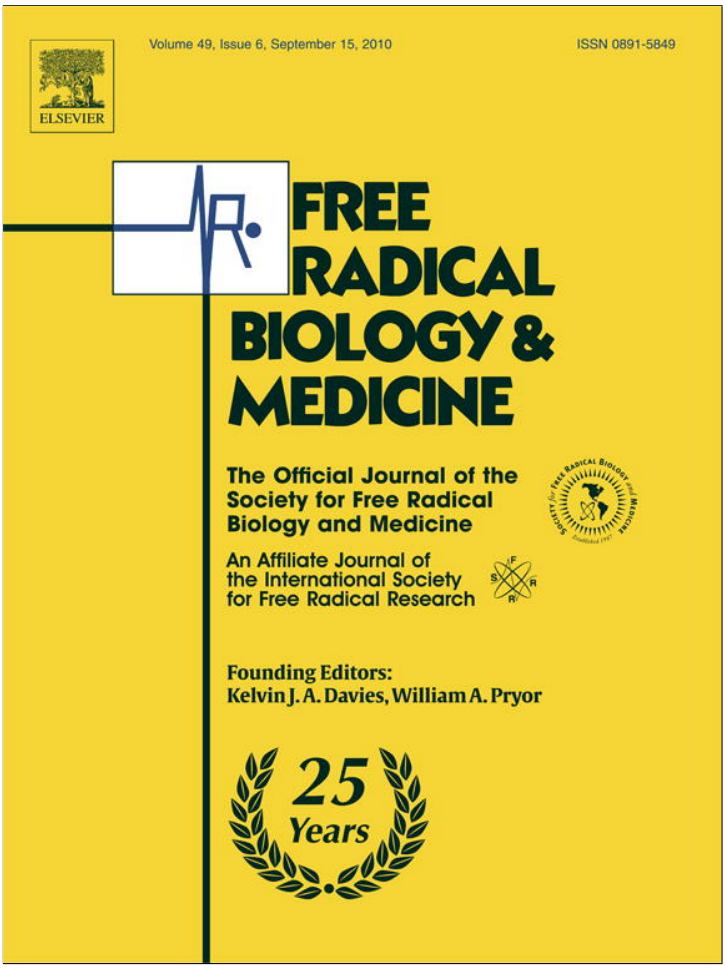

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Original Contribution

Electron-transfer processes induced by the triplet state of pterins in aqueous solutions

M. Laura Dántola [a], Mariana Vignoni [a], Constanza González [a], Carolina Lorente [a], Patricia Vicendo [b], Esther Oliveros [b,*], Andrés H. Thomas [a,*]

[a] Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CCT La Plata–CONICET, 1900 La Plata, Argentina

[b] Laboratoire des Interactions Moléculaires Réactivité Chimique et Photochimique, UMR 5623-CNRS/UPS, Université Paul Sabatier (Toulouse III), F-31062 Toulouse Cédex 9, France

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ABSTRACT

Pterins (Pt) are heterocyclic compounds widespread in living systems. They participate in relevant biological processes, such as metabolic redox reactions, and can photoinduce the oxidation of biomolecules through electron-transfer mechanisms. We have investigated the electron-transfer pathways initiated by excited states of pterin (Ptr) and 6-methylpterin (Mep), selected as model compounds. The experiments were carried out in aqueous solutions under continuous UV-A irradiation, in the presence and in the absence of ethylenediaminetetraacetic acid (EDTA), used as an electron donor. The reactions were followed by UV/Vis spectrophotometry, HPLC, and an enzymatic method for H_2O_2 determination. The formation of the superoxide anion ($O_2^{\bullet-}$) was investigated by electron paramagnetic resonance–spin trapping. The triplet excited states of Ptr and Mep are efficient electron acceptors, able to oxidize a Pt molecule in its ground state. The resulting radical anion ($Pt^{\bullet-}$) reacts with dissolved O_2 to yield $O_2^{\bullet-}$, regenerating the pterin. In the presence of EDTA, this reaction competes efficiently with the anaerobic reaction between $Pt^{\bullet-}$ and $EDTA^{\bullet+}$, yielding the corresponding stable dihydroderivatives H_2Pt. The effects of EDTA and dissolved O_2 concentrations on the efficiencies of the different competing pathways were analyzed.

Pterins, a family of heterocyclic compounds (Fig. 1), are present in biological systems in multiple forms and play various roles ranging from pigments to enzymatic cofactors for numerous redox and one-carbon transfer reactions [1,2]. Interest in the photochemistry and photophysics of pterin derivatives has been increasing steadily during the past decade, because of the implication of these compounds in various photobiological processes. Under UV-A excitation (320–400 nm), these biomolecules can fluoresce, undergo photooxidation to produce various products, and generate reactive oxygen species such as singlet oxygen ($^1O_2$) [3].

Some pterin derivatives (e.g., biopterin, 6-carboxypterin) accumulate in the skin of patients affected by vitiligo, a depigmentation disorder, in which the protection against UV radiation fails because of the lack of melanin [4]. Various studies performed on this disease indicate that excited states of pterins are photogenerated in vivo [5,6]. In addition, the in vivo photodegradation of folic acid, a conjugated pterin, has also been demonstrated in independent investigations [7].

Solar radiation induces modifications to genomic DNA and is implicated in the generation of human skin cancers [8,9]. Although nucleobases absorb very weakly above 300 nm, both UV-B (280–320 nm) and UV–A radiation can induce modifications in DNA through photosensitized reactions [10]. This indirect action may be mediated 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 state of the photosensitizer to pyrimidine bases leads to the formation of pyrimidine dimers [11,12]. Photosensitized oxidations also contribute to DNA damage induced by UV-A radiation. These processes involve the generation of radicals (type I mechanism), e.g., via electron transfer or hydrogen abstraction, and/or the production of $^1O_2$ (type II mechanism) [13].

It was demonstrated for the first time in 1997 [14] that, upon excitation with UV-A radiation, pterins are able to photoinduce DNA damage. Taking into account indirect evidence, the mechanism involved in this process was proposed to be an electron transfer. Later studies provided additional evidence on the photosensitizing capability of pterins [15,16]. In very recent studies performed with nucleotides as substrates, it was demonstrated that pterin (Ptr) can

Abbreviations: Pt, pterin derivative; Ptr, pterin; Mep, 6-methylpterin; EDTA, ethylenediaminetetraacetic acid; $O_2^{\bullet-}$, superoxide anion; H_2Pt, dihydropterin derivative; $^1O_2$, singlet oxygen; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; EPR, electron paramagnetic resonance; Φ, quantum yield; P_0, incident photon flux; P_a, photon flux absorbed; H_2Mep, 6-methyl-7,8-dihydropterin; Φ_{ISC}, quantum yield of intersystem crossing.

