Oxidation of 2′-Deoxyadenosine 5′-Monophosphate Photoinduced by Lumazine

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UV radiation induces damages to the DNA molecule and its components through photosensitized reactions. Among these processes, photosensitized oxidations may occur through electron transfer or hydrogen abstraction (type I mechanism) and/or the production of singlet molecular oxygen ($^1O_2$) (type II mechanism). Lumazines are an important family of heterocyclic compounds present in biological systems as biosynthetic precursors and/or products of metabolic degradation. To evaluate the capability of lumazines to act as photosensitizers through type I mechanism, we have investigated the oxidation of 2′-deoxyadenosine 5′-monophosphate (dAMP) photosensitized by the specific compound called lumazine (pteridine-2,4(1,3*H*)-dione; Lum) in aqueous solutions under UV irradiation. The photochemical reactions were followed by UV/vis spectrophotometry, HPLC, electrochemical measurement of dissolved O_2, and an enzymatic method for H_2O_2 determination. The effect of pH was evaluated and the participation of oxygen was investigated. In aerated solutions, oxidation of dAMP photoinduced by the acid form of Lum (pH 5.5) takes place through a type I mechanism, in which the excitation of Lum is followed by an electron transfer from dAMP molecule to the Lum triplet excited state. During the process, O_2 is consumed and H_2O_2 is generated, whereas the photosensitizer is not consumed. In contrast, no evidence of a photochemical reaction induced by the basic form of Lum (pH 10.5) was observed.

Introduction

Solar radiation induces modifications to genomic DNA and is implicated in the generation of human skin cancers.[1,2] UV radiation at wavelengths lower than 300 nm damages DNA as a result of the direct excitation of the nucleobases.[3] On the other hand, although nucleobases absorb very weakly above 300 nm, both UV-B (280−320 nm) and UV-A (320−400 nm) radiations can induce modifications to DNA through photosensitized reactions.[3,4] This indirect action may be mediated by endogenous or exogenous sensitizers.

The chemical changes to DNA and its components via photosensitized reactions can take place through different mechanisms. Energy transfer from the triplet state of the photosensitizer to pyrimidine bases leads to the formation of pyrimidine dimers.[4−6] Photosensitized oxidations also contribute to DNA damage induced by UV radiation. These processes involve the generation of radicals (type I), e.g., via electron transfer or hydrogen abstraction, and/or the production of singlet molecular oxygen ($^1O_2$) (type II).[7] The nucleobases are the preferential DNA substrates of type I oxidation.[3] Although guanine is the main target because of its low ionization potential, adenine is also a target in type I sensitized oxidations, being more reactive than pyrimidine bases.[8] On the other hand, guanine is the only DNA component that significantly reacts with $^1O_2$.[9]

Pteridines in their multiple forms are widespread in biological systems and play different roles ranging from pigments to cofactors for numerous redox and one-carbon transfer reactions.[10,11] Within the pteridine family,[12] pterins are those compounds derived from 2-aminopteridine-4(3*H*)-one (pterin, Ptr) and lumazines are those derived from pteridine-2,4(1,3*H*)-dione (lumazine, Lum).

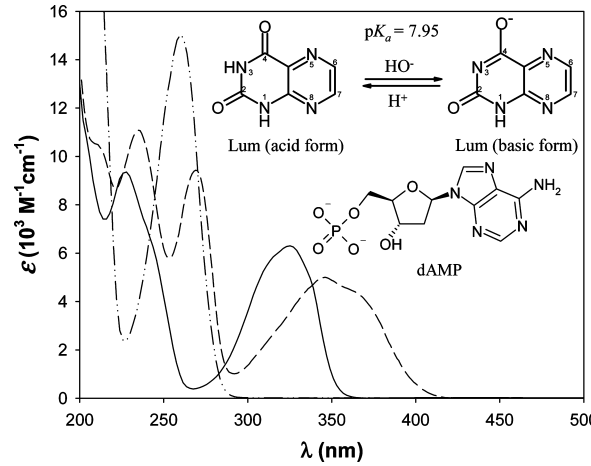

Figure 1. Molecular structure of Lum and dAMP, and the corresponding absorption spectra in air-equilibrated aqueous solutions: (solid line) acid form of Lum (pH 5.5); (dashed line) basic form of Lum (pH 10.5); and (dashed-dotted lines) dAMP.

