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Selective quenching of triplet excited states of pteridines

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M. Paula Denofrio,[a] Peter R. Ogilby,[b] Andrés H. Thomas[c] and Carolina Lorente*[c]

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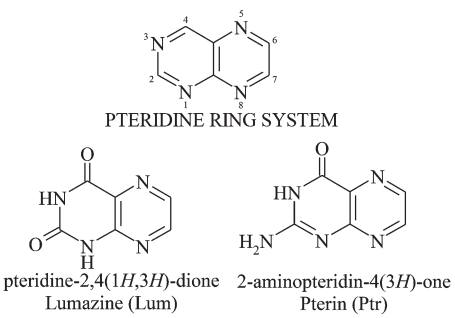
Steady-state and time-resolved studies on quenching of excited states of pterin (Ptr) and lumazine (Lum) in the presence of iodide in aqueous solution have been performed. In contrast to the typical iodide enhancement in the triplet state population, iodide promotes a fast non-radiative $T_1 \rightarrow S_0$ transition for both Ptr and Lum. In this work, we present evidence for the effective iodide-induced deactivation of singlet and triplet excited states, with rate constants close to the diffusion-controlled limit (between 3×10^9 M^{-1} s^{-1} and 1×10^{10} M^{-1} s^{-1}). The longer lifetimes of the triplet excited states over the singlet excited states increase the probability of deactivation ($k_q^T \tau_T^0 \gg k_q^S \tau_S^0$). Therefore, at micromolar concentrations of iodide, where the deactivation of the singlet excited state is negligible, an efficient deactivation of the triplet excited states is observed. This selective deactivation of the excited triplet state is an analytical tool for the study of photosensitized reactions where pteridines are involved.

Introduction

Pteridines are a family of heterocyclic compounds widely distributed in living systems. The pteridine ring system is composed of fused pyrazine and pyrimidine rings (Scheme 1). According to the substituent in position 2, pteridines can be divided into two groups: lumazines and pterins.

The photophysical and photochemical properties of pteridines (PTs) have been investigated in detail in aqueous solution.[1] The absorption spectra of these compounds show typically two main absorption bands in the range between 200 and 400 nm (Fig. 1). UV-A photoexcitation of PTs is followed by efficient intersystem crossing to the triplet excited states, which have been proposed to participate in photosensitized reactions. Photosensitization of biological substrates by PTs can involve electron transfer[2] or energy transfer to molecular oxygen, to yield singlet oxygen ($^1O_2$ ($^1\Delta_g$)).[3–5] The main mechanism of the oxidation of nucleotides[6,7] and amino acids[8,9] photosensitized by PTs in neutral and acidic aqueous solutions involves an initial step in which an electron is transferred from the biomolecule (M) to the pteridine triplet state (^{3}PT*), thus yielding the corresponding radical ion pair. The biomolecule radical cation ($M^{\bullet+}$) undergoes further reactions, at the



Scheme 1 Chemical structures of pteridines.

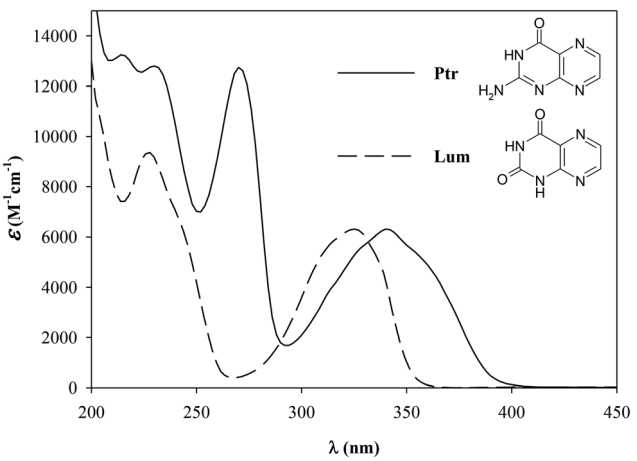
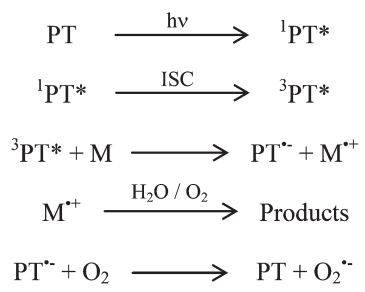


Fig. 1 Absorption spectra of Ptr (solid line) and Lum (dashed line) in air-equilibrated aqueous solutions at pH 5.5.