* Corresponding authors. A.H. Thomas is to be contacted at fax: +54 221 4254642. E. Oliveros, fax: +33 5 61558155.

E-mail addresses: oliveros@chimie.ups-tlse.fr (E. Oliveros), athomas@inifta.unlp.edu.ar (A.H. Thomas).

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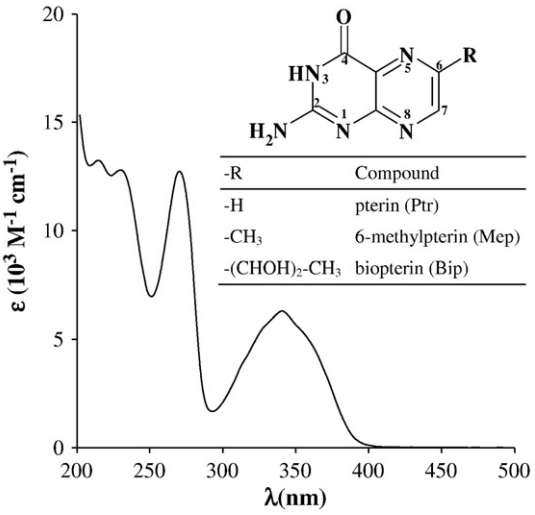

Fig. 1. Molecular structures of pterin derivatives and absorption spectra of Ptr in neutral and slightly acidic aqueous solutions. Spectra of Mep solutions are almost identical to those shown for Ptr.

act as a photosensitizer through both type I and type II mechanisms [17,18]. For the oxidation of 2′-deoxyadenosine 5′-monophosphate and 2′-deoxyguanosine 5′-monophosphate photoinduced by the neutral form of Ptr in aqueous solutions, the predominant mechanism is type I and would involve an initial electron transfer from the nucleotide to the triplet excited state of Ptr. In the following step, the resulting radical anion would reduce dissolved O_2 to regenerate the sensitizer and form the superoxide anion ($O_2^{\bullet-}$).

A similar mechanism was proposed for the autocatalytic photooxidation of 7,8-dihydrobiopterin [19] and for the photosensitization of nucleotides by lumazine ([20], M.P. Denofrio et al., manuscript in preparation), a compound chemically related to pterins. Therefore this type of mechanism might be a general pathway of photosensitization of biomolecules by pterins and related heterocycles. However, although the formation of nucleotide radicals was demonstrated [18], many aspects of the mechanism remain unknown. In particular, despite the biological relevance of $O_2^{\bullet-}$ due to its participation in the physiopathology of many diseases [21], the photochemical production of $O_2^{\bullet-}$ by pterins has not been demonstrated.

In the mechanism discussed in the previous paragraphs, the photosensitizer is not consumed. In contrast, it has been suggested that some pterins in the presence of electron donors undergo photoreduction, yielding the corresponding dihydropterin derivative, which in turn is reduced to a tetrahydropterin [22]. Although a general mechanism for the photoreduction of pterins has not been suggested, obviously an electron-transfer process must be involved, but it is clear that the overall mechanism should be different from that proposed for the photosensitized oxidation of nucleotides.

This work was aimed at a better understanding of the photoinduced electron-transfer mechanisms wherein pterins act as photosensitizers. In particular, we have investigated the production of $O_2^{\bullet-}$, the effect of dissolved O_2 on the efficiencies of the processes, and the experimental conditions needed to achieve photoreduction of pterins.

Materials and methods

Chemicals

Ptr and 6-methylpterin (Mep) were purchased from Schircks Laboratories (Jona, Switzerland) and used without further purification. Superoxide dismutase (SOD; bovine erythrocytes), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), ammonium acetate buffer, and tris (hydroxymethyl)aminomethane (Tris) were provided by Sigma. Ethylenediaminetetraacetic acid (EDTA) was purchased from Merck. KI was provided by Laboratorios Cicarelli. DMPO was purified by activated carbon and stored at −20 °C before use.

The pH of the aqueous solutions of pterins was adjusted by adding drops of HCl or NaOH solution from a micropipette. The concentrations of the acid and base used for this purpose ranged from 0.1 to 2 M. The ionic strength was approximately 10^{-3} M in the experiments performed in the absence of quenchers (DMPO, EDTA, KI).

Electron paramagnetic resonance–spin trapping experiments

Electron paramagnetic resonance (EPR) setup

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 $O_2^{\bullet-}$

Nitrones are common reagents for the detection and identification of transient radicals because of their ability to form persistent radical adducts that are detectable and fingerprintable by EPR spectroscopy [23,24]. In our experiments, DMPO was used as a spin trap [25].

Samples (500 μl) contained 9×10^{-5} to 10^{-4} M Ptr, 5×10^{-2} M DMPO 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 various times from the beginning of the irradiation and until a plateau was observed in the EPR signal intensity (after 2 to 3 min under our experimental conditions).

To confirm the formation of $O_2^{\bullet-}$ upon Ptr photolysis, similar EPR–spin trapping experiments were performed in the presence of SOD (22 U/ml). This enzyme catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2 and O_2 [26,27]. Control experiments in the presence of SOD denatured by heating for 30 min at 90 °C were also performed [28].

Steady-state irradiation

UV irradiation

The continuous photolyses of Ptr and Mep were carried out in aqueous solutions placed in quartz cells (1-cm optical pathlength) at room temperature. Rayonet RPR lamps emitting at 350 nm (Southern N.E. Ultraviolet Co., Branford, CT, USA) were employed for irradiating. The experiments were performed in the presence and in the absence of O_2. Deaerated and O_2-saturated solutions were obtained by bubbling with Ar and O_2 for 20 min, respectively.

Quantum yield determinations

The quantum yields of reactant disappearance (Φ_{-R}) and photoproduct formation (Φ_P) were determined in experiments performed under different conditions. Values were obtained using the following equations:

$$\Phi_{-R} = -[(d[R]/dt)_0]/P_a, \quad (1)$$

$$\Phi_P = [(d[P]/dt)_0]/P_a, \quad (2)$$

where $(d[R]/dt)_0$ and $(d[P]/dt)_0$ (mol L^{-1} s^{-1}) are the initial rates of reactant consumption and photoproduct formation, respectively, and P_a (einsteins L^{-1} s^{-1}) is the photon flux absorbed by the reactant. The initial rates were obtained from the slopes of the corresponding plots of concentration vs irradiation time.