Lumazine derivatives are present in cells, since 6,7-dimethyl-8-ribityllumazine is the biosynthetic precursor of riboflavin (vitamin B2). Riboflavin is itself the precursor of flavin mononucleotide and flavin adenine dinucleotide, essential cofactors for a wide variety of redox enzymes.[13] Lumazine derivatives are found in the urine as the main degradation products from the metabolic degradation of all reduced pterins.[14,15] Lum presents different acid−base equilibria in aqueous solutions. The only relevant equilibrium at physiological pH involves the neutral form and the monoanion (Figure 1), with a pK_a value of 7.95.[16]

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The participation of pteridine compounds in photobiological processes has been suggested or demonstrated in past decades, and interest in the photochemistry and photophysics of these compounds has subsequently increased. Both classes of pteridine derivatives, pterins and lumazines, are able to photogenerate reactive oxygen species, in particular $^1O_2$.[17−19] It has been known for more than a decade that several pterins are able to photoinduce chemical changes and cleavage to double-stranded DNA.[20−22] In later studies performed with nucleotides as substrates, it was demonstrated that Ptr can act as photosensitizer through both type I and type II mechanisms.[23,24]

In a very recent work the photosensitizing activity of Lum, using 2′-deoxyguanosine 5′-monophosphate (dGMP) and HeLa cells as targets, was investigated.[25] In this study, kinetic analysis of steay-state experiments suggested that, although reaction with $^1O_2$ contributes to the photosensitized oxidation of dGMP, the main mechanism is via an initial electron transfer from the nucleotide to excited states of Lum.

Nucleotides or nucleosides bearing adenine are interesting substrates for studying photosensitized reactions via type I mechanism due to several reasons. In the first place, the investigation of the photoinduced oxidation of adenine in isolated and cellular DNA is difficult due to the drawbacks of the analytical methods used to assess the oxidative damage and to the long-distance charge transport in double-stranded DNA from adenine radical ($dAMP^{\bullet+}$) to guanine (dGMP).[26−28] In the second place, if a given photosensitizer produces $^1O_2$, its presence in the media does not interfere with the analysis of the electron-transfer process because adenine is not oxidized by this reactive oxygen species[9] (actually the rate constant of the chemical reaction is extremely low[23]). Finally, nucleotides have high solubility in H_2O and are easily quantified by chromatographic methods.

In this work we report oxidation of 2′-deoxyadenosine 5′-monophosphate (dAMP) photosensitized by Lum in aqueous solution under UV-A and UV-B radiation (Figure 1). We have evaluated the effect of pH, investigated the participation of the different excited states of Lum, and analyzed the products. Mechanistic aspects of the results obtained are discussed.

Experimental Section

General. Lum (purity >99%, Schircks Laboratories, Switzerland) was used without further purification after checking for impurities by HPLC. Superoxide dismutase (SOD) from bovine erythrocytes, 2′-deoxyadenosine 5′-monophosphate (dAMP), and ammonium acetate (NH_4OAc) (Sigma Chemical Co) were of the highest purity available (>98%) and were used without further purification. Methanol (MeOH) and KI were purchased from J. T. Baker and Laboratorios Cicarelli, respectively. Other chemicals were from Sigma Chemical Co. Solutions were prepared dissolving Lum and dAMP in water at pH 11 (10^{-3} M KOH). The final pH of the solutions was adjusted by neutralization with HCl solutions (0.1−0.2 M) added with a micropipet. The ionic strength was ca. 10^{-3} M in all experiments. Concentration ranges used for the experiments of Lum and dAMP were 60−160 and 100−300 μM, respectively.

Steady-State Irradiation. ***Irradiation Setup.*** Aqueous solutions containing Lum and dAMP were irradiated in 1 cm path length quartz cells at room temperature with Rayonet RPR lamps with emission centered at 300 or 350 nm (bandwidth ∼20 nm) (Southern N.E. Ultraviolet Co.). To avoid irradiation below 300 nm, where the nucleotide absorbs (Figure 1), a cutoff filter was placed between the lamp of 300 nm and the cell. A cutoff filter was obviously not necessary for the lamp of 350 nm. The experiments were performed in the presence and absence of air. Oxygen-free solutions were obtained by bubbling with Ar gas for 20 min. The measurements were carried out under conditions of reduced environmental light.

Actinometry. Aberchrome 540 (Aberchromics Ltd.), the anhydride form of the (*E*)-*R*-(2,5-dimethyl-3-furylethylidene)(isopropylidene)succinic acid, was used as an actinometer for the measurements of the incident photon flux (P_0) at the excitation wavelength. The method for the determination of P_0 has been described in detail elsewhere.[29,30] Values of the photon flux absorbed (P_a), were calculated from P_0 ($P_0^{350} = 6.6 \times 10^{-4}$ einstein L^{-1} min^{-1}, $P_0^{300} = 7.1 \times 10^{-5}$ einstein L^{-1} min^{-1}) according to the Lambert−Beer law ($P_a = P_0\,(1-10^{-A})$, where A is the absorbance of the sensitizer at the excitation wavelength).