[a]*IIB-INTECH-UNSAM-CONICET Av. Intendente Marino Km 8, 2. CC 164 (7130) Chascomús, Buenos Aires, Argentina*
[b]*Center for Oxygen Microscopy and Imaging, Department of Chemistry, University of Aarhus, DK-8000 Århus, Denmark*
[c]*INIFTA, Departamento de Química, Facultad de Ciencias Exactas, UNLP, CCT-La Plata, CONICET. Casilla de Correo 16, Suc. 4, 1900-La Plata, Argentina. E-mail: clorente@inifta.unlp.edu.ar*



Scheme 2 Mechanism proposed for the degradation of biomolecules (M) photosensitized by pteridines (PT).

same time as the radical anion of the pteridine ($PT^{\bullet-}$) reacts with molecular oxygen, yielding both a superoxide anion ($O_2^{\bullet-}$) and the pteridine in the ground state (PT) (Scheme 2).

The study of photosensitized reactions involves several strategies to resolve the mechanisms involved in these processes in living systems. The photosensitized oxidations may take place *via* the type I or type II mechanism,[10] but distinguishing between these two types is not always easy. The participation of excited states in photosensitized reactions is clear, but the nature of the excited state often needs further analyses. The experimental conditions may be altered to determine which mechanism is involved. For example, comparative kinetics analysis of the photosensitizing process by varying the O_2 concentration or the nature of the solvent is a useful methodology to determine the type of the mechanism involved.[11] Nevertheless, the assessment of which excited state, singlet or triplet, is implicated in the process requires other experimental tools. Selective deactivation of a specific excited state gives the possibility of better elucidating which state is involved in the observed photosensitized reaction.

Iodide (I^-) is an enhancer of spin forbidden processes (intersystem crossing) in organic molecules as a consequence of increased spin–orbit coupling produced by a heavy atom.[12,13] In contrast to the typical enhancement of the $S_1 \rightarrow T_1$ process, it has been reported that the $T_1 \rightarrow S_0$ process can also be enhanced by iodide.[12,13] Consequently, for systems characterized by the reactions of triplet excited states, the addition of I^- could change the final result. For example, selective quenching of triplet excited states is observed in flavins; I^--mediated deactivation of the triplet excited states is more efficient than the corresponding I^--mediated deactivation of excited singlet states.[14,15] This phenomenon has been used to investigate the role of the excited states of flavin molecules in photochemical mechanisms. The same approach was used to evaluate the participation of triplet excited states of pterins in their photoreduction.[16] We previously observed that I^- inhibited reactions photosensitized by pteridines[8,17] and we inferred that the phenomenon was due to the selective quenching of triplet states by I^-. However, to our knowledge, the quenching of triplet excited states of pteridines by I^- has yet to be explicitly shown.

In the present work, we examined the behavior of the singlet and the triplet excited states of lumazine (Lum) and pterin (Ptr) in aqueous solution in the presence of I^-. In addition, the effect of I^- on the oxidation of 2′-deoxyguanosine 5′-monophosphate (dGMP) and 2′-deoxyadenosine 5′-monophosphate (dAMP) photosensitized by Ptr was studied, and a simple experimental tool to evaluate the participation of triplet excited states of PTs in photosensitizing processes is proposed.

Experimental

General

Pteridines were purchased from Schircks Laboratories (Jona, Switzerland) and used without further purification (purity >99%). Potassium iodide (KI), deuterated water (D_2O) and nucleotides (dGMP, dAMP) were purchased from Sigma-Aldrich (purity >99.0%).

The pH of aqueous solutions was adjusted by adding drops of 0.1–0.2 M aqueous NaOH or HCl solutions with a micropipette. The ionic strength was *ca.* 10^{-3} M in all experiments.

Laser flash photolysis

Time-resolved absorption experiments. Time-resolved absorption experiments were performed as previously described.[18] 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 output of a steady-state Xe lamp that was spectrally resolved using a monochromator. To increase the signal-to-noise ratio, data from ~250 independent laser pulses were typically averaged.

Singlet oxygen experiments. Samples were irradiated (309 nm) with the laser described in the previous paragraph. $^1O_2$ 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, and acquired using a computer for storage and analysis. To improve signal-to-noise ratios, data recorded from 10 to 100 independent laser pulses were generally averaged.

Fluorescence spectroscopy

Fluorescence measurements were performed on air-equilibrated aqueous solutions containing Ptr or Lum using Single-Photon-Counting apparatus (FL3 TCSPC-SP, Horiba Jobin Yvon) that has been previously described.[19]

Steady-state measurements. The sample solution in a quartz cell was irradiated with a 450 W Xenon source through an excitation monochromator. The fluorescence, after passing through an emission monochromator, was monitored at 90° with respect to the incident beam using a room-temperature PMT. Corrected fluorescence spectra obtained by excitation at 350 nm were recorded between 360 and 700 nm, and the total fluorescence intensities (I_F) were calculated by integration of the fluorescence band centered at *ca.* 450 nm. To avoid inner

filter effects, the absorbance of the solutions at the excitation wavelength was maintained below 0.10.

Time-resolved studies. A LED (emission maximum at 341 nm, pulse duration <1 ns; fwhm = 14 nm) was employed for time-resolved studies. The emitted photons, after passing through the monochromator, were detected using a TBX-04 photomultiplier tube module and counted using a Fluoro-Hub-B module. The selected counting time window for the measurements reported in this study was 0–200 ns.

Steady-state irradiation

Solutions containing Ptr or Lum and a substrate were irradiated in 1 cm path length quartz cells at room temperature with Rayonet RPR3500 lamps (Southern N.E. Ultraviolet Co.) with emission centered at 350 nm (fwhm ~ 20 nm). Photolysis experiments were performed in aerated and O_2-saturated solutions. O_2-saturated solutions were obtained by bubbling for 10 min with O_2.

Analysis of irradiated solutions

UV/vis analysis. Electronic absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer. Measurements were made using quartz cells of 0.4 or 1 cm optical pathlength. The absorption spectra of the solutions were recorded at regular intervals of irradiation time.

High-performance liquid chromatography (HPLC). Chromatographic analysis was performed using a Prominence instrument from Shimadzu (solvent delivery module LC-20AT, online degasser DGU-20A5, autosampler SIL-20A HT, column oven CTO-10AS VP, photodiode array detector SPD-M20A). A Synergi Polar-RP column (150 × 4.6 mm, 4 μm, Phenomenex) was used for product separation. 10 mM NH_4OAc aqueous solution (pH 6.8) was used as the mobile phase. HPLC runs were monitored by UV/visible spectroscopy at different wavelengths.

Results

Quenching of the triplet states of pterin by iodide

Laser flash excitation at 355 nm of deaerated solutions of Ptr at pH 5.5 showed a strong transient absorption in the 400–600 nm spectral region with a lifetime of 3.4 (±0.1) μs. The transient signal could be assigned to the triplet state of Ptr based on the following results: (i) an increase in its decay rate in the presence of O_2, (ii) a spectrum and lifetime (τ) comparable to those previously reported for the triplet states of Ptr[20] and similar compounds such as methylpterin,[21] biopterin[22] and 6-carboxypterin.[23]

Experiments performed in the presence of I^- showed that the rate of transient signal decay increased with an increase in the I^- concentration. Typical traces obtained for the quenching of ^{3}Ptr* by I^- are shown in Fig. 2(a). The rate of the decrease in ^{3}Ptr* concentration is given by eqn (1):

$$-\mathrm{d}[^3\mathrm{Ptr}^*]/\mathrm{d}t = k_q^T\,[^3\mathrm{Ptr}^*][I^-] + \sum k[^3\mathrm{Ptr}^*] \qquad (1)$$

