Mechanism of photooxidation of folic acid sensitized by unconjugated pterins

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Folic acid, or pteroyl-L-glutamic acid (PteGlu), is a precursor of coenzymes involved in the metabolism of nucleotides and amino acids. PteGlu is composed of three moieties: a 6-methylpterin (Mep) residue, a *p*-aminobenzoic acid (PABA) residue, and a glutamic acid (Glu) residue. Accumulated evidence indicates that photolysis of PteGlu leads to increased risk of several pathologies. Thus, a study of PteGlu photodegradation can have significant ramifications. When an air-equilibrated aqueous solution of PteGlu is exposed to UV-A radiation, the rate of the degradation increases with irradiation time. The mechanism involved in this "auto-photo-catalytic" effect was investigated in aqueous solutions using a variety of tools. Whereas PteGlu is photostable under anaerobic conditions, it is converted into 6-formylpterin (Fop) and *p*-aminobenzoyl-L-glutamic acid (PABA-Glu) in the presence of oxygen. As the reaction proceeds and enough Fop accumulates in the solution, a photosensitized electron-transfer process starts, where Fop photoinduces the oxidation of PteGlu to Fop, and H_2O_2 is formed. This process also takes place with other pterins as photosensitizers. The results are discussed with the context of previous mechanisms for processes photosensitized by pterins, and their biological implications are evaluated.

Introduction

Folic acid, or pteroyl-L-glutamic acid (PteGlu), is a conjugated pterin widespread in biological systems. Its chemical structure is composed of three moieties: a 6-methylpterin (Mep) residue, a *p*-aminobenzoic acid (PABA) residue, and a glutamic acid (Glu) residue (Fig. 1). In living systems, PteGlu is present in multiple forms including molecules attached to several glutamate residues and dihydro and tetrahydro pterin derivatives. Folate is the generic term for this large family of chemically similar compounds.

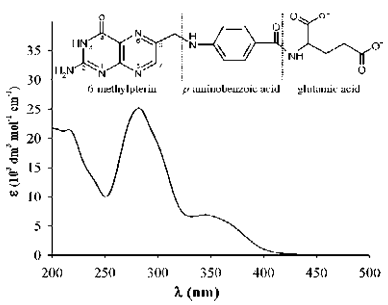

Fig. 1 Molecular structure and absorption spectrum of the predominant form of PteGlu in neutral aqueous solutions.

Coenzymes derived from PteGlu belong to the vitamin B group and facilitate the transfer of one-carbon units from donor molecules in metabolic pathways leading to the biosynthesis of nucleotides.[1] These coenzymes also participate in the metabolism of several amino acids.[1] Folate requirements increase in periods of rapid cell division and growth, and, as such, it is imperative that pregnant women, for example, keep folate concentrations at an appropriate level.[2] Folate deficiency in pregnant women has clearly been shown to be related to neural tube defects (NTD), such as spina bifida and anencephaly.[3,4] Furthermore, a deficit of folate leads to megaloblastic anemia in children and adults,[5] coronary heart disease,[6] cancer,[7] neurological disorders,[8] and fertility problems.[9–11] PteGlu is inexpensive to produce, more stable than most members of folate's family and efficiently metabolized into biologically active derivatives such as 5-methyl-tetrahydrofolic acid. Due to these properties, PteGlu is used in tablet form and in fortified foods for dietary supplementation.[1,12]

The absorption spectrum of PteGlu shows bands in the UV-B (280–320 nm) and UV-A (320–400 nm) spectral regions (Fig. 1) and, as such, this molecule can be excited by solar radiation. Different studies have suggested that UV-A exposure and the photolysis of PteGlu derivatives lead to increased risk of NTD, and that skin pigmentation is an effective mechanism of protection against folate depletion. Epidemiological data have shown that the prevalence of NTD is higher in light-skinned people.[13,14] In addition, the correlation between NTD and UV-A exposure[15] has been described in amphibian larvae[16] and in women who had used artificial tanning sunbeds during the first weeks of pregnancy.[17]