Actinometry

Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurements of the incident photon flux (P_0) at the excitation wavelength. Aberchrome 540 is the anhydride form of (*E*)-α-(2,5-dimethyl-3-furylethylidene)(isopropylidene)succinic acid, which, under irradiation in the spectral range 316–366 nm, leads to a cyclized form. The reverse reaction to ring opening is induced by visible light (436–546 nm). A value of 5.0 ($\pm$0.3) $\times 10^{-6}$ einsteins L^{-1} s^{-1} was obtained for P_0 in the irradiation setup described above. 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 according to the Lambert–Beer law:

$$P_a = P_0(1-10^{-A}), \quad (3)$$

where A is the absorbance of the reactant at the excitation wavelength.

UV/Vis spectrophotometry

Electronic absorption spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer. Measurements were made using quartz cells of 1-cm optical pathlength. The absorption spectra of the solutions were recorded at regular intervals of irradiation time.

High-performance liquid chromatography

A high-performance liquid chromatograph, Prominence from Shimadzu (solvent delivery module LC-20AT, online degasser DGU-20A5, communications bus module CBM-20, autosampler SIL-20A HT, column oven CTO-10AS VP, and photodiode array detector SPD-M20A), was employed for monitoring the reaction. A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping, 150 × 4.6 mm, 4 μm; Phenomenex) was used for product separation. Solutions containing 3% methanol and 97% 10 mM ammonium acetate (pH 7.0) were used as mobile phase.

Detection and quantification of H_2O_2

H_2O_2 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 [31,32]. 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 SAIC (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 H_2O_2 solutions prepared from commercial standards were employed for obtaining the corresponding calibration curves.

In all cases in which H_2O_2 was detected and quantified using the technique described in the previous paragraph, controls with catalase, the enzyme that catalyzes the decomposition of H_2O_2 to H_2O and O_2, were carried out. Catalase was added after irradiation and before mixing the analyzed solution with the reactants. Thus, the absence of absorbance at 505 nm in these controls confirmed the formation of H_2O_2 in the studied reactions.

Fluorescence measurements

Steady-state fluorescence measurements were performed in quartz cells (1-cm optical pathlength) using a PerkinElmer LS 50B spectrofluorimeter. Fluorescence spectra of aqueous solutions of Ptr and Mep were obtained by excitation at 340 nm and were recorded between 350 and 650 nm. The spectra were corrected for differences in instrumental response and light scattering. For determining the quenching of fluorescence of Ptr and Mep by iodide (I^-), emission spectra of Ptr and Mep solutions (17 μM) were recorded in the absence and in the presence of KI (0 to 35 mM). The total fluorescence intensities were calculated by integration of the corresponding fluorescence bands.

Results and discussion

Pterin photolysis: electron paramagnetic resonance–spin trapping experiments

To characterize the formation of $O_2^{\bullet-}$ upon photolysis of Ptr, EPR analyses were performed in the presence of a spin trap (DMPO). As shown in Fig. 2a, the irradiation of a solution containing Ptr (10^{-4} M) and DMPO (5×10^{-2} M) led to the immediate formation of an EPR signal, which increased with the irradiation time. The intensity of the EPR signal reached a maximum after 2 to 3 min of irradiation (Fig. 2b). The EPR spectrum is characterized by hyperfine coupling constants $a^N = 14.2$ G, $a^H_\beta = 11.2$ G, and $a^H_\alpha = 1.25$ G, corresponding to the adduct DMPO–OOH [33].This result suggests that DMPO has trapped $O_2^{\bullet-}$. Control experiments were carried out in the absence of Ptr and, as expected, no signal corresponding to the adduct DMPO–OOH was registered.

To confirm the involvement of the $O_2^{\bullet-}$ in the formation of the DMPO–OOH adduct, similar experiments were performed in the presence of SOD (see Materials and methods). As shown in Fig. 2c, the addition of this enzyme caused an important decrease in the EPR signal. In addition, when thermally denatured SOD was added to the Ptr solution, no significant decrease in the EPR signal corresponding to the spin adduct DMPO–OOH was detected (data not shown), which is

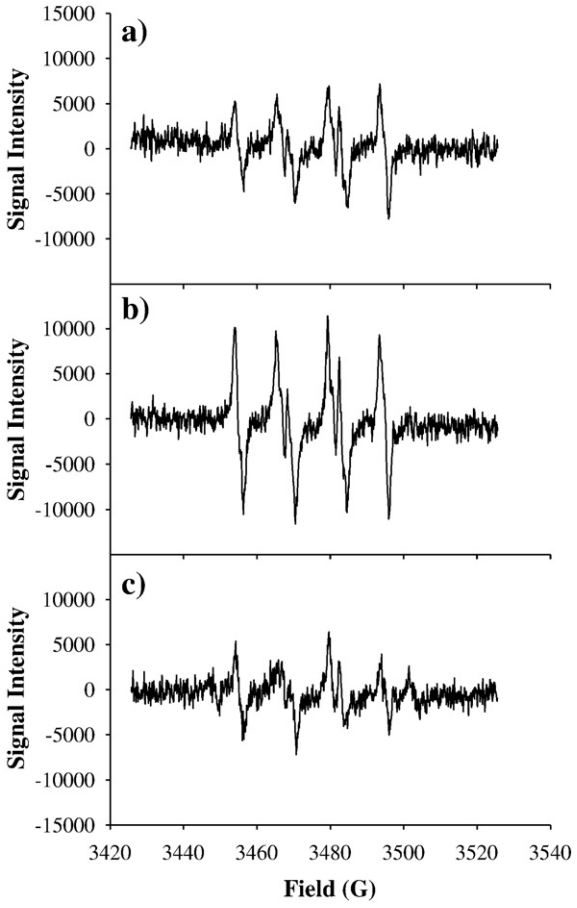

Fig. 2. Detection of the DMPO–OOH adduct by EPR analysis upon aerobic irradiation of an aqueous solution of Ptr (100 μM) at pH 7.0 and DMPO (50 mM). (a) After 40 s of irradiation, (b) after 3 min of irradiation, and (c) same as (b) with added SOD (22 U/ml).

further evidence of the production of $O_2^{\bullet-}$ [28]. To exclude the participation of Tris as an electron donor in the formation of $O_2^{\bullet-}$ upon Ptr photolysis, similar EPR–spin trapping experiments were performed in water. Under such conditions, the EPR signal corresponding to the DMPO–OOH adduct was also observed. Therefore, we can conclude that UV-A irradiation of Ptr induces the production of $O_2^{\bullet-}$. Similar results were obtained in the case of Mep.

Two main mechanistic hypotheses may be proposed. The first is the direct reduction of O_2 by excited Ptr (Reaction (4)). However, to the best of our knowledge, this type of pathway has not been proposed for pterins. The second is an electron transfer between Ptr in its ground state and electronically excited Ptr to yield the corresponding pair of radical ions ($Ptr^{\bullet+}$ and $Ptr^{\bullet-}$) (Reaction (5)). This process has been reported from flash-photolysis studies [34,35]. Pterin radical anion is in equilibrium with its protonated form (Reaction (6)) [34,36], whereas, considering the behavior of other related heterocycles such as purines [37–39], the radical cation is very likely in equilibrium with its deprotonated form (Reaction (7)). It is well established that dissolved O_2 quenches organic radical anions, leading to the formation of $O_2^{\bullet-}$ [40,41]. If this is the case, $O_2^{\bullet-}$ detected by EPR–spin trapping would be the product of the reaction between $Ptr^{\bullet-}$ (or eventually $Ptr(H)^{\bullet}$) and O_2 (Reactions (8) and (8′)). Taking into account that Reaction (5) has been suggested to take place from triplet states of pterins [34,35] and that the triplet state of Ptr may be responsible for the photooxidation of purine nucleotides through an electron-transfer mechanism [17,18], it can be assumed that this excited state participates in the photoproduction of $O_2^{\bullet-}$ as well (*vide infra*):

$$Ptr^{*} + O_2 \rightarrow Ptr^{\bullet+} + O_2^{\bullet-} \quad (4)$$

$$Ptr^{*} + Ptr \rightarrow Ptr^{\bullet+} + Ptr^{\bullet-} \quad (5)$$

$$Ptr^{\bullet-} + H^{+} \rightleftarrows Ptr(H)^{\bullet} \quad (6)$$

$$Ptr^{\bullet+} \rightleftarrows Ptr(-H)^{\bullet} + H^{+} \quad (7)$$

$$Ptr^{\bullet-} + O_2 \rightarrow Ptr + O_2^{\bullet-} \quad (8)$$

$$Ptr(H)^{\bullet} + O_2 \rightarrow Ptr + HO_2^{\bullet} \quad (8')$$

$$O_2^{\bullet-} + H_3O^{+} \rightleftarrows HO_2^{\bullet} + H_2O \quad (9)$$

$$O_2^{\bullet-} + HO_2^{\bullet} \rightarrow HO_2^{-} + O_2 \quad (9')$$

$$Ptr^{\bullet+} + O_2^{\bullet-} \rightarrow Ptr + O_2 \quad (10)$$

$$^{3}Ptr^{*} \rightarrow Ptr \quad (11)$$

$$^{3}Ptr^{*} + {}^{3}O_2 \rightarrow Ptr + {}^{1}O_2 \quad (12)$$

$$^{3}Ptr^{*} + {}^{3}O_2 \rightarrow Ptr + O_2 \quad (12')$$

$$Ptr^{\bullet-} + Ptr^{\bullet+} \rightarrow 2\,Ptr \quad (13)$$

$$Ptr(H)^{\bullet} + Ptr(-H)^{\bullet} \rightarrow 2\,Ptr \quad (14)$$

$$2Ptr(H)^{\bullet} \rightarrow PtrH_2 + Ptr \quad (15)$$

Pterin photolysis: effect of SOD on H_2O_2 production

The photochemistry of Ptr and pterin derivatives bearing substituents that cannot be easily oxidized, such as Mep, has been previously studied [42,43]. Excitation in air-equilibrated solutions leads to oxidation, yielding nonpterinic photoproducts (cleavage of pterin moiety) and H_2O_2. The quantum yields of reactant disappearance at pH 5.5 were reported to be 8.2×10^{-4} and 2.4×10^{-4}, respectively.

The H_2O_2 detected during the photolysis of Ptr and Mep can be the product of the spontaneous disproportionation of $O_2^{\bullet-}$, with its conjugated acid $HO_2^{\bullet}$ (Reactions (9) and (9′)) [44]. However, considering that radical ion recombination may be quite efficient (Reaction (10)), the fraction of the generated $O_2^{\bullet-}$ yielding H_2O_2 can be very low. Therefore, we performed experiments in the presence of SOD, this enzyme being able to catalyze the $O_2^{\bullet-}$ disproportionation, thus increasing the efficiency of H_2O_2 production.

In irradiation experiments carried out in air-equilibrated solutions, as expected, the rate of H_2O_2 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 (Reaction (10)) that consume most $O_2^{\bullet-}$ formed in the absence of SOD. It should be noted that the time windows of these experiments cannot be directly compared to those of EPR measurements because the experimental conditions are different; i.e., as explained under Materials and methods, the irradiation sources and the geometry are not the same.

In addition, results showed that the rate of Ptr consumption was higher in the presence of SOD than in its absence. This fact is in agreement with the proposed mechanism: SOD, by removing $O_2^{\bullet-}$, prevents electron back-transfer from $O_2^{\bullet-}$ to $Ptr^{\bullet+}$ (Reaction (10)) and thus stimulates pterin consumption.

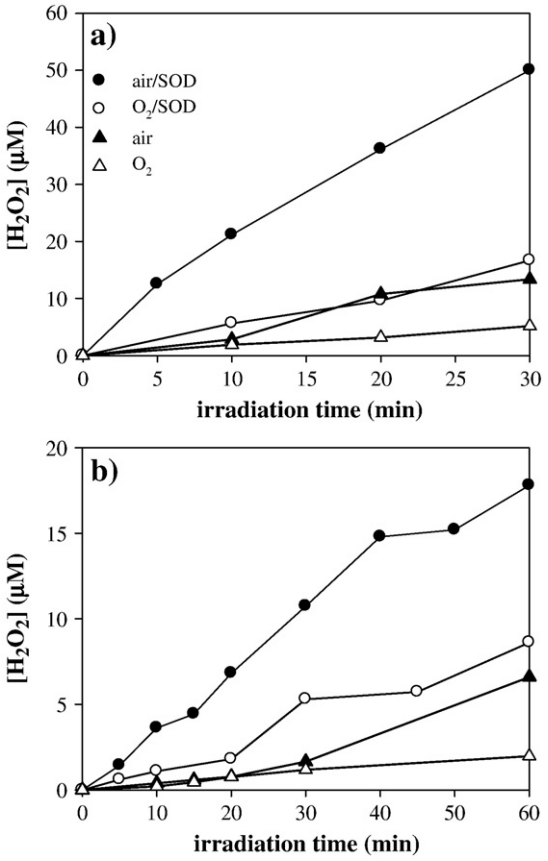

Fig. 3. Evolution of the H_2O_2 concentration as a function of the elapsed irradiation time in solutions of (a) Ptr (100 μM) and (b) Mep (100 μM). Experiments were performed in air-equilibrated and O_2-saturated aqueous solutions (pH 5.5), in the absence and in the presence of SOD (50 U/ml).

Effect of the oxygen concentration on the efficiency of H_2O_2 production

To assess the possible mechanisms proposed for the formation of $O_2^{\bullet-}$ (*vide supra,* Reaction (4) and/or Reactions (5) and (8)) and to investigate the participation of the Ptr triplet excited state, a set of experiments was performed in O_2-saturated solutions. Surprisingly in experiments carried out in the absence of SOD, instead of an increase, O_2 caused a significant decrease in the rate of H_2O_2 production (Fig. 3). This behavior was even more notable in the presence of SOD. This experimental result may be interpreted on the basis of the following mechanistic analysis. As O_2 does not quench singlet excited states of pterins [45,46], but is an efficient quencher of their triplet states [43,47,48] (*vide infra*), the behavior observed is in favor of the participation of the Ptr triplet excited state in the studied process. Assuming this is the case, the species Ptr* in Reactions (4) and (5) should be the Ptr triplet state (^{3}Ptr*). Three additional ^{3}Ptr* deactivation pathways compete with the electron-transfer process: intersystem crossing to the ground state (Reaction (11)), energy transfer to O_2 to yield $^1O_2$ (Reaction (12)), and deactivation by O_2 (Reaction (12′)).

Photosensitized production of $^1O_2$ (Reaction (12)) has been demonstrated for a group of pterin derivatives, including Ptr and Mep, by monitoring the near-infrared $^1O_2$ luminescence in steady-state experiments [43,48]. The quantum yields of $^1O_2$ production (Φ_Δ) were determined to be 0.18 and 0.10 in air-equilibrated aqueous solutions at pH 5.5 for Ptr and Mep, respectively.

If $O_2^{\bullet-}$ were formed predominantly by direct electron transfer from ^{3}Ptr* to O_2 (Reaction (4)), the efficiency of H_2O_2 production would be expressed as $k_4[O_2]/(k_{O2}[O_2]+k_5[Ptr]+k_{11})$ (with $k_{O2}=k_4+k_{12}+k_{12'}$), and it should increase with O_2 concentration in the presence or in the absence of SOD (or remain unchanged if $k_{O2}[O_2] >> k_5[Ptr]+k_{11}$). On the other hand, if $O_2^{\bullet-}$ were mainly formed through the two consecutive steps represented by Reactions (5) and (8), enhanced quenching of ^{3}Ptr* by O_2 should decrease the relative efficiency of Reaction (5) ($k_5[Ptr]/(k_{O2}[O_2]+k_5[Ptr]+k_{11})$). Consequently the rate of H_2O_2 formation should decrease with O_2 concentration (or remain unchanged if $k_{O2}[O_2] << k_5[Ptr]+k_{11}$). Therefore the comparative experiments carried out in air-equilibrated and O_2-saturated solutions strongly suggest that Reaction (5) is the first step in the mechanism of $O_2^{\bullet-}$ formation.

To obtain additional evidence on this point, we calculated the quantum yields of H_2O_2 formation (Φ_{H2O2}) under various experimental conditions. A simple competition between quenching of ^{3}Ptr* by Ptr (Reaction (5)) and by O_2 (Reactions (4), (12), and (12′)) (i.e., k_{11} negligible) was assumed. Taking into account that the O_2 concentration in O_2-saturated solutions is fivefold higher than in air-equilibrated solutions, one can expect the recovery of Φ_{H2O2} in O_2-saturated solutions, with a fivefold increase in pterin concentration. We obtained the following values for Φ_{H2O2}: $9\ (\pm1)\times10^{-4}$ ([Ptr] = 30 μM, air), $3.8\ (\pm0.3)\times10^{-4}$ ([Ptr] = 30 μM, O_2), and $10\ (\pm2)\times10^{-4}$ ([Ptr] = 150 μM, O_2). These results clearly support the hypothesis that quenching of ^{3}Ptr* by O_2 competes with a process in which Ptr in its ground state participates and leads to the formation of H_2O_2.

Experiments similar to those described in the previous and this section were carried out with Mep and the same behavior was observed (Fig. 3b): (i) in air-equilibrated solutions SOD caused an important increase in H_2O_2 production and (ii) under irradiation in the presence or in the absence of SOD, the rate of H_2O_2 formation was faster in air-equilibrated than in O_2-saturated solutions. Therefore, we can conclude that Reactions (5) and (8) should be the predominant pathway of $O_2^{\bullet-}$ production.

Photolysis in the absence of O_2

The participation of radicals of heterocyclic compounds in various types of reactions has been reported. Just to mention a few examples: (i) in flavins, disproportionation of the conjugate acid of the radical anion leads to the fully reduced dihydroflavin [49]; (ii) the radical cation of norharmane, a β-carboline, undergoes dimerization [50]. These types of reactions have not been reported for radicals of pterins. Both Ptr and Mep are photostable in the absence of O_2 [42,43], even after several hours of irradiation ($\lambda_{ex}>320$ nm) as confirmed in this work. Therefore, recombination processes, such as those represented by Reactions (13) and (14), recover the reactants, and the photoreduction of Ptr or Mep via the disproportionation of radicals (Reaction (15)) is negligible under our experimental conditions.

Effect of an electron donor

To investigate how the system behaves in the presence of an electron donor we performed photolysis experiments with solutions containing a pterin derivative and EDTA. This compound was chosen as an electron donor for several reasons: it is very soluble in H_2O, its absorption in the UV-A range is very weak, and it has been already used in previous studies on photoreduction of pterins [22] and other photosensitizers [51–53].

In the absence of O_2, for both studied compounds (Ptr and Mep), significant spectral changes consisting in blue shifts of the corresponding low-energy bands were observed. Spectral analysis was similar to that reported in previous studies [22] and suggested the presence of dihydropterins as the main photoproducts [54]. For Mep the formation of the corresponding dihydropterin derivative (6-methyl-7,8-dihydropterin; H_2Mep) was confirmed by means of HPLC, using a standard solution. For Ptr this was not possible because 7,8-dihydropterin is not commercially available because of its instability. In experiments carried out at various initial concentrations of EDTA, Mep and H_2Mep concentrations were determined as a function of irradiation time (Fig. 4). The concentration profiles showed that H_2Mep was formed with a yield of about 75%, compared to Mep consumption. Other pterinic products different from H_2Mep were not detected by HPLC analysis. A fraction of H_2Mep formed during the reaction may be consumed in secondary reactions. To the best of our knowledge, the photochemistry of H_2Mep has not been studied, but considering the photolability, even in the absence of O_2, of other dihydropterins (such as 6-formyl-7,8-dihydropterin [55] and 7,8-dihydrobiopterin [19]), photochemical transformations of H_2Mep may also contribute to consumption of this product.

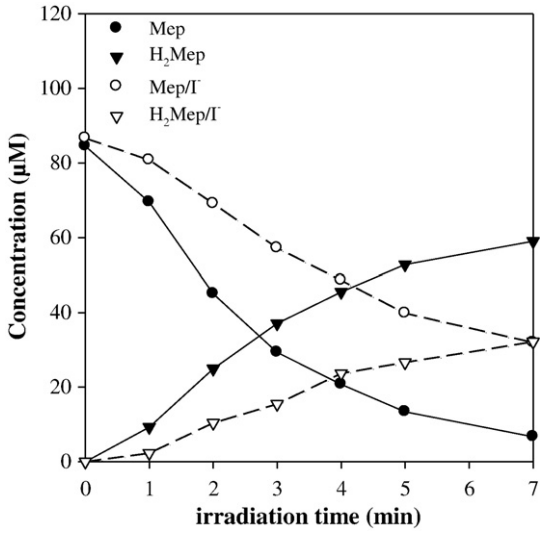

Fig. 4. Anaerobic photolysis of Mep in the presence of EDTA. Mep and H_2Mep concentrations as a function of the elapsed irradiation time. $[Mep]_0$ 85 μM, [EDTA] 30 mM, pH 5.5. Open symbols and dashed lines: experiments performed in the presence of iodide (400 μM).

These results must be considered in connection with previous studies performed with nucleotides [17,18], wherein no photoreduction of Ptr was reported. Therefore, it seems that, under anaerobic conditions, substrates with very low ionization potential, such as EDTA ($E_{(EDTA^{\bullet+}/EDTA)} = 0.4$ V vs Normal Hydrogen Electrode (NHE)) [56], induce the photoreduction of pterins, whereas this process does not take place with oxidizable substrates with higher ionization potential, such as purine nucleotides (NT) ($E_{(NT^{\bullet+}/NT)} > 1.33$ V vs NHE) [57].

The quantum yield of Mep consumption (Φ_{-Mep}) in deaerated solutions was determined for various EDTA concentrations. The plot of Φ_{-Mep} as a function of the initial EDTA concentration increases to reach a plateau (Fig. 5), which is expected taking into account a reaction between the electron donor and a Mep excited state steadily formed upon irradiation.

In a similar set of experiments, also carried out in the presence of various EDTA concentrations but *in air-equilibrated solutions*, no photoreduction was detected and no consumption of Ptr or Mep was registered. In these experiments, H_2O_2 was formed and its concentration increased as a function of irradiation time (Fig. 6). Although H_2Mep was not detected in these experiments, its formation and further reaction with H_2O_2 must be considered. In fact, the oxidation of H_2Mep by H_2O_2 takes place and has been previously studied [54]. However, we can discard this reaction in our experiments because: (i) the main products are non-pterinic compounds [54]; subsequently if a proportion of Mep (or Ptr) were converted into H_2Mep and the latter, in turn, reacted with H_2O_2, consumption of Mep should be observed; and (ii) if H_2Mep were formed, the rate of its oxidation, calculated from the value of the corresponding bimolecular rate constant reported in Ref. [53], would be negligible under the experimental conditions used in the present work.

These results suggest that the pterin radical anion reacts with dissolved O_2, regenerating the pterin and yielding $O_2^{\bullet-}$, which in turns gives H_2O_2 as a measurable product. When the concentration of H_2O_2 exceeded approx 180 μM, Mep started being consumed when high EDTA concentrations were present (Fig. 6). This was very probably due to a drastic reduction in the O_2 concentration in the solution, leading to practically anaerobic conditions and consequently to the behavior discussed above and exemplified in Fig. 4.

In addition, the Φ_{H2O2} determined in air-equilibrated solutions at various EDTA concentrations showed an asymptotic behavior (Fig. 5), similar to that observed in deaerated solutions. This result is discussed in the next section.

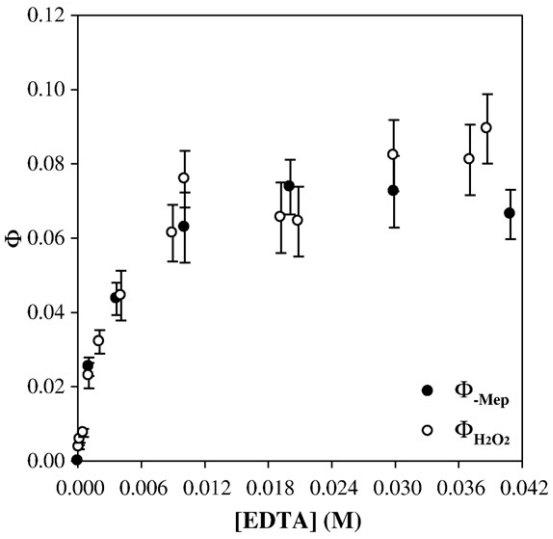

Fig. 5. Quantum yields of Mep consumption (Φ_{-Mep}) and H_2O_2 formation (Φ_{H2O2}) as function of the EDTA concentration. Φ_{-Mep} and Φ_{H2O2} were determined under anaerobic and aerobic conditions, respectively. $[Mep]_0$ 85 μM, pH 5.5.

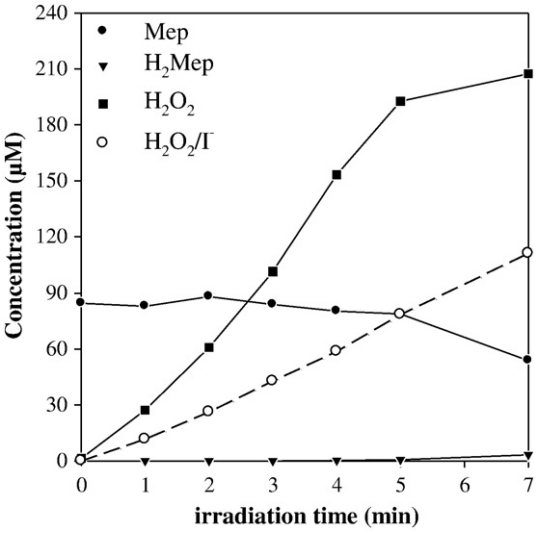

Fig. 6. Aerobic photolysis of Mep in the presence of EDTA. Mep, H_2Mep, and H_2O_2 concentrations as a function of the elapsed irradiation time. $[Mep]_0$ 85 μM, [EDTA] 30 mM, pH 5.5. Open symbols and dashed line: experiments performed in the presence of iodide (400 μM).

Based on the results obtained for the photolysis of Mep in the presence of EDTA, the mechanistic pathways may be described by Reactions (16)–(24). In both deaerated and aerated solutions, the radical anion of Mep ($Mep^{\bullet-}$) and the radical cation of EDTA ($D^{\bullet+}$) are formed by electron transfer from EDTA to the Mep triplet state (^{3}Mep*) (Reaction (19)). $Mep^{\bullet-}$ and $D^{\bullet+}$ are in equilibrium with their corresponding neutral forms: $Mep(H)^{\bullet}$ and $D(-H)^{\bullet}$, respectively (Reactions (20) and (21)). Alternatively, these neutral radicals may be formed by a proton transfer between the radical ion pair within the solvent cage. Then, the following reactions depend on the O_2 concentration:

(a) *Under anaerobic conditions*, a hydrogen transfer between the neutral radicals $D(-H)^{\bullet}$ and $Mep(H)^{\bullet}$ (Reaction (22)) may lead to the formation of H_2Mep and the oxidized donor D(−2H) (in the case of EDTA, ethenediaminetetraacetic acid should be formed).

(b) *In air-equilibrated solutions*, trapping of $Mep^{\bullet-}$ by O_2 (Reaction (23)) competes with Reactions (21) and (22). In the former reaction, $Mep^{\bullet-}$ is oxidized back to Mep, and $O_2^{\bullet-}$ is formed. Although in air-equilibrated solutions both reactions are possible, Reaction (23) seems to be predominant because no consumption of Mep but an efficient production of H_2O_2 resulting from $O_2^{\bullet-}$ disproportionation was observed. In addition, it has been reported for amines, including EDTA, that the corresponding radicals formed after one-electron oxidation and deprotonation ($D(-H)^{\bullet}$) are able to reduce O_2 to $O_2^{\bullet-}$ (Reaction (24)) [51–53]. The relevance of the latter reaction is discussed in the next section.

$$Mep \xrightarrow{h\nu} {}^1Mep^* \quad (16)$$

$${}^1Mep^* \xrightarrow{ISC} {}^3Mep^* \quad (17)$$

$${}^3Mep^* + O_2 \rightarrow Mep + {}^1O_2 \quad (18)$$

$${}^3Mep^* + O_2 \rightarrow Mep + O_2 \quad (18')$$

$${}^3Mep^* + D \rightarrow Mep^{\bullet-} + D^{\bullet+} \quad (19)$$

$$D^{\bullet+} \rightleftarrows D(-H)^{\bullet} + H^+ \quad (20)$$

$$H^+ + Mep^{\bullet -} \rightleftarrows Mep(H)^{\bullet} \quad (21)$$

$$Mep(H)^{\bullet} + D(-H)^{\bullet} \rightarrow H_2Mep + D(-2H) \quad (22)$$

$$Mep^{\bullet -} + O_2 \rightarrow Mep + O_2^{\bullet -} \quad (23)$$

$$D(-H)^{\bullet} + O_2 \rightarrow D(-2H) + HO_2^{\bullet}\left(H^+ + O_2^{\bullet -}\right) \quad (24)$$

(c) *In O_2-saturated solutions,* the formation of H_2O_2 was slower than under air-equilibrated conditions (Fig. 7), as in the absence of EDTA (Fig. 3). This result suggests again that competitive quenching processes of ^{3}Mep* by O_2 (Reactions (18) and (18′)) decrease the relative efficiency of the electron-transfer reaction between the electron donor (in this case EDTA) and ^{3}Mep* (Reaction (19)) and reaction at the origin of the formation of $Mep^{\bullet -}$ and thus of $O_2^{\bullet -}$ (Reaction (23)) and H_2O_2 (Reactions (9) and (9′)). In addition, as expected, no consumption of Mep was registered.

Evaluation of the participation of the triplet excited states of pterins

To confirm the participation of triplet states of pterins in the electron-transfer processes, experiments in the presence of 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 values of the rates of the different deactivation pathways (non-radiative decays to ground state, intersystem crossing) [58]. Therefore, in some cases, the presence of I^- causes an increment in the quantum yields of triplet state formation, whereas in other cases, a decrease is observed. I^- quenches flavin triplet states much more efficiently than the corresponding excited singlet states. This property has been used to investigate the role of the excited states of flavin molecules in photochemical mechanisms [59,60]. Moreover, 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^- [61].

To apply this methodology to our system, we first evaluated the capability of I^- to deactivate the singlet excited states of Ptr and Mep by fluorescence quenching experiments. Only a moderate quenching was registered under the experimental conditions used ([Mep] or [Ptr] 18 μM, [I^-] 0–35 mM, pH 5.5); i.e., a decrease of ca. 50% of the fluorescence was measured at a concentration of 10 mM I^- (Fig. 8). At I^- concentrations lower than 1 mM, the fluorescence quenching was negligible.

Solutions containing Mep and EDTA were irradiated in the presence of I^- at concentrations of 100 to 500 μM. Under anaerobic conditions, I^- slowed down the consumption of Mep and the formation of H_2Mep (Fig. 4). Likewise, in air-equilibrated solutions, the rate of H_2O_2 formation was much lower than that registered in the absence of I^- (Fig. 6). Thermal or photochemical reactions of H_2O_2 with I^- were discarded by performing control experiments in the dark and under UV-A irradiation ([H_2O_2] 100 μM, [I^-] 500 μM). No consumption of H_2O_2 was registered for more than 20 min after mixing of the reagents. Therefore results of photolysis in the presence of I^- (Figs. 4 and 6) are in agreement with experiments performed in O_2-saturated solutions and support our assumption that the first step in the reaction manifold occurring under both aerobic and anaerobic conditions is the electron transfer from the EDTA molecule to ^{3}Mep* (Reaction (19)).

The behavior of the quantum yields $\Phi_{-\mathrm{Mep}}$ and Φ_{H2O2} presented in Fig. 5 can be discussed further under this assumption. In an experiment performed under steady irradiation, the rate of ^{3}Mep* formation is given by Eq. (25),

$$d\left[^3Mep^*\right]/dt = P_a\ \Phi_{ISC}, \quad (25)$$

where P_a is the photon flux absorbed by Mep and Φ_{ISC} is the quantum yield of intersystem crossing. In the presence of a large excess of EDTA, all ^{3}Mep* formed should react with EDTA (Reaction (19)) to yield $Mep^{\bullet -}$, so that the rate of $Mep^{\bullet -}$ formation will be also given by Eq. (25). If, in the absence of O_2, all $Mep^{\bullet -}$ formed is consumed to yield

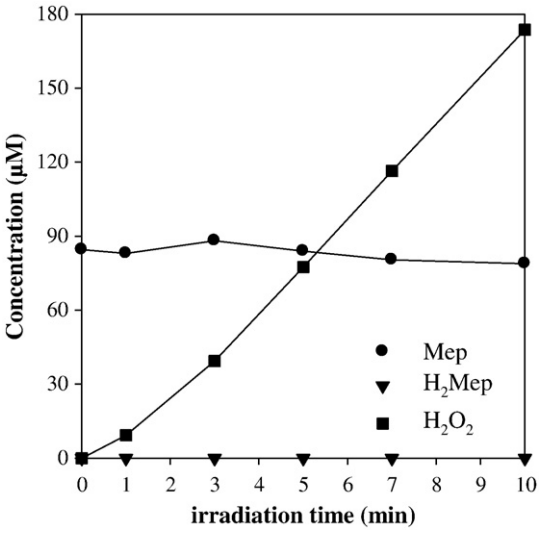

Fig. 7. Evolution of the concentrations of Mep, H_2Mep, and H_2O_2 during irradiation of O_2-saturated solutions of Mep in the presence of EDTA. $[Mep]_0$ 85 μM, [EDTA] 30 mM, pH 5.5.

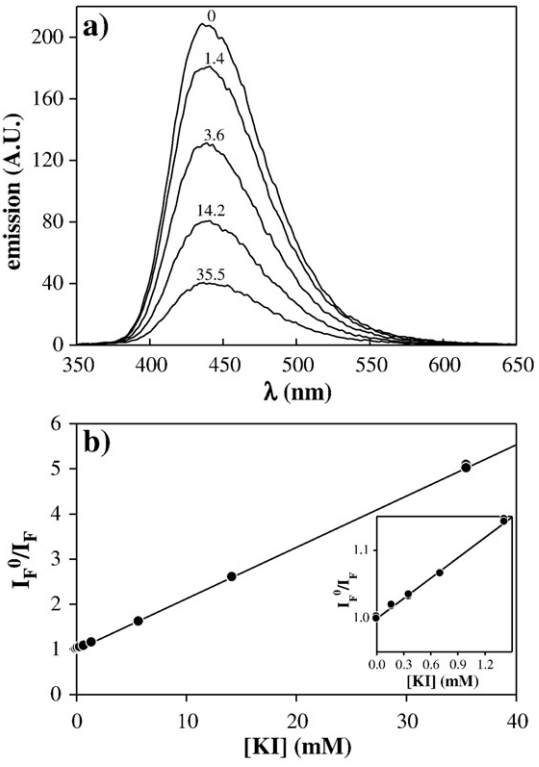

Fig. 8. Quenching of the fluorescence of Mep by I^-. (a) Fluorescence spectra (λ_{ex} = 350 nm) of a solution of Mep (17 μM, pH 5.5) in the absence and in the presence of various concentrations of KI; the KI concentration (mM) appears above each spectrum. (b) Stern–Volmer plots of the fluorescence intensities (I_F).

H_2Mep through Reactions (21) and (22), the corresponding rate of Mep consumption should be also given by Eq. (25). Likewise, considering Reactions (23) and (24) as the sources of $O_2^{\bullet-}$ in the presence of O_2, and Reactions (9) and (9′) for the conversion of $O_2^{\bullet-}$ into H_2O_2, the corresponding rate of H_2O_2 formation should be also given by Eq. (25). If this analysis is correct, $\Phi_{-\mathrm{Mep}}$ and Φ_{H2O2}, at high EDTA concentrations, should be equal to Φ_{ISC}. To the best of our knowledge, Φ_{ISC} has not been reported for Mep; however, previous studies on $^1O_2$ production suggested a value of 0.10 for this parameter [43], which is very close to the asymptotic value reached in plots of $\Phi_{-\mathrm{Mep}}$ and Φ_{H2O2} vs [EDTA].

Conclusions

We have investigated the electron-transfer pathways initiated by the excited states of pterin and 6-methylpterin, selected as model pterinic compounds (Pt). In neutral or slightly acidic aqueous solutions, triplet states of pterins ($^3Pt^*$) initiate a series of competing reactions that can be summarized as follows (protonation equilibria were omitted for clarity):

$$^3Pt^* \rightarrow Pt \quad (a)$$

$$^3Pt^* \rightarrow O_2\ Pt \quad (b)$$

$$^3Pt^* + D \rightarrow Pt^{\bullet-} + D^{\bullet+} \quad (c)$$

$$Pt^{\bullet-} + D^{\bullet+} \rightarrow Pt + D \quad (d)$$

$$Pt^{\bullet-} + O_2 \rightarrow Pt + O_2^{\bullet-} \quad (e)$$

$$Pt^{\bullet-} + D^{\bullet+} \rightarrow \rightarrow H_2Pt + D(-2H) \quad (f)$$

$$D(-H)^{\bullet} + O_2 \rightarrow D(-2H) + H^+ + O_2^{\bullet-} \quad (g)$$

$$O_2^{\bullet-} + D^{\bullet+} \rightarrow O_2 + D \quad (h)$$

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

In addition to intersystem crossing to the ground state (Reaction (a)), $^3Pt^*$ can be deactivated by dissolved O_2 (Reaction (b)). Alternatively, $^3Pt^*$ can react with an electron donor (D) (Reaction (c)), which can be Pt itself or a different compound. Three different pathways are possible for the resulting radical anion ($Pt^{\bullet-}$): electron back-transfer to $D^{\bullet+}$ (Reaction (d), main reaction in the absence of O_2 and of an electron donor other than Pt itself), electron transfer to O_2 (Reaction (e)), or reaction with $D^{\bullet+}$ to yield a dihydroderivative (Reaction (f), main reaction in the presence of the electron donor EDTA under anaerobic conditions). An additional source of $O_2^{\bullet-}$ is the reduction of O_2 by a neutral form of $D^{\bullet+}$ (Reaction (g), D = EDTA). Finally, $O_2^{\bullet-}$ can react with $D^{\bullet+}$ (Reaction (h), D = EDTA) or undergo disproportionation yielding H_2O_2 and O_2 as final products (Reaction (i)).

Acknowledgments

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