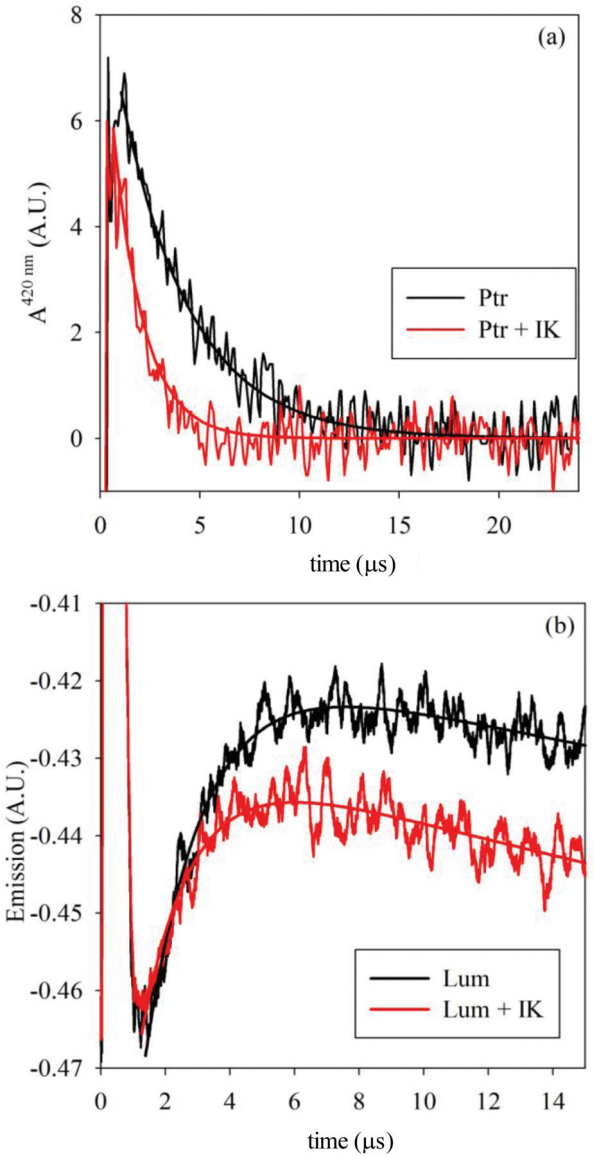


Fig. 2 Laser flash photolysis experiments. (a) Transient absorption decay registered at 420 nm in the absence and in the presence of I^- (57 μM); experiments performed in O_2-free solutions, [Ptr] = 80 μM, pH = 5.5, excitation wavelength 355 nm. (b) Signal of the near-infrared $^1O_2$ luminescence in the absence and in the presence of I^- (43 μM); experiments performed in air-equilibrated D_2O solutions, [Lum] = 80 μM, pD 5.5; excitation wavelength 309 nm.

where k_q^T is the bimolecular rate constant for quenching of Ptr by I^-, and $\sum k[^3\mathrm{Ptr}^*]$ accounts for all other channels of triplet decay (*e.g.*, radiative and non-radiative deactivation, self quenching with another Ptr molecule in the ground state, *etc.*). Therefore, quenching of the Ptr triplet state by I^- may be evaluated by a Stern–Volmer analysis (eqn (2)):

$$\tau_T^0/\tau_T = 1 + k_q^T\tau_T^0\,[I^-] \qquad (2)$$

where τ_T^0 and τ_T are the Ptr triplet lifetimes in the absence and in the presence of I^-, respectively and $[I^-]$ is the I^- concentration (mol L^{-1}). The value of k_q^T obtained from the slope of

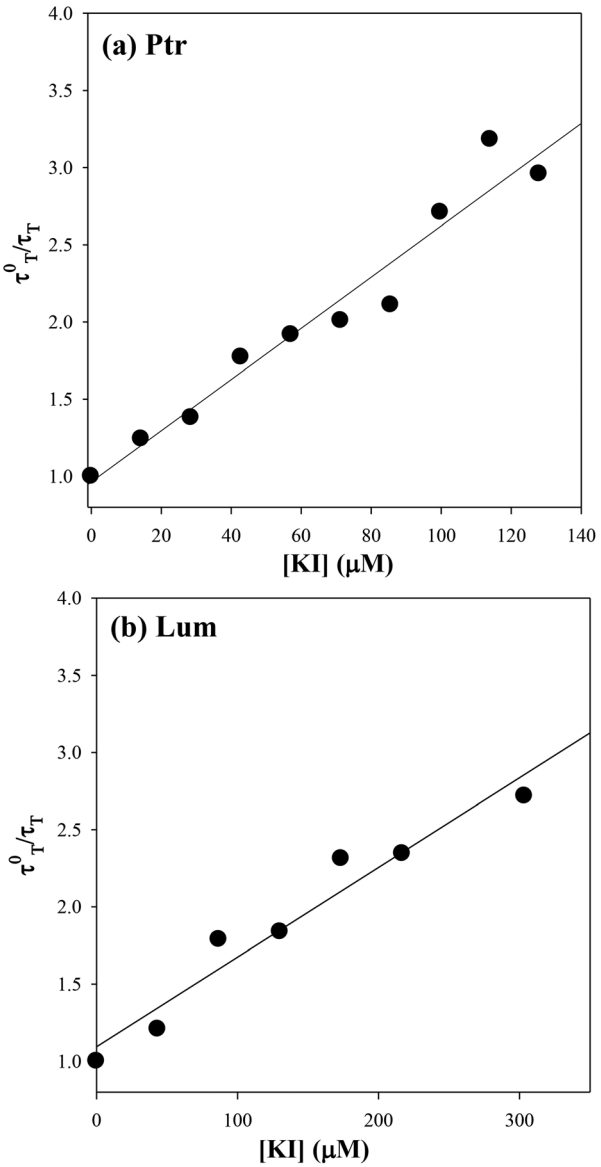


Fig. 3 Stern–Volmer plots of the quenching of (a) ^{3}Ptr* by I^-, at pH 5.5 (λ_{IR} = 355 nm, λ_{AN} = 420 nm) and (b) ^{3}Lum* by I^-, at pD 5.5 (λ_{IR} = 309 nm, λ_{AN} = 1270 nm).

the Stern–Volmer plot (Fig. 3(a)) is 4.9(±0.6) × 10^9 M^{-1} s^{-1}. The data indicate that I^- deactivates ^{3}Ptr* with a rate constant characteristic of the diffusion-controlled limit.