The photosensitivity of PteGlu has been known since the late 1940s.[18] In 1978, Branda and Eaton proposed that one of the main functions of skin pigmentation is to avoid photolysis of folate.[19] This novel and striking hypothesis was based on two pieces of evidence: (i) light-skinned patients undergoing photochemotherapy (*i.e.*, psoralen plus UV-A) showed lower serum folate levels than healthy controls, and (ii) a 30–50% loss of folate in human plasma was observed after *in vitro* exposure to simulated sunlight. Recent reports indicate that both *in vitro* and *in vivo* exposure of human blood to UV-A radiation leads to photodegradation of folate.[20,21]

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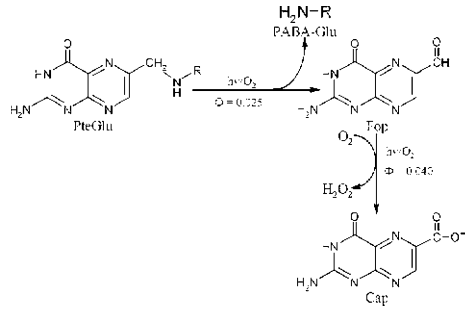
Based on some of the studies mentioned above, Jablonski and Chaplin published an intriguing concept about the evolution of human skin coloration.[22,23] This hypothesis proposes that humans with dark skin have been positively selected in regions with high solar intensities, because pigmentation, by protecting against folate photolysis, would prevent NTD and deficiency of spermatogenesis.

Due to the biological implications of the photodegradation of folate in humans, the photochemical behavior of PteGlu as a model compound becomes very interesting. In the last decade, several studies have analyzed the products of photodegradation of PteGlu in aqueous solutions.[24–28] Briefly, in the absence of oxygen, PteGlu is photostable. However, excitation of PteGlu in air-equilibrated solutions leads to cleavage and oxidation of the molecule, yielding 6-formylpterin (Fop) and *p*-aminobenzoylglutamic acid (PABA-Glu) as photoproducts (Scheme 1). In turn, Fop is transformed into 6-carboxypterin (Cap) upon further photooxidation. Thus, the presence of oxygen is a required component in PteGlu photodegradadtion.



Scheme 1 Photooxidation of PteGlu in air-equilibrated aqueous solutions under UV-A irradiation. R refers to the PABA-Glu conjugate (see Fig. 1).

The PteGlu absorption band centered at *ca.* 355 nm (Fig. 1) corresponds to the typical low-energy band of pterins.[29] Therefore, upon UV-A irradiation, excited states of the pterin moiety are readily formed. In contrast to most unconjugated pterins (derivatives with small substituents instead of the PABA unit), PteGlu has a very low fluorescence quantum yield ($\Phi_F < 5 \times 10^{-3}$)[30] and does not sensitize the production of singlet oxygen ($^1O_2$).[31] These properties suggest that the substituent (PABA-Glu) in PteGlu acts as an "internal quencher" that efficiently deactivates the singlet excited states of the pterin moiety. This has been attributed to intramolecular photo-induced electron transfer from the PABA ring to the pterin moiety.[32–34] It has been suggested that this internal electron transfer is a key step in the photo-degradation of PteGlu.[32] In this context of an electron transfer reaction, the observed oxygen effect on PteGlu degradation could involve the trapping of radicals and/or radical ions by oxygen.

It was reported in 2000 that, when an air-equilibrated aqueous solution of PteGlu is exposed to UV-A radiation, the rate of PteGlu degradation increases with irradiation time.[26] This "auto-photo-catalytic" effect, which has since been confirmed,[28] could imply that the main mechanism of PteGlu photodegradation reported in different systems could involve a photosensitized reaction (*i.e.*, photochemical or photophysical alteration occurring in one molecular entity as a result of initial absorption of radiation by another molecular entity called the photosensitizer).[35]

Given the important biological and medical ramifications of PteGlu photodegradation, we set out to examine the mechanism involved in this process. In particular, the oxidation of PteGlu photosensitized by unconjugated pterins was investigated. The results are discussed within the context of previous mechanisms for processes photosensitized by pterins, and their biological implications are evaluated.