UV/Vis Analysis. UV−visible absorption spectra were registered on a Varian Cary-3 spectrophotometer or on a Hewlett-Packard 8452A diode array spectrophotometer. Measurements were made in quartz cells of 0.4 and 1 cm optical path length. The absorption spectra of the solutions were recorded at regular intervals of irradiation time, and the signals were averaged and smoothed with the Varian software. Experimental difference (ED) spectra were obtained by subtracting the spectrum at time $t = 0$ from the subsequent spectra recorded at different times t. Each ED spectrum was normalized yielding the normalized experimental difference (NED) spectrum.

High-Performance Liquid Chromatography (HPLC). System Prominence equipment from Shimadzu (solvent delivery module LC-20AT, online degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, column oven CTO-10AS VP, and photodiode array detector SPD-M20A) was used to monitor and quantify the photosensitized reactions and photoproducts. For reactants and products separation we used (i) a Pinnacle-II C18 column (250 × 4.6 mm, 5 μm; Restek) with a solution containing a mixture of 2% acetronitrile and 98% of a 20 mM potassium phosphate aqueous solution (pH 5.5) as mobile phase and (ii) a Sinergy Polar-RP column (150 × 4.6 mm, 5 μm; Phenomenex) with 10 mM NH_4OAc aqueous solution (pH 6.8) as the mobile phase. HPLC runs were monitored by UV/vis spectroscopy at different wavelengths.

Determination of O_2 Concentration. The O_2 consumption during irradiation was measured with an O_2-selective electrode (Consort c932). The solutions and the electrode were placed in a closed glass cell of 130 mL. In a given experiment, for determining the relationship between O_2 and dAMP consumptions ($\Delta[O_2]/\Delta[dAMP]$), the solution was analyzed by HPLC, before and after irradiation, to obtain the initial and final dAMP concentrations, respectively.

Superoxide ($O_2^{\bullet-}$) Investigation. Solutions containing Lum and dAMP were irradiated in the presence of SOD (∼340 U/mL) at pH 5.5. Results of UV/vis spectrophotometric analysis, HPLC, and H_2O_2 determination were compared with those obtained in the absence of SOD.

Detection and Quantification of H_2O_2. For the determination of H_2O_2, a Cholesterol Kit (Wiener Laboratorios S.A.I.C.) was used. H_2O_2 was quantified after reaction with 4-aminophenazone and phenol.[31,32] Briefly, 400 μL of irradiated solution was added to 1.8 mL 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 H_2O_2 solutions prepared from commercial standards were employed for obtaining the corresponding calibration curves.

Fluorescence Measurements. Steady-state and time-resolved fluorescence measurements were performed on aqueous solu-

tions of Lum, using Edinburgh EAI-FS/FL900 SPC equipment. The quartz cells (1 cm path length) used for the measurements were thermoregulated at 25.0 ± 0.2 °C. Corrected fluorescence spectra obtained by excitation at the absorption maxima (high pressure Xe lamp, 419 W) were recorded between 350 and 700 nm, and total fluorescence intensities (I_F) were calculated by integration of the fluorescence band centered at ca. 450 nm.

A N_2 excitation lamp (1.2 bar, 6.3 kV, 40 kHz) was employed for time-resolved studies. The single-photon counting range of the equipment was 500 ps to 500 μs, and the selected counting time window was 0−100 ns for the measurements reported. Emission decays were monitored at 460 nm after excitation at 324 and 347 nm, for acid and alkaline forms, respectively. Lifetimes were obtained from the monoexponential decays observed after deconvolution from the lamp background signal, using the Edinburgh Analytical Instruments proprietary software, as previously described in detail.[33,34]

Results

Irradiation of Solutions Containing Lum and dAMP. Air-equilibrated solutions containing Lum and dAMP were exposed to UV radiation for different periods of time (up to 80 min). To avoid interference between the acid and the basic forms of Lum, the experiments were performed in the pH range 5.0−5.8, where Lum is present at more than 99% in its acid form, and, independently, in the pH range 10.2−10.7, where Lum is present at more than 99% in the basic form. Due to the dependence of the Lum spectrum on the pH (Figure 1), acidic solutions were irradiated at 300 nm, whereas alkaline solutions were irradiated at 350 nm. Under these experimental conditions, Lum was excited, whereas dAMP did not absorb radiation. The photochemical reactions were followed by UV−visible spectrophotometry and HPLC.

Reactions between Lum and dAMP in the absence of UV irradiation were discarded after control experiments were performed by keeping solutions containing both compounds in the dark. These experiments were carried out under the same conditions used in the irradiation experiments ([Lum] = 60−160 μM, [dAMP] = 100−300 μM, pH 5.5 and 10.5 and time 0−240 min). In another set of control experiments, dAMP (∼100 μM) solutions were irradiated in the absence of Lum at 300 and 350 nm. Under both irradiation conditions, no chemical modifications of the nucleotide were detected, thus excluding spurious effects of direct light absorption by dAMP.

In acidic media, significant changes in the absorption spectra of the solutions containing Lum and dAMP were registered after exposure to UV radiation. Examples of the changes observed in the absorption spectra are shown in Figure 2. These changes revealed that, upon irradiation, the characteristic band assigned to dAMP (240−280 nm) decreased in intensity, whereas product(s) absorbing at wavelengths longer than 290 nm were formed.

Under the same pH conditions, the concentration profiles of Lum and dAMP were determined by HPLC. Examples are shown in Figure 3. A decrease of the dAMP concentration was observed as a function of irradiation time, whereas the Lum concentration did not change in the analyzed time-window. The results are consistent with the absorption spectra shown in Figure 2. In the HPLC analysis of the irradiated solutions, several products were detected, most of them having shorter retention times than both dAMP and Lum. Therefore, these products should be very polar substances, most probably because of the incorporation of oxygen into their structures. This would be expected for a photooxygenation reaction of dAMP.

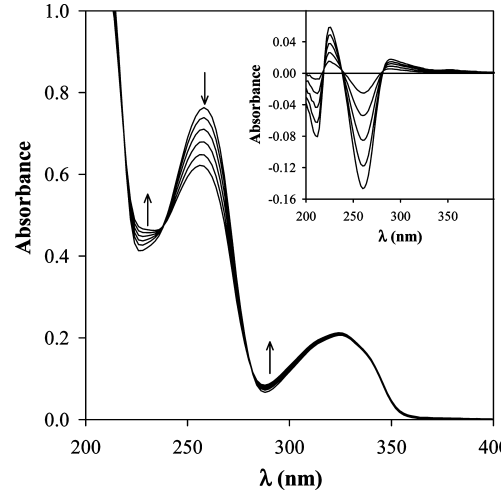

Figure 2. Time evolution of the absorption spectra of air-equilibrated solutions of dAMP irradiated in the presence of Lum. Spectra were recorded every 10 min, optical path length = 0.4 cm. Arrows indicate the changes observed at different wavelengths. Inset: Experimental difference spectra. $[Lum]_0$ = 80 μM, $[dAMP]_0$ = 120 μM, pH 5.5.

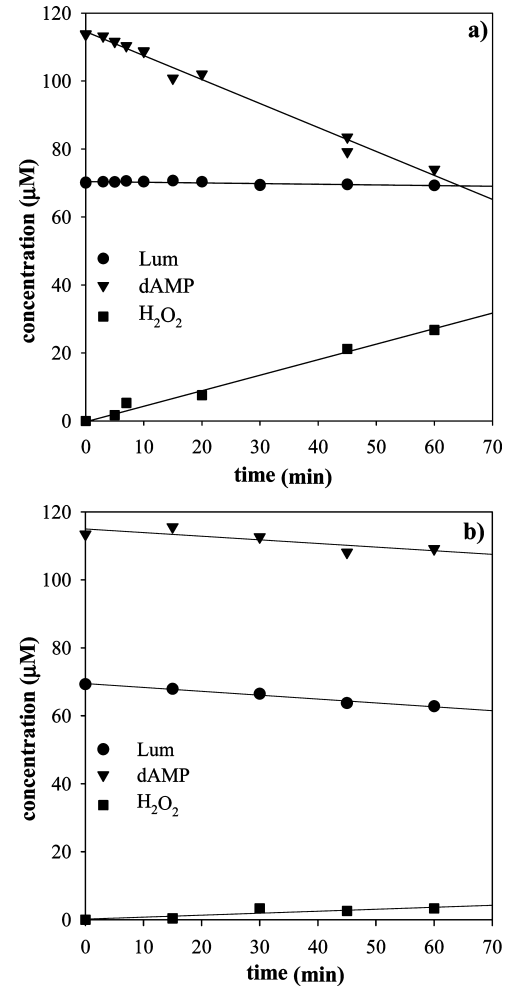

Figure 3. Evolution of the dAMP, Lum, and H_2O_2 concentrations in air-equilibrated aqueous solutions under UV irradiation as a function of time: (a) λ_{exc} = 300 nm, pH 5.5 and (b) λ_{exc} = 350 nm, pH 10.5. $[Lum]_0$ = 70 μM, $[dAMP]_0$ = 115 μM. Errors on an individual data point are ca. ±4 μM.

On the other hand, very small spectral changes were detected in experiments carried out at pH 10.5 (data not shown). In addition, under this pH condition the decrease of the dAMP

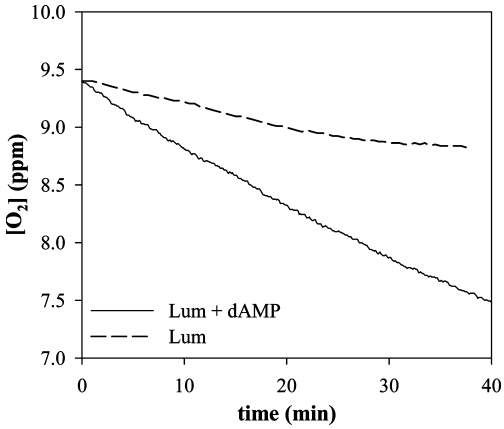

Figure 4. Evolution of the O_2 concentration in irradiated solutions containing Lum and dAMP as a function of time. pH 5.5, $[Lum]_0 = 160\ \mu M$, $[dAMP]_0 = 247\ \mu M$. Control experiment: photolysis of Lum (170 μM) at pH 5.5 in the absence of dAMP.

concentration was very slow in comparison with that registered in acidic media (Figure 3b). These results show that, whereas the acid form of Lum is able to photosensitize and induce chemical modification of the nucleotide dAMP, this is not the case for the basic form of Lum. Notice that the slight decrease on Lum concentration can be attributed to the photolysis of Lum itself.[19]

Solutions containing Lum (~60 μM) and dAMP (~120 μM) at both pH conditions, previously purged with Ar, were irradiated. No significant changes were observed in the absorption spectra of the solutions after more than 80 min of irradiation. HPLC measurements showed that, in these experiments lacking oxygen, the dAMP concentration did not decrease. Accordingly, no photoproducts were detected.

The evolution of the O_2 concentration during the irradiation of air-equilibrated solutions was monitored by using an oxygen electrode in a closed cell. In acidic solutions containing dAMP and Lum the O_2 concentration decreased as a function of irradiation time. Figure 4 illustrate a plot of O_2 concentration versus irradiation time for dAMP (247 μM) and Lum (160 μM) in air-equilibrated solutions. Control experiments in the absence of dAMP were performed in order to check the consumption of O_2 resulting from the photolysis of Lum itself.[19] This result strongly suggests that the process described in the previous paragraphs consists of the oxidation of dAMP. In contrast, no significant O_2 consumption was observed in alkaline media. This observation supports the hypothesis that only the acid form of Lum acts as an efficient photosensitizer.

In several experiments performed at pH 5.5, the O_2 concentration was monitored with the oxygen electrode and the concentration of dAMP was measured by HPLC, before and after irradiation. The relationship between O_2 and dAMP consumptions ($\Delta[O_2]/\Delta[dAMP]$) was calculated for different irradiation times (45−90 min) and a value of 1.3 ± 0.2 was obtained.

The formation of H_2O_2 was investigated in acidic solutions containing Lum and dAMP irradiated in the presence of O_2. H_2O_2 was found to be generated and its concentration increased as a function of irradiation time. In the experiment shown in Figure 3a and in others performed with different initial concentrations of Lum and dAMP, the ratio of the experimental initial rates of H_2O_2 formation and dAMP consumption is 0.65 ± 0.05. On the other hand, in alkaline media H_2O_2 was also detected (Figure 3b), but its rate of formation was very low in comparison with that registered at pH 5.5.

Mechanistic Analysis. Results presented so far (consumption of dAMP and O_2, production of H_2O_2, and constant concentration of Lum during the reaction) clearly demonstrate that Lum photosensitizes the oxidation of dAMP under UV irradiation at pH 5.5. Although the results are compatible with both type I and type II mechanisms, one can presume that the former is predominant since it is accepted that adenine does not react significantly with $^1O_2$. To unambiguously discard the participation of $^1O_2$ in our reaction system we set out to perform kinetic analysis. The method for the assessment of the role of $^1O_2$ in the photosensitized oxidation of nucleotides has been described in detail elsewhere.[24]

Briefly, taking into account the previously reported value of the rate constant of the chemical reaction (k_r) between $^1O_2$ and dAMP,[23] the contribution of $^1O_2$ to the photosensitized oxidation of dAMP by Lum can be evaluated by comparing the experimental initial rate of dAMP consumption to the initial rate of the reaction between $^1O_2$ and dAMP calculated from eq 1.[35]

$$(d[dAMP]/dt) = -k_r[^1O_2][dAMP] \quad (1)$$

The steady-state concentration of $^1O_2$ during irradiation of a solution containing Lum and dAMP is given by eq 2:

$$[^1O_2] = P_a\Phi_\Delta/(k_d + k_t^{Lum}[Lum] + k_t^{dAMP}[dAMP]) \quad (2)$$

where P_a and Φ_Δ are the estimated photon flux absorbed by Lum and the Lum-sensitized quantum yield of $^1O_2$ production, respectively, k_d is the overall rate constant of $^1O_2$ deactivation that reflects the effect of solvent, and k_t^{Lum} and k_t^{dAMP} are the overall rate constants of $^1O_2$ quenching by Lum and dAMP, respectively.

The initial rate of the reaction between $^1O_2$ and dAMP for a given experiment, calculated by using eq 1, $(d[dAMP]/dt)_{calc}$, was compared to the corresponding initial rate of dAMP consumption experimentally determined by HPLC analysis, $(d[dAMP]/dt)_{exp}$. As expected, results obtained for different initial dAMP and Lum concentrations showed that, in all cases, $(d[dAMP]/dt)_{calc}$ was negligible in comparison to $(d[dAMP]/dt)_{exp}$. Therefore, a contribution of $^1O_2$ can be discarded and a mechanism via electron transfer can be assumed, which confirms the hypothesis previously proposed on the capability of Lum to photosensitize through mechanism type I.[25]

It was recently reported that the oxidation of dGMP photoinduced by Lum is inhibited at high O_2 concentration.[25] To check if this effect also takes place with dAMP as substrate, a new set of experiments was performed in O_2-saturated solutions at pH 5.5 and the results were compared with those performed in air-equilibrated solutions. Concentration profiles clearly showed that the rate of Lum-sensitized dAMP disappearance is greater in air-saturated solutions than in O_2-saturated solutions (Figure 5). Since $^1O_2$ is formed by energy transfer from the triplet state of the sensitizer to dissolved O_2, these results rule out again the involvement of a type II mechanism.

Quenching of the triplet state of Lum by O_2 has already been studied by steady-state methods.[19] On the other hand, although it was demonstrated that O_2 does not deactivate singlet excited states of pterins,[36,37] no previous studies on interaction between Lum singlet excited states and O_2 have been reported. Therefore quantum yields of fluorescence were calculated in different O_2 concentrations. Similar values were obtained in argon-saturated, air-equilibrated, and oxygen-saturated Lum solutions (Table 1),

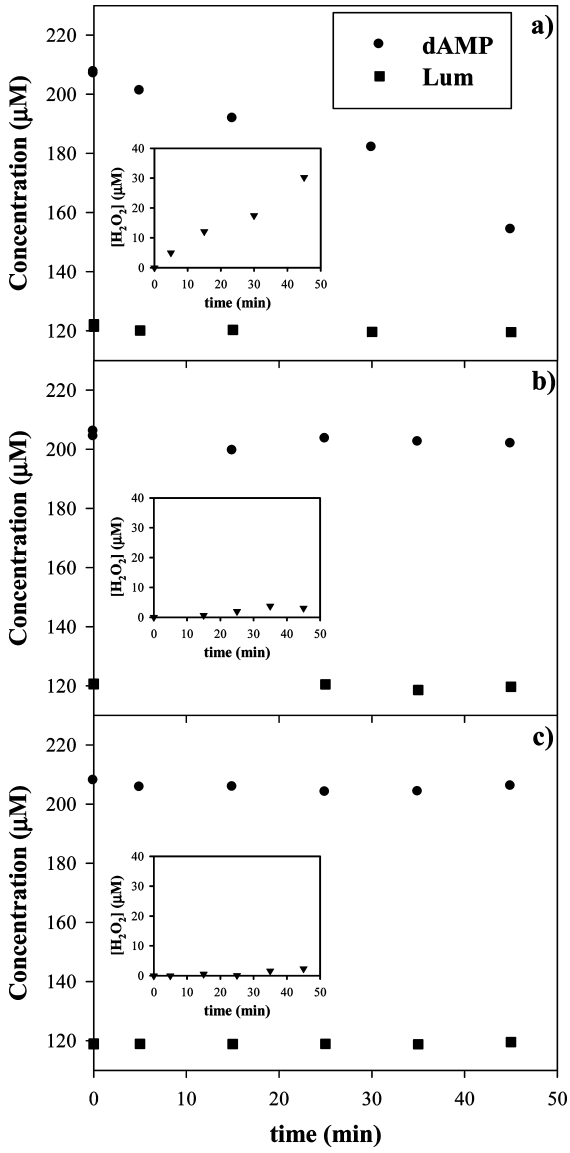

Figure 5. Time-evolution of the dAMP, Lum, and H_2O_2 concentrations in aqueous solutions under UV irradiation (300 nm): (a) air-equilibrated solutions; (b) O_2-saturated solutions; and (c) air-equilibrated solutions in the presence of KI (200 μM). $[Lum]_0 = 120$ μM, $[dAMP]_0 = 210$ μM, pH 5.5. Errors on an individual data point are ca. ± 4 μM.

TABLE 1: Fluorescence Properties of Lum: Fluorescence Quantum Yields (Φ_F) in Argon-Saturated, Air-Equilibrated, and Oxygen-Saturated Aqueous Solutions, and Fluorescence Lifetimes (τ_F) in Air-Equilibrated Aqueous Solutions[a]

	Φ_F			
	air	O_2	Ar	τ_F (ns)
acid form	0.051 ± 0.005	0.054 ± 0.005	0.051 ± 0.005	5.5 ± 0.3
basic form	0.21 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	7.6 ± 0.3

[a] Measurements were carried out for the acid form (pH 5.5, $\lambda_{exc} = 325$ nm) and the basic form (pH 10.5, $\lambda_{exc} = 345$ nm).

thus indicating that O_2 does not quench the singlet excited state of Lum. In addition time-resolved experiments were performed and a first-order rate law was observed for the fluorescence decays. Fluorescence lifetimes (τ_F) were calculated by averaging at least three values (Table 1). Therefore the inhibition of the photosensitized process observed at high O_2 concentrations cannot be due to quenching of the singlet excited states.

After discarding the involvement of singlet excited states of Lum and $^1O_2$, the participation of Lum triplet states in the photosensitized oxidation of dAMP can be assumed. To explore with more detail this point, experiments in the presence of iodide (I^-) were performed. This anion is able to interact with both singlet and triplet excited states of organic compounds. The resulting effects on the photophysical behavior of a given compound depend on the relative increase in the rates of the different deactivation pathways (nonradiative decays to ground state, intersystem crossing).[38] Therefore, in some cases, the presence of I^- causes an increase of the quantum yields of triplet state formation, whereas in others, a decrease. The efficiency of I^- to quench flavin triplet states is much higher than the efficiency to quench the corresponding excited singlet states. This property has been used to investigate the role of the excited states of flavin molecules in photochemical mechanisms.[39,40] The same approach was used to evaluate the participation of triplet excited states of pterins in their photoreduction.[41,42] Furthermore, in studies of room temperature phosphorescence of pterins adsorbed on paper, it was observed that the nonradiative decay from the lowest triplet state of pterins is enhanced by I^-.[43]

To apply this methodology to our system, we first evaluated the capability of I^- to deactivate the singlet excited states of Lum by fluorescence quenching experiments ($[Lum] = 72$ μM, $[I^-] = 0-40$ mM, pH 5.5). A significant quenching was registered at I^- concentrations higher than 2 mM, e.g. a decrease of ca. 50% of the fluorescence was measured at a concentration of 25 mM of I^-. On the other hand, at I^- concentrations lower than 1 mM, the fluorescence quenching was negligible.

Photosensitization experiments were carried out in air-equilibrated aqueous solutions at pH 5.5 in the presence of I^- at concentrations (100−300 μM) for which no quenching of singlet excited states exists. Results revealed that, under these conditions, the rate of nucleotide consumption is much slower than that in the absence of I^- (Figure 5c). These results are in agreement with experiments performed in oxygen-saturated solutions and validate the participation of triplet states of Lum in the first steps of the photosensitized process.

According to the evidence shown thus far, the photosensitized oxidation should start with an electron transfer from the nucleotide to the triplet excited state of Lum. It is well-established that, in a typical type I process, ground state O_2 will readily quench an organic radical anion to produce the superoxide anion ($O_2^{\bullet-}$).[44,45] The detected H_2O_2 (vide supra) can then be the product of the spontaneous disproportionation of $O_2^{\bullet-}$.[46]

Therefore, to investigate the participation of $O_2^{\bullet-}$ in the mechanism, experiments at pH 5.5 were carried out in the presence of superoxide dismutase (SOD), an enzyme that catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2 and O_2.[47] For each irradiation time, four independent experiments were performed, the concentrations were averaged, and the standard deviation was calculated. The data showed a significant increase in the rate of dAMP consumption when SOD was present in the solution (Figure 6). These results indicate that $O_2^{\bullet-}$ is involved in the photosensitized process and provide further evidence for the existence of an electron transfer reaction. In addition, this result suggests that elimination of $O_2^{\bullet-}$ inhibits a step that prevents the photoinduced oxidation of dAMP.

Taking into account the results presented thus far, we propose that the Lum-sensitized oxygenation/oxidation of dAMP is an electron transfer mediated process that can be summarized as shown in eqs 3−12.

$$\text{Lum} \xrightarrow{h\nu} {}^1\text{Lum}^* \qquad (3)$$

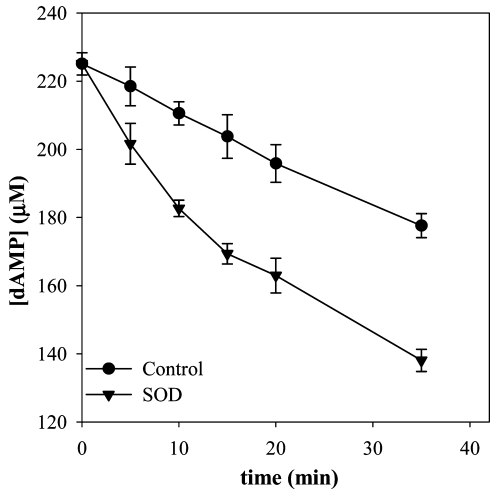

Figure 6. Evolution of dAMP concentration in irradiated solutions containing Lum and dAMP as a function of irradiation time. Experiments were performed in quadruplicate in the absence (●) and in the presence (▼) of SOD (340 U/mL). $[Lum]_0 = 155\ \mu M$, $[dAMP]_0 = 225\ \mu M$, pH 5.5.

$$^1Lum^* \rightarrow {}^3Lum^* \quad (4)$$

$$^3Lum^* \rightarrow Lum \quad (5)$$

$$^3Lum^* + {}^3O_2 \rightarrow Lum + {}^1O_2 \quad (6)$$

$$^3Lum^* + dAMP \rightarrow Lum^{\bullet -} + dAMP^{\bullet +} \quad (7)$$

$$Lum^{\bullet -} + dAMP^{\bullet +} \rightarrow Lum + dAMP \quad (8)$$

$$Lum^{\bullet -} + O_2 \rightarrow Lum + O_2^{\bullet -} \quad (9)$$

$$2H^+ + 2O_2^{\bullet -} \rightarrow H_2O_2 + O_2 \quad (10)$$

$$dAMP^{\bullet +} + O_2^{\bullet -} \rightarrow dAMP + O_2 \quad (11)$$

$$dAMP^{\bullet +} + O_2 \rightarrow dAMP(ox) \quad (12)$$

After excitation of Lum and formation of its triplet excited state, ^{3}Lum* (reactions 3 and 4), three reaction pathways compete for the deactivation of the latter: intersystem crossing to singlet ground state (reaction 5), energy transfer to molecular oxygen leads to the regeneration of Lum and the formation of $^1O_2$ (reaction 6), and electron transfer between dAMP and ^{3}Lum* leads to the formation of the corresponding radical ions, $Lum^{\bullet -}$ and $dAMP^{\bullet +}$ (reaction 7). In experiments performed with air-equilibrated solutions, the rate of the latter reaction is significant and a considerable consumption of dAMP is observed (Figure 3a). In contrast, in oxygen-saturated solutions reaction 6 is dominant, the proportion of ^{3}Lum* reacting with the nucleotide is much lower, and, consequently, the rate of dAMP consumption is also much slower.

In the following step, the radical ions may recombine (reaction 8), which explains the absence of dAMP consumption under anaerobic conditions. Alternatively, the electron transfer from $Lum^{\bullet -}$ to O_2 regenerates Lum and forms $O_2^{\bullet -}$ (reaction 9). This radical may disproportionate with its conjugated acid $HO_2^{\bullet}$ to form H_2O_2 (summarized by reaction 10) or react with the $dAMP^{\bullet +}$ to regenerate dAMP (reaction 11). SOD accelerates the former reaction (vide supra), and therefore fast elimination of $O_2^{\bullet -}$ through this pathway prevents reaction 11. In consequence, in the presence of SOD enhancement of the photosensitized oxidation of the nucleotide is observed experimentally (Figure 6). Finally a group of processes, represented schematically by reaction 12 and that might include the reactions of $dAMP^{\bullet +}$ with O_2 and H_2O, leads to the oxidation of dAMP and consumption of O_2.[48]

Conclusions

The photosensitization of dAMP by Lum in aqueous solution under UV iradiation was investigated. We have shown that when an aerated solution containing dAMP and the acid form of Lum (pH 5.5), the predominant form at physiological pH, was exposed to UV radiation the nucleotide was consumed, whereas the photosensitizer (Lum) concentration did not change significantly. During this process, O_2 was consumed and H_2O_2 was generated. Since lumazines, although in low concentrations, are widespread in living systems, these results are relevant from a biological point of view. In contrast, no evidence of a photochemical reaction induced by the basic form of Lum (pH 10.5) was observed.

Mechanistic analysis indicates that the Lum-sensitized oxygenation/oxidation of dAMP does not involve $^1O_2$ as an intermediate. In contrast, it is an electron transfer mediated process. In this mechanism, the excitation of Lum is followed by an electron transfer from the dAMP molecule to the Lum triplet excited state, leading to the formation of the corresponding ion radicals ($Lum^{\bullet -}$ and $dAMP^{\bullet +}$). In the following step, the electron transfer from $Lum^{\bullet -}$ to O_2 regenerates Lum and forms the superoxide anion. The latter may disproportionate with its conjugated acid ($HO_2^{\bullet}$) to form H_2O_2 or react with $dAMP^{\bullet +}$ to regenerate dAMP.

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