Quenching of the triplet states of lumazine by iodide

Similar transient absorption experiments could not be performed for Lum in acidic media because the absorbance of the acidic form of Lum at 355 nm is very weak (Fig. 1). For that reason several attempts were carried out to detect ^{3}Lum* 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 ^{3}Lum* by I^- was studied indirectly through the formation of $^1O_2$, detected by its phosphorescence in the near-infrared (NIR).[7] D_2O was used as a solvent since the lifetime of $^1O_2$ (τ_Δ) is much longer in D_2O than in H_2O.[24]

For a given set of experimental conditions, the $^1O_2$ concentration after the laser flash increased due to its formation by energy transfer from ^{3}Lum* to dissolved O_2, reached a maximum value and then decreased as a consequence of interactions with the molecules in the surrounding medium (Fig. 2(b)). This behavior is expressed in eqn (3):

$$I = A1\ \exp(-t/\tau_\Delta) - A2\ \exp(-t/\tau_T) \quad (3)$$

where I is the time-dependent signal intensity registered by the NIR detector, and τ_Δ and τ_T are the lifetimes of $^1O_2$ and of ^{3}Lum*, respectively, under the experimental conditions used.

By performing our experiments in D_2O, a solvent in which the lifetime of $^1O_2$ is comparatively long,[24] we can more readily decouple the two kinetic components in eqn (3). At all I^- concentrations used, our fits to the data yield a τ_Δ value 63 (± 2) μs, and this is consistent with published values for the $^1O_2$ lifetime in D_2O.[24,25] Most importantly, these data also confirm the expectation that, at the highest concentrations of I^- used at our experimental concentrations (3×10^{-4} M), quenching by I^- is still negligible compared with deactivation by the solvent (D_2O). Likewise, quenching by Lum at 8×10^{-5} M is negligible. This is expressed in eqn (4):

$$k_D \gg (k_T^{Lum}[Lum] + k_T^{I^-}[I^-]) \quad (4)$$

where k_D is the rate constant of deactivation by the solvent (1.6×10^4 s^{-1}),[25] k_T^{Lum} and $k_T^{I^-}$ are the bimolecular rate constants of $^1O_2$ total quenching by Lum and I^- (7.2×10^5 and 8.7×10^5 s^{-1} M^{-1}, respectively).[17,26]

Using eqn (3), the τ_T value was obtained from the $^1O_2$ phosphorescence trace for each I^- concentration. In the absence of I^-, a τ_T of 1.8 (±0.3) μs was obtained in air-equilibrated solutions. With these iodide-dependent values of τ_T, the quenching of the Lum triplet state may be evaluated by a Stern–Volmer analysis (eqn (2)). The value of k_q^T obtained from the slopes of the Stern–Volmer plot (Fig. 3(b)) was 3.2 (±0.5) × 10^9 M^{-1} s^{-1}, indicating that I^- likewise deactivates ^{3}Lum* with a rate constant characteristic of the diffusion-controlled limit.

Quenching of the singlet excited states

Previous studies have demonstrated that the intensity of the pterin fluorescence, at acidic pH, is significantly reduced by some anions through a proton-transfer mechanism.[27] This process is favored by anions of weak acids, such as phosphate and acetate, whereas anions of strong acids, such as chloride and nitrate, do not quench the fluorescence of PTs. HI is an acid stronger than HCl; consequently, proton transfer can be discarded as the predominant mechanism of fluorescence quenching by I^-. On the other hand, electron transfer has been proposed to be the main mechanism of quenching of fluorescence of PTs by nucleotides.[28]

Fluorescence quenching by I^- in aqueous solution at pH 5.5 has already been reported for Ptr[29] and for Lum[17] and

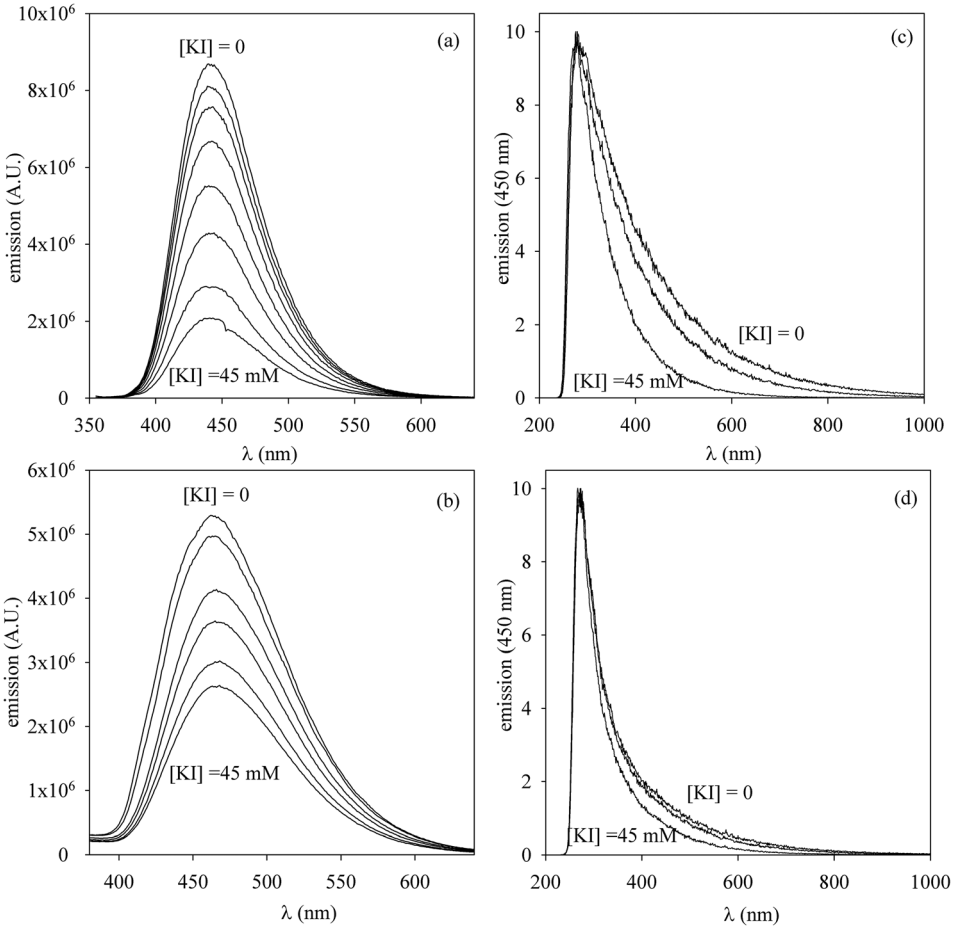


Fig. 4 Corrected fluorescence spectra of (a) Ptr, (λ_{exc} = 350 nm, pH = 5.5) and (b) Lum (λ_{exc} = 325 nm, pH = 5.5) in the absence and in the presence of different concentrations of I^-. Fluorescence decays of the (c) Ptr and (d) Lum emission in the absence and in the presence of different concentrations of I^- (λ_{exc} = 341 nm, λ_{an} = 450 nm).

fluorescence quenching for other pterin derivatives has likewise been reported.[30] Only steady-state measurements have been performed in these studies and, to the best of our knowledge, no results corresponding to time-resolved experiments have been reported. In the present work, we monitored the fluorescence intensity and lifetime of Ptr and Lum in aqueous solutions (pH 5.5) at different I^- concentrations.

The fluorescence spectra of the acidic forms of Ptr and Lum were recorded in the presence and in the absence of I^-. A set of experiments were carried out in aqueous solutions of a given pteridine derivative ($<30 \times 10^{-6}$ mol L^{-1}) containing different concentrations of I^- (0 to 0.05 mol L^{-1}) at pH 5.5. In all cases, a strong decrease in the fluorescence intensity was observed, but the shape of the fluorescence spectra remained unaffected, indicating that the acidic form of the PTs is responsible for the emission. The spectra obtained for the quenching of the Ptr and Lum fluorescence by I^- are presented in Fig. 4.

In time-resolved experiments, first-order kinetics were observed for all fluorescence decays of Ptr and Lum at various I^- concentrations. The corresponding lifetimes (τ_S) decreased strongly as a function of I^- concentration. Typical traces obtained for the quenching of the fluorescence of Ptr by I^- are shown in Fig. 4.

For both Ptr and Lum, the decrease of the integrated fluorescence intensity and the fluorescence lifetimes was evaluated as collisional quenching by a Stern–Volmer analysis: if a dynamic (collisional) process is operating, the quenching is described with the following equation:[31]

$$I_F^0/I_F = \tau_S^0/\tau_S = 1 + k_q^S \tau_S^0 [I^-] \quad (5)$$

where I_F^0 and I_F are the integrated fluorescence intensities and τ_S^0 and τ_S are the fluorescence lifetimes in the absence and in the presence of a quencher, respectively, k_q^S is the bimolecular quenching rate constant (L mol^{-1} s^{-1}), and $[I^-]$ is the I^- concentration (mol L^{-1}). Therefore, if I_F^0/I_F *versus* $[I^-]$ and τ_S^0/τ_S *versus* $[I^-]$ are linear and have the same slope, a purely dynamic process can be assumed. The Stern–Volmer analyses of the results obtained in both steady-state and time-resolved experiments for the quenching of the Ptr and Lum fluorescence by I^- are shown in Fig. 5. The corresponding k_q^S values, both in steady-state and time-resolved experiments, are listed in Table 1.

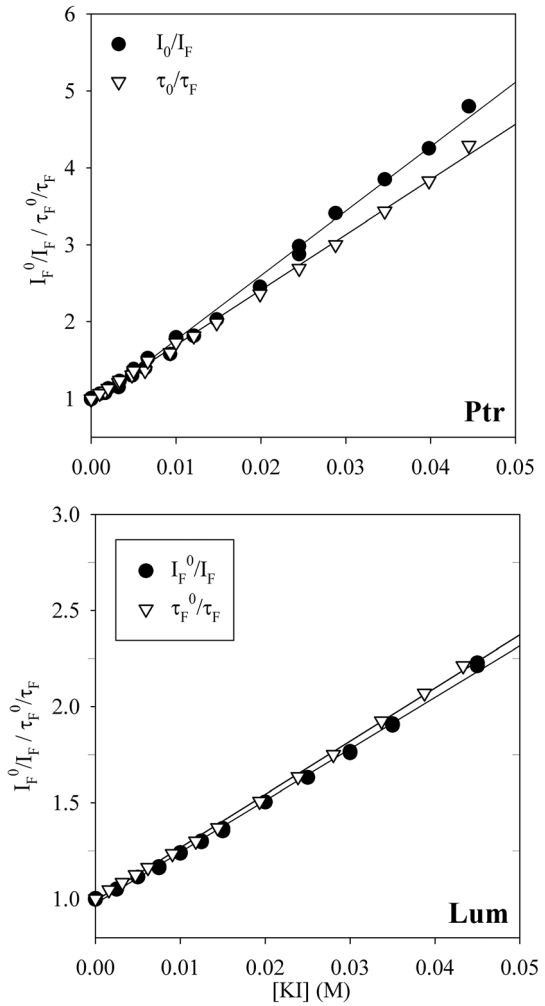


Fig. 5 Stern–Volmer plots of the quenching of fluorescence of Ptr and Lum by I^-, from both time-resolved and steady-state experiments, in acid media. [Ptr] = 21 μM; [Lum] = 18 μM.

Table 1 Summary of experimental fluorescence lifetimes (τ^S), bimolecular fluorescence quenching rate constants (k_q^S), triplet excited state lifetimes (τ^T), and bimolecular triplet excited state quenching rate constants (k_q^T) in aqueous solution (pH = 5.5)

	τ^S (10^{-9} s)	k_q^S (10^9 M^{-1} s^{-1})	τ^T (10^{-6} s)	k_q^T (10^9 M^{-1} s^{-1})
Lum	5.5 ± 0.3	4.9 ± 0.6[a] 4.2 ± 0.5[b]	1.8 ± 0.3	3.2 ± 0.5
Ptr	7.6 ± 0.3	10 ± 1[a] 9 ± 1[b]	3.4 ± 0.5	4.9 ± 0.6

[a] Steady state experiments. [b] Time resolved experiments.

These results indicate that the quenching of the fluorescence of Ptr and Lum by I^- is indeed a dynamic process and no association of I^- with PTs occurs in the analyzed concentration ranges. The results presented in this work, in connection with previous studies, strongly support the hypothesis that singlet excited states of PTs can be deactivated by both proton and electron transfer, the latter being the predominant one for I^-.

Selective quenching of triplet excited states by I^-

Both triplet and singlet excited states of Ptr and Lum are deactivated by I^-. The values of the bimolecular quenching rate constants obtained through our Stern–Volmer analyses and the lifetimes of the excited states are summarized in Table 1. All k_q values are relatively large for both the triplet and singlet excited states, and are close to the diffusion controlled limit (Table 1).

The longer lifetimes of the triplet excited states increase the probability of deactivation compared to the singlet excited states. The concentration of I^- needed for the identical extent of deactivation of singlet and triplet excited states can be calculated using eqn (2) and (5). For a given ratio of deactivation, the concentration of I^- is 242 and 252 times higher to deactivate the singlet excited states than the triplet excited states of Ptr and Lum, respectively. Taking into account these results, a concentration of 1–5 × 10^{-4} M of I^- deactivates more than 50% of the triplet excited states, but less than 2% of the singlet excited states.

Inhibition of the photosensitizing process by PTs

At micromolar I^- concentrations, both ^{3}Ptr* and ^{3}Lum* are efficiently deactivated by I^-, whereas the corresponding deactivation of excited singlet states is negligible. Both Ptr and Lum have been reported as sensitizers of the degradation of nucleotides,[6,17,32] amino acids,[8,9] DNA[33] and proteins.[34,35] According to these results, the addition of I^- in a suitable concentration can selectively deactivate the triplet states of PTs and, as such, it is possible to determine whether the PT singlet or triplet states are responsible for the reaction in a given system.

As an example, photosensitization experiments were carried out in air-equilibrated aqueous solutions of Ptr and the nucleotides, 2′-deoxyguanosine 5′-monophosphate (dGMP) and 2′-deoxyadenosine 5′-monophosphate (dAMP), at pH 5.5 in the absence and in the presence of I^- at a concentration of 200 μM.

Studies of photosensitized degradation of dGMP by Ptr were previously reported,[6] concluding that a type I oxidation mechanism predominates at acidic pH. A new set of experiments were performed to evaluate the consumption of dGMP in solutions containing Ptr exposed to continuous UV-A radiation (350 nm). Concentration profiles for Ptr and dGMP were obtained by HPLC analysis (Fig. 6a). In the absence of I^-, dGMP, as expected, was consumed. In the presence of I^- (200 μM), the rate of consumption of dGMP was much slower than that in its absence. At this concentration of I^- the singlet excited state was not significantly affected, in agreement with a mechanism in which the first step involves the triplet excited state.

Equivalent experiments were performed with dAMP, a nucleotide that does not react with $^1O_2$.[6] The mechanism involved in the dAMP oxidation photosensitized by pteridines was previously reported as an electron transfer mediated process.[17,32] Concentration profiles of Ptr and dAMP (Fig. 6b)

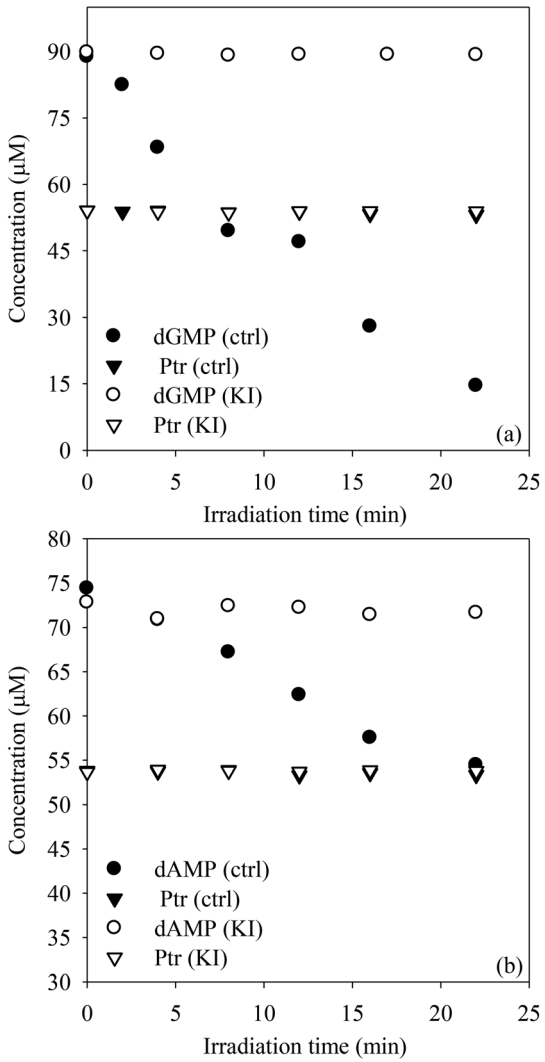


Fig. 6 Evolution of Ptr and a given nucleotide concentrations in air-equilibrated aqueous solutions as a function of the elapsed UV irradiation time (concentrations were determined by HPLC analysis) in the absence and in the presence of I^- (200 μM). (a) dGMP, (b) dAMP (pH = 5.5, λ_{exc} = 350 nm).

demonstrated that this nucleotide is consumed when a solution containing Ptr and dAMP (pH 5.5) was exposed to UV-A radiation (350 nm). As the only possibility of dAMP degradation is through a photosensitized reaction *via* the electron transfer mechanism, the addition of I^- (200 μM) resulted in a decrease in the rate of dAMP consumption, as expected, indicating that the electron transfer initiating step occurs from the ^{3}Ptr*.

Conclusions

In the present work, bimolecular rate constants for the deactivation of the singlet and triplet excited states of Ptr and Lum were obtained, finding that all have values in the range of diffusion controlled limit. The longer lifetimes of the triplet excited states of PTs allow their deactivation at concentrations of I^- of 10^{-3}–10^{-4} M, whereas the deactivation of the PT singlet excited states to a significant extent only starts at the much higher I^- concentration of 10^{-2} M. These results support the use of I^- to discriminate the participation of triplet excited states of PTs in the photosensitizing process. The addition of I^- to solutions of nucleotides or amino acids inhibits the electron transfer from these biomolecules to the PT triplet excited states, thereby hindering the PT photosensitized degradation of these molecules.

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