Experimental

General

Folic acid (PteGlu), *p*-aminobenzoyl-L-glutamic acid (PABA-Glu), 6-methylpterin (Mep), 6-formylpterin and 6-carboxypterin, purchased from Schircks Laboratories, were of the highest purity available (>95%) and were used without further purification. KI was provided by Laboratorios Cicarelli. Superoxide dismutase (SOD) from bovine erythrocytes (lyophilized powder, ≥95% biuret, ≥3000 units per mg protein), *p*-aminobenzoic acid (PABA) and other chemicals were from Sigma Aldrich and used as received. The pH of aqueous solutions was adjusted by adding drops of 0.1–0.2 M aqueous NaOH or HCl solutions with a micropipette. In all experiments the ionic strength was *ca.* 10^{-3} M.

Steady-state irradiation

Irradiation apparatus. Aqueous solutions were irradiated in 1 cm path length quartz cells at room temperature with a Rayonet RPR lamp (Southern N.E. Ultraviolet Co.) with emission centered at 350 nm (bandwidth ~ 20 nm). Photolysis experiments were performed in deoxygenated, aerated, and oxygen-saturated solutions. Deoxygenated and O_2-saturated solutions were obtained by bubbling for 20 min with Ar and O_2 gas, respectively.

Actinometry. Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurements of the incident photon flux (P_0) at the excitation wavelength ($P_0^{350} = 5.1\ (\pm 0.4) \times 10^{-6}$ einstein $L^{-1}\ s^{-1}$). 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 method for the determination of P_0 has been described in detail elsewhere.[36,37] Values of the photon flux absorbed (P_a) were calculated from P_0 using the expression:

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

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

Analysis of irradiated solutions

UV/vis analysis. Electronic absorption spectra were recorded on a Varian Cary-3 or Hewlett–Packard Model 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. Chromatography of the reaction systems was performed using an instrument from Shimadzu (Prominence, solvent delivery module LC-20AT, on-line degasser DGU-20A5, auto sampler SIL-20A HT, column oven CTO-10AS VP and photodiode array detector SPD-M20A).

A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping, 150 × 4.6 mm, 4 μm, Phenomenex) was used for product separation, the elution being achieved with: (i) 3% methanol, 97% aqueous solution of ammonium acetate (10 mM, pH = 7.0); or (ii) 5% acetonitrile, 95% aqueous solution of sodium citrate (10 mM, pH = 4.0). Aqueous solutions of commercial standards were employed for obtaining calibration curves of reactants and products.

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.[38,39] 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.

Laser flash photolysis

Time-resolved absorption experiments were performed as previously described.[40] Briefly, the frequency-tripled output (355 nm) of a Quanta-Ray GCR 230 Nd:YAG laser operating at the repetition rate of 10 Hz was used as the excitation source (pulse fwhm ~ 5 ns). Transient species thus produced were monitored using the spectrally-resolved output of a steady-state Xe lamp. To increase the signal-to-noise ratio, data from ~250 independent laser pulses were typically averaged.

Results and discussion

Photolysis of PteGlu in air and O_2-saturated solutions

Air-equilibrated aqueous solutions of folic acid (PteGlu) at concentrations over the range 20–1000 μM were irradiated for different periods of time. Identification and quantification of reactant and products by HPLC analysis was in agreement with previous reports: PteGlu was oxidized into 6-formylpterin (Fop) and *p*-aminobenzoylglutamic acid (PABA-Glu) and the rate of this process increased with irradiation time (Fig. 2). The subsequent oxidation of Fop into 6-carboxypterin (Cap) was also observed.

In the photooxidative degradation of other pterin systems, including Fop, H_2O_2 has been observed to play a role.[41] To the best of our knowledge, evidence of H_2O_2 formation during the photolysis of PteGlu has yet to be reported. In our present experiments, this reactive oxygen species was detected upon irradiation of air-equilibrated PteGlu solutions, its concentration increasing with irradiation time (Fig. 2). Upon the photooxidation of Fop to produce Cap (Scheme 1), H_2O_2 is produced in a 1 : 1 stoichiometry[42] (*i.e.*, one molecule of H_2O_2 generated for each molecule of Fop consumed). If the H_2O_2 detected in the irradiated PteGlu solutions were generated only as a consequence of Fop photooxidation, its concentration at a given time should be equal to [Cap]. On the other hand, if H_2O_2 were also produced with the same stoichiometry in the photochemical conversion of PteGlu into Fop, its concentration, at a given time, should be equal to [Fop] + 2[Cap] (*i.e.*, moles of H_2O_2 formed = moles of PteGlu converted into Fop + moles of Fop converted into Cap). Mass balance calculated in different experiments (as an example, see inset of Fig. 2) showed that the latter hypothesis is likely to be correct, indicating that H_2O_2 is also generated in the same reaction in which Fop is formed from PteGlu.

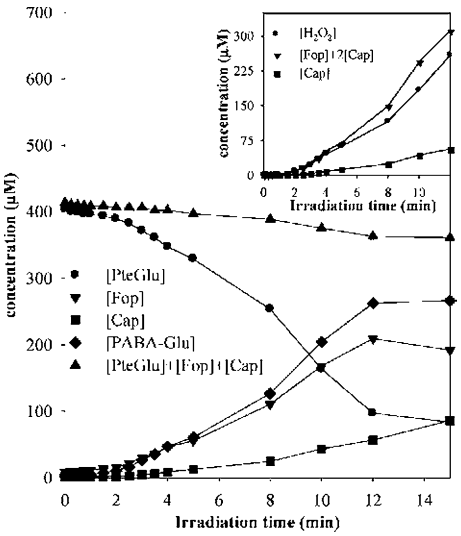


Fig. 2 Time evolution of reactant and photoproduct concentrations in air-equilibrated aqueous solutions of PteGlu under UV-A irradiation. $[PteGlu]_0 = 400$ μM, pH = 5.5. Inset: time evolution of H_2O_2 concentration and comparison with oxidized product formation. Errors on an individual data point are ~±4 μM.

The stoichiometric relationship between H_2O_2 released and PteGlu consumed suggests that H_2O_2 is a final product and does not play a role as a reactive intermediate in the mechanism of the photosensitized process. This fact was confirmed by a series of control experiments: (i) no changes in the composition were detected in solutions containing H_2O_2 (>1 mM) and PteGlu (115 μM) kept in the dark for several hours; (ii) the same rates of PteGlu consumption (and Fop formation) were registered when PteGlu (115 μM) solutions with and without H_2O_2 (>1 mM) were irradiated under otherwise identical conditions.

The H_2O_2 detected can be the product of the spontaneous disproportionation of superoxide anion ($O_2^{\bullet-}$), with its conjugate acid $HO_2^{\bullet}$.[43] Therefore, to investigate the participation of $O_2^{\bullet-}$ in the mechanism, experiments in air-equilibrated solutions 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.[44] The data showed a significant increase in the rates of PteGlu consumption and H_2O_2 formation when SOD was present in the solution (Fig. 3). These results (i) indicate that $O_2^{\bullet-}$ is involved in the photoinitiated process, (ii) provide evidence for the existence of electron transfer reactions, and (iii) indicate that $O_2^{\bullet-}$ may act to prevent or inhibit PteGlu degradation.

A complementary set of experiments was performed in O_2-saturated solutions and the results were compared with those performed under air-saturated conditions. The data obtained clearly showed that the rate of PteGlu disappearance is *greater* in air-saturated solutions than in O_2-saturated solutions (Fig. 4). Although this is a non-linear system, it is worth mentioning that the inhibition is about a factor of 5, which corresponds to the concentration ratio of O_2 in air- and O_2-saturated solutