents signal intensity), were registered using different scan ranges.

Results and discussion

Production of O₂^{•-} photoinduced by Lum

In previous EPR-spin trapping studies, it was shown that when air-equilibrated aqueous solutions containing Ptr were irradiated, O₂^{•-} was formed, even in the absence of substrates that could act as electron donors.²⁵ The process responsible for the O₂^{•-} is initiated by an electron transfer between Ptr in its ground state and electronically excited Ptr to yield the corresponding pair of radical ions (Ptr^{•+} and Ptr^{•-}). In a second step O₂^{•-} is formed by the reduction of the dissolved O₂ by Ptr^{•-}, which is a common reaction of organic radical anions.^{26,27}

To characterize the formation of O₂^{•-} upon photolysis of Lum, EPR analyses were performed in the presence of a spin trap

(DMPO). The irradiation of a solution (pH 7.2) containing Lum (10⁻⁴ M) and DMPO (5 × 10⁻² M) led to the immediate formation of an EPR signal, which increased with the irradiation time (Fig. 2a–2c). The intensity of the EPR signal reached a maximum after 4 min of irradiation (Fig. 2c). The EPR spectrum was characterized by hyperfine coupling constants $a^N = 14.2$ G, $a^H_\beta = 11.2$ G, $a^H_\alpha = 1.25$ G, corresponding to the adduct DMPO-OOH.²⁸ This result suggests that DMPO has trapped O₂^{•-}. Control experiments were carried out in the absence of Lum and, as expected, no signal corresponding to the adduct DMPO-OOH was registered. To confirm the involvement of the O₂^{•-} in the formation of the DMPO-OOH adduct, similar experiments were performed in the presence of SOD (Experimental section). The addition of this enzyme caused an important decrease of the EPR signal (Fig. 2d). Therefore, these results indicate that UV irradiation of Lum in aqueous solution induces the production of O₂^{•-}.

Fig. 2 Detection of the DMPO-OOH adduct by EPR analysis upon aerobic irradiation of an aqueous solution of Lum (100 μM) at pH 7.2 and DMPO (50 mM), in the absence (a–d) and in the presence (e–h) of dAMP (200 μM): (a,e) after 40 s of irradiation, (b,f) after 2 min of irradiation, (c,g) after 4 min of irradiation, and (d,h) same as (c,g) with added SOD (22 U mL⁻¹).

In an independent series of experiments, air-equilibrated solutions of Lum were steadily irradiated in the presence and in the absence of SOD and the concentrations of H₂O₂ and Lum were determined as a function of time. The rate of H₂O₂ formation

in the presence of SOD was much higher than that measured in its absence (Fig. 3). This result is in agreement with the data obtained from EPR–spin trapping experiments, but also implies the existence of recombination reactions that consume most $O_2^{\cdot-}$ formed in the absence of SOD. In addition, results showed that the rate of Lum consumption was higher in the presence of SOD than in its absence. This fact may be interpreted as follows: SOD, by removing $O_2^{\cdot-}$, prevents back electron transfer from $O_2^{\cdot-}$ to Lum^{++} and thus stimulates Lum consumption.

Fig. 3 Evolution of the H_2O_2 concentration as a function of the elapsed irradiation time in solutions of Lum (160 μM). Experiments were performed in air-equilibrated and O_2 -saturated aqueous solutions (pH = 5.5), in the absence and in the presence of SOD (50 U mL^{-1}).

To better understand the mechanism of $O_2^{\cdot-}$ production in the absence of electron donors, a series of irradiation experiments were performed in O_2 -saturated solutions. In photolysis experiments carried out in the absence, as well as in the presence of SOD, the increase in the O_2 concentration caused a significant decrease in the rate of H_2O_2 production (Fig. 3). This behavior is an indirect evidence of the participation of Lum triplet excited state in the studied process because O_2 does not quench singlet excited states of Lum,¹⁵ but is an efficient quencher of its triplet excited state.¹⁶ The effect of the O_2 concentration is discussed in more detail below.

The mechanism represented by reactions 1–12 summarizes the possible reaction pathways. After excitation of Lum and formation of its triplet excited state, $^3Lum^*$ (reactions 1 and 2), several reaction pathways compete for the deactivation of the latter: monomolecular deactivation pathways (e.g., radiative and nonradiative energy losses, reaction 3), quenching by O_2 (reactions 4, 4' and 4''), and electron transfer from Lum in its ground state (reaction 5) to yield the corresponding pair of radical ions ($Lum^{\cdot-}/Lum^{++}$). This latter type of self-quenching processes have been described for flavins.^{29,30} Considering the behavior of other related heterocycles such as pterins,^{31,32} purines^{33,34} and flavins,^{29,30} it is very likely that the radical anion and the radical cation of Lum are in equilibrium with the protonated and deprotonated forms ($Lum(H)^{\cdot}/Lum(-H)^{\cdot}$), respectively (reactions 6 and 7). Under anaerobic conditions, the recombination of the two Lum radicals (reaction 8) results in a low quantum yield of Lum consumption.¹⁶

In the presence of O_2 , photosensitized production of 1O_2 (reaction 4') by Lum has been demonstrated with pH sensitive quantum yields of 0.44 and 0.08 in air-equilibrated acidic and alkaline aqueous solutions, respectively.¹⁶ It was also shown that Lum is poorly reactive with 1O_2 .

Superoxide anion ($O_2^{\cdot-}$) may be produced by direct electron transfer from $^3Lum^*$ to O_2 (reaction 4'') or by electron transfer from $Lum^{\cdot-}$ (reaction 9) formed through reaction 5. If $O_2^{\cdot-}$ were formed predominantly by the former pathway (reaction 4''), the relative efficiency of the production of $O_2^{\cdot-}$ (and of H_2O_2 resulting from $O_2^{\cdot-}$ disproportionation, reaction 11, see below) would be expressed by:

$$k_4''[O_2]/(k_q^{O_2}[O_2] + k_5[Lum] + k_3), \text{ with } k_q^{O_2} = k_4 + k_4' + k_4''$$

This efficiency should then increase when the O_2 concentration increases (or remain unchanged if $k_q^{O_2}[O_2] \gg k_5[Lum] + k_3$). However, the experimental evidence shows that the rate of H_2O_2 formation decreases with the O_2 concentration (Fig. 3). Therefore reaction 5 should be the predominant initiating step in the $O_2^{\cdot-}$ formation, in agreement with the expression giving the relative efficiency of this reaction ($k_5[Lum]/(k_q^{O_2}[O_2] + k_5[Lum] + k_3)$).

Reaction 10 (recombination of the Lum radical cation, or its deprotonated form, with $O_2^{\cdot-}$) explains the low quantum yields of Lum consumption ($\Phi_{-Lum} = (2 \pm 1) \times 10^{-4}$ at pH 5.5)¹⁶ and of H_2O_2 production under aerobic conditions (Fig. 3). Alternatively, $O_2^{\cdot-}$ disproportionation with its conjugated acid $HO_2^{\cdot}$ (represented by the overall reaction 11) leads to the formation of H_2O_2 . Whereas reaction 10 is predominant and reaction 11 is negligible in the absence of SOD, the latter reaction becomes significant in its presence and H_2O_2 is produced in larger amounts. Finally, reactions of $Lum^{++}/Lum(-H)^{\cdot}$ with O_2 and/or the solvent, schematically represented by reaction 12, lead to the oxidation of Lum.

Production of $\text{O}_2^{\bullet-}$ photoinduced by Lum in the presence of dAMP

In contrast to the results obtained with solutions containing only Lum (Fig. 3), in the presence of nucleotides, such as dAMP or dGMP, the photosensitizer, under UV-A or UV-B irradiation, generates significant amounts of H_2O_2 , comparable to the consumption of nucleotides.^{14,15} This fact, in the context of an electron transfer-initiated mechanism, suggests the formation of $\text{O}_2^{\bullet-}$ in the photosensitized process. Therefore, to investigate this point further, EPR-spin trapping experiments were performed in the presence of dAMP and compared to those carried out in its absence. For different irradiation times, the signals corresponding to the DMPO-OOH adduct obtained in the solutions containing dAMP (200 μM) were much more intense than those registered in its absence (Fig. 2e–2g). To the best of our knowledge, this is the first time that direct evidence is shown in favor of the formation of $\text{O}_2^{\bullet-}$ in the oxidation of a nucleotide photosensitized by a pteridine derivative.

It was reported that no photosensitization of dAMP by Lum takes place in the absence of O_2 and that in air-equilibrated solutions the dAMP consumption is faster in the presence of SOD.¹⁵ Thus, this result shows again that recombination reactions consuming $\text{O}_2^{\bullet-}$ and recovering dAMP are taking place in the absence of SOD. Therefore, taking into account these previous results and those obtained in EPR experiments in this work, reactions 13 to 17 may be proposed to complete the reaction scheme (reactions 1 to 12), in the presence of dAMP. Reaction 13 is an additional and the predominant source of $\text{Lum}^{\bullet-}$, which, in turn, increases the $\text{O}_2^{\bullet-}$ production, *via* reaction 9, explaining the increase in the DMPO-OOH EPR signal in the presence of dAMP (Fig. 2). Depending on the pH, the dAMP radical cation may deprotonate (reaction 14). The recombination between $\text{Lum}^{\bullet-}$ and the dAMP radical/radical cation (reactions 15 and 15') may explain the absence of dAMP photosensitization by Lum under anaerobic conditions. Finally, dAMP radicals can react with $\text{O}_2^{\bullet-}$ to recover the substrate (reactions 16 and 16') or yield products (reaction 17).

Results shown so far suggest that the triplet excited state of Lum plays a key role in the photosensitized oxidation of dAMP. However, direct evidence for the interaction between the $^3\text{Lum}^*$

and the ground state of dAMP has yet to be presented. Dissolved O_2 also plays a key role and is consumed in this process, the rate of consumption of the nucleotide being lower in O_2 -saturated than in air-equilibrated solutions. Moreover, comparison of the photosensitizing activity of Lum under different pH conditions revealed that the photoinduced oxidation of dAMP is much faster in acidic media, where Lum exists in its neutral form, than in alkaline media, where Lum exists as a monoanion (Fig. 1).¹⁵ Therefore in order to get more insight into the mechanism of the photosensitized oxidation of dAMP by Lum, the quenching of $^3\text{Lum}^*$ by O_2 and dAMP was investigated in both media by laser flash photolysis.

Quenching of Lum triplet excited state by O_2

Alkaline media. Laser flash excitation at 355 nm of de-aerated Lum solutions at pH 10.5 showed a strong transient absorption in the 400–600 nm spectral region. The absorption decays were clearly biexponential, with lifetimes of 2.1 (± 0.5) μs and 15 (± 3) μs (Fig. 4a). The short-lived transient could be assigned to $^3\text{Lum}^*$ based on the following results: (i) increase in its decay rate in the presence of O_2 , (ii) lifetime in the absence of added quenchers (τ_T^0) comparable to those reported for the triplet states of other related heterocyclic compounds such as pterins.^{31,35} The long-lived

Fig. 4 Laser flash photolysis experiments. (a) Transient absorption decay registered at 420 nm in the absence of quencher; experiments performed in O_2 -free solutions, $[\text{Lum}] = 220 \mu\text{M}$, $\text{pH} = 10.5$, excitation wavelength 355 nm. (b) Signal of the near-infrared $^1\text{O}_2$ luminescence; experiments performed in air-equilibrated D_2O solutions at $\text{pD} 5.5$; excitation wavelength 309 nm, $[\text{Lum}] = 80 \mu\text{M}$.

transient could be a radical produced from the triplet excited state (reactions 5 to 7).

We have determined the τ_T values as a function of the concentration of dissolved O_2 from the decay of the short-lived transient. The corresponding rate equation for the decrease in $^3Lum^*$ concentration is given by:

$$-d[^3Lum^*]/dt = k_{T,obs}[^3Lum^*] = (1/\tau_T)[^3Lum^*] \quad (18)$$

where $k_{T,obs}$ is the observed rate constant of the $^3Lum^*$ decay under the given experimental conditions (s^{-1}); in the absence of added quencher $k_{T,obs} = k_3 + k_5[Lum] = 1/\tau_T^0$; in the presence of O_2 , $k_{T,obs} = k_q^{O_2}[O_2] + 1/\tau_T^0 = 1/\tau_T$.

The rate constant of quenching of $^3Lum^*$ by O_2 ($k_q^{O_2}$) was calculated by a Stern–Volmer analysis of the τ_T values as a function of the concentration of O_2 according to eqn (19).

$$1/\tau_T = 1/\tau_T^0 + k_q^{O_2}[O_2] \quad (19)$$

The corresponding Stern–Volmer plot at pH 10.5 is shown in Fig. 5a and a $k_q^{O_2}$ value of $1.2 (\pm 0.2) \times 10^9 M^{-1} s^{-1}$ was obtained.

Fig. 5 Laser flash photolysis experiments: quenching of the Lum triplet state by dissolved O_2 . (a) Experiments performed in H_2O solutions at pH 10.5; τ_T values were calculated analyzing the transient absorbance ΔA vs. t (Fig. 4a); excitation wavelength 355 nm, analysis wavelength 420 nm, $[Lum] = 220 \mu M$. (b) Experiments performed in D_2O solutions at pD 5.5; τ_T values were calculated analyzing the near-infrared 1O_2 luminescence (Fig. 4b); excitation wavelength 309 nm, $[Lum] = 80 \mu M$.

Acidic media. Similar experiments could not be performed in acidic media because the absorbance of the acid form of Lum at 355 nm is very weak (Fig. 1). Therefore several attempts were carried out to detect $^3Lum^*$ by excitation at 309 nm. However, in our experimental setup, the energy of the laser at that wavelength

is much lower than that at 355 nm and, consequently, absorption transients could not be detected either. Therefore the quenching of $^3Lum^*$ by O_2 was studied through the formation of 1O_2 , detected by its emission in the near-infrared (NIR) (see Experimental). D_2O was used as a solvent since the lifetime of 1O_2 (τ_A) is much longer in D_2O than in H_2O .³⁶

For a given set of experimental conditions, the 1O_2 concentration after the laser flash increased due to its formation by energy transfer from $^3Lum^*$ to dissolved O_2 , reached a maximum value and decreased because of its relaxation to the ground state by both radiationless and radiative pathways (Fig. 4b). Quenching must also be considered if a substance present in the solution is able to deactivate or trap 1O_2 . These typical signals can be fitted with the following equation:

$$I = A1\exp(-t/\tau_A) - A2\exp(-t/\tau_T) \quad (20)$$

where I is the signal registered by the NIR detector, which is proportional to the 1O_2 concentration; $A1$ and $A2$ are pre-exponential factors, τ_A and τ_T are the lifetimes of 1O_2 and of $^3Lum^*$, respectively, under the experimental conditions used.

In our case, τ_A was more than one order of magnitude longer than τ_T , and was calculated by fitting monoexponential decays ($I = A1\exp(-t/\tau_A)$) obtained for time windows longer than 200 μs . Then the average τ_A value obtained ($63 \pm 2 \mu s$) was introduced in eqn (20) and τ_T was calculated for each O_2 concentration, analyzing signals within time windows shorter than 50 μs . A Stern–Volmer analysis of the τ_T values as a function of the concentration of O_2 according to eqn (19) (Fig. 5b) gave a $k_q^{O_2}$ value of $7.0 (\pm 1) \times 10^8 M^{-1} s^{-1}$, about 40% lower than in alkaline media. The value of τ_T^0 in acidic media was estimated from the y-intercept of eqn (19) and a value of $2.6 (\pm 0.5) \mu s$ was obtained, which is close to that in alkaline media.

Quenching of Lum triplet excited state by dAMP

Alkaline media. Laser flash photolysis experiments performed in the presence of dAMP at pH 10.5 (in the absence of O_2) showed that this nucleotide does not quench $^3Lum^*$ up to a concentration of 680 μM , *i.e.* the lifetime of the short-lived transient did not decrease with an increase in the quencher concentration (Fig. 6). Likewise, the lifetime of the long-lived component did not change within the range of dAMP concentrations used. These results are in agreement with the fact that dAMP is not photosensitized by Lum in alkaline media¹⁵ and support the hypothesis that interaction of $^3Lum^*$ with the nucleotide is the first step of the photosensitized oxidation mechanism in acidic media.

Acidic media. As indicated in the previous section (*Quenching of $^3Lum^*$ by O_2*), the transient absorption of $^3Lum^*$ could not be detected by laser flash photolysis in acidic media, and the quenching of $^3Lum^*$ by dAMP was also studied through the formation of 1O_2 following the same procedure as described above. This analysis in air-equilibrated aqueous solutions (pD 5.5) showed that, under our experimental conditions, 1O_2 was not quenched by dAMP, which was expected considering previous studies on quenching of 1O_2 by nucleotides.¹² The τ_A value ($63 \pm 2 \mu s$) was introduced in eqn (20) and the τ_T value was calculated for each dAMP concentration. In the absence of dAMP, a τ_T of $1.6 (\pm 0.3) \mu s$ was obtained in air-equilibrated solutions.

Fig. 6 Laser flash photolysis experiments: Stern–Volmer plots of the quenching of the Lum triplet state by dAMP (slope of the plots: $k_q^{\text{dAMP}} \tau_T^0$, with τ_T^0 triplet lifetime in the absence of dAMP). Open triangles: experiments performed at pH 10.5 in O_2 -free H_2O solutions (τ_T values were calculated analyzing ΔA vs. t signals); excitation wavelength 355 nm, analysis wavelength 420 nm, $[\text{Lum}] = 220 \mu\text{M}$. Black triangles: experiments performed at pH 5.5 in air-equilibrated D_2O solutions (τ_T values were calculated by analyzing the near-infrared $^1\text{O}_2$ luminescence with eqn (19), see text); excitation wavelength 309 nm, $[\text{Lum}] = 80 \mu\text{M}$.

The rate constant of $^3\text{Lum}^*$ quenching by dAMP ($k_q^{\text{dAMP}} = k_{13} + k_{13'}$) was calculated from the Stern–Volmer analysis of the data using eqn (21):

$$1/\tau_T' = 1/\tau_T + k_q^{\text{dAMP}}[\text{dAMP}] \quad (21)$$

where τ_T is the lifetime of $^3\text{Lum}^*$ in air-equilibrated solution in the absence of dAMP and τ_T' is the lifetime with added dAMP ($=1/(k_q^{\text{dAMP}}[\text{dAMP}] + k_q^{\text{O}_2}[\text{O}_2] + 1/\tau_T^0)$).

The value of k_q^{dAMP} obtained at pH 5.5 from the slope of the Stern–Volmer plot (Fig. 6) was $6 (\pm 2) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. This result provides direct evidence for the interaction of $^3\text{Lum}^*$ with dAMP, but also indicates that the quenching in acidic media is much more efficient than in alkaline media. This fact explains why the oxidation of dAMP photosensitized by Lum is much faster in acidic than in alkaline media, as previously reported.¹⁵

The strong variation of k_q^{dAMP} with the pH may be due to the differences in the charges of the molecules. Acid (neutral) and alkaline (monoanionic) forms of Lum have charges of 0 and -1 , respectively (Fig. 1). Coulombic repulsion between alkaline forms of Lum and dAMP, molecule with negative charges in the phosphate moiety, very likely hinders the interaction necessary to allow the quenching process to proceed. Therefore, this repulsion, which does not exist for the acid form, may diminish the efficiency of the dynamic quenching *via* electron transfer and then makes inefficient the photosensitized chemical oxidation of the substrate.

Effect of O_2 concentration on the dAMP photosensitization

Knowing the values of $k_q^{\text{O}_2}$ and k_q^{dAMP} , the competition between the quenching of $^3\text{Lum}^*$ by dAMP and by O_2 can be analyzed for various experimental conditions, *i.e.* at different pH, and dAMP and O_2 concentrations under continuous irradiation of Lum.

Table 1 Lifetimes of $^3\text{Lum}^*$, rate constants of its quenching by dAMP and O_2 and fraction of $^3\text{Lum}^*$ quenched by dAMP in acidic and alkaline aqueous solutions

	τ_T^0 (μs)	k_q^{dAMP} ($\text{M}^{-1} \text{ s}^{-1}$)	$k_q^{\text{O}_2}$ ($\text{M}^{-1} \text{ s}^{-1}$)	f^{dAMP}
pH = 5.5	2.6 ± 0.5	$6 (\pm 2) \times 10^8$	$7 (\pm 1) \times 10^8$	0.172 (air) 0.086 (O_2)
pH = 10.5	2.1 ± 0.5	very small	$1.2 (\pm 0.2) \times 10^9$	0

The fraction of $^3\text{Lum}^*$ quenched by dAMP under aerobic conditions (f^{dAMP}) is given by:

$$f^{\text{dAMP}} = k_q^{\text{dAMP}}[\text{dAMP}] / (k_q^{\text{dAMP}}[\text{dAMP}] + k_q^{\text{O}_2}[\text{O}_2] + 1/\tau_T^0) \quad (22)$$

Values of $k_q^{\text{dAMP}}[\text{dAMP}]$, $k_q^{\text{O}_2}[\text{O}_2]$ and $1/\tau_T^0$ were estimated for different experimental conditions; typically for those used in ref. 15 ($[\text{dAMP}] \sim 200 \mu\text{M}$, pH 5.5), where the inhibition of the photosensitization of dAMP in O_2 -saturated solutions was reported for the first time. Calculations show that the decrease of the f^{dAMP} value is about a factor of 2 (Table 1) when the O_2 concentration increases from the value in air-equilibrated to that in O_2 -saturated solutions. However, the decrease in the dAMP consumption observed experimentally is higher than that corresponding to a factor of 2. This result is related to the fact that quenching of $^3\text{Lum}^*$ by dAMP represents only the first step in a series of subsequent and competing reactions leading to dAMP consumption. The fraction of dAMP^{*+} formed by electron transfer (reaction 13) that undergoes a further transformation resulting in a permanent chemical change in the dAMP molecule has to be compared under air- and O_2 -saturated conditions. Under the latter conditions, less dAMP^{*+} and Lum^{*-} are produced. However, higher O_2 concentration will result in a more efficient trapping of Lum^{*-} by O_2 (reaction 9) and therefore in a higher relative efficiency of O_2^{*-} production. Consequently, recombination reactions regenerating dAMP (reaction 16) should compete more favorably with the formation of oxidation products from the dAMP radicals (reaction 17). In the final balance, the effect of the O_2 concentration on dAMP consumption is higher than the effect expected considering only the efficiency of the first step of the process (quenching of $^3\text{Lum}^*$ by dAMP).

Mass spectrometry analysis of photoproducts

Aqueous solutions containing dAMP and Lum at pH 5.5 were analyzed by HPLC coupled to ESI mass spectrometry, before and after irradiation. The mass analysis was carried out in both positive and negative ion modes (ESI^+ and ESI^- , respectively). In non-irradiated solutions, as expected, the signals corresponding to the intact molecular ions of dAMP and Lum as $[\text{M} + \text{H}]^+$ species at m/z 332.2 Da and 165.2 Da were observed in ESI^+ mode. In addition, under the same conditions the adduct $[\text{M} + \text{Na}]^+$ was also detected at m/z 187.2 Da for Lum. Likewise, in ESI^- mode the intact molecular ions of the photosensitizer and substrate as $[\text{M} - \text{H}]^-$ species were observed at m/z 163.1 Da and 329.9 Da, respectively.

Comparison of the areas of chromatographic peaks of irradiated and non-irradiated solutions revealed that whereas dAMP was consumed during the photolysis, Lum did not change its concentration. In addition, in irradiated solutions two photoproducts (arbitrarily named P1 and P2) were detected, the areas of the

corresponding peaks increasing with irradiation time. Analysis by ESI mass spectrometry revealed that the molecular weight of P1 is 347 (signals at m/z 346 and 348 in ESI⁻ and ESI⁺ modes, respectively), whereas the molecular weight of P2 is 329 (signals at m/z 328 and 330 in ESI⁻ and ESI⁺ modes, respectively).

The molecular weight of P1 corresponds to 8-oxo-7,8-dihydro-2'-deoxyadenosine 5'-monophosphate (8-oxo-dAMP, Scheme 1). This fact is important since 8-oxo-7,8-dihydro 2'-deoxyadenosine (8-oxo-dAdo) has been proposed as a product of the photosensitized oxidation of 2'-deoxyadenosine (dAdo) in DNA *via* a type I mechanism.³⁷ Moreover, the efficient conversion of the radical cation of dAdo into 8-oxo-dAdo has been reported.³⁸ Therefore, the results obtained in ESI mass analysis support the hypothesis proposed of an electron transfer from dAMP to excited Lum (reaction 13).

Scheme 1 Chemical structures of 8-oxo-dAMP (1) and 8-P-dAMP (2).

Finally, the molecular weight of P2 might correspond to a tetracyclic compound (8-P-dAMP) recently detected by ESI mass spectra of irradiated solutions containing dAMP and pterin (Ptr).¹² Scheme 1 shows the chemical structure proposed for 8-P-dAMP, but more information is needed for a complete characterization of this compound. To the best of our knowledge this is the first time that separation of this compound (by HPLC, in our case) is carried out before mass spectrometry analysis, confirming that it is a product of the photosensitized reaction, instead of a compound formed during the ionization of the mass spectrometry analysis.

Conclusions

The mechanisms involved in electron transfer initiated oxidations of biomolecules photosensitized by lumazine (Lum), a compound belonging to a family of heterocycles present in biological systems, were studied. 2'-Deoxyadenosine 5'-monophosphate (dAMP) was used as a target because this nucleotide does not react significantly with singlet oxygen (¹O₂), reactive species produced by Lum under UV irradiation.

Using EPR and H₂O₂ analyses, we have shown, to the best of our knowledge for the first time, that O₂^{•-} is produced during the oxidation of a nucleotide (dAMP) photosensitized by a pteridine derivative (Lum). Production of O₂^{•-} occurs by electron transfer from Lum^{-•} to O₂, and therefore requires as a prior step quenching of ³Lum* by dAMP to produce the radical ion pair (dAMP^{•+}/Lum^{-•}). Time-resolved transient absorption experiments and NIR analysis of ¹O₂ phosphorescence by laser flash photolysis provided evidence that dAMP does not quench ³Lum* in alkaline aqueous solutions, and subsequently Lum does not photosensitize dAMP oxidation in this medium. In acidic or neutral solutions, competitive quenching of ³Lum*

by dAMP and O₂ results in an apparent contradictory effect of the O₂ concentration: O₂ is required and consumed in the process, but the rate of consumption of the nucleotide is lower in O₂-saturated than in air-equilibrated solutions. Two main products are formed by photosensitized oxidation of dAMP by Lum, as shown by HPLC coupled to mass spectrometry: 8-oxo-7,8-dihydro-2'-deoxyadenosine 5'-monophosphate (8-oxo-dAMP) and a second compound that might be 8-P-dAMP but is not yet fully characterized.

The mechanism involved in the photosensitized process is summarized in Scheme 2. After UV-A or UV-B excitation of Lum and formation of its triplet excited state (³Lum*), three reaction pathways compete for the deactivation of the latter: intersystem crossing to singlet ground state, energy transfer to O₂ leading to the regeneration of Lum and the production of ¹O₂, and electron transfer between dAMP and ³Lum* yielding the corresponding pair of radical ions (Lum^{-•} and dAMP^{•+}). In the following step, the electron transfer from Lum^{-•} to O₂ regenerates Lum and forms O₂^{•-}. The latter undergoes disproportionation with its conjugated acid (HO₂[•]) yielding H₂O₂ and O₂, in competition with recombination reactions with dAMP^{•+} (and/or deprotonated dAMP^{•+}) regenerating dAMP and O₂ (reactions 16 and 16'). Finally, processes that may include reactions of dAMP^{•+} with H₂O and O₂ lead to the formation of dAMP oxidation products.

Scheme 2 Mechanism of the oxidation of dAMP photosensitized by Lum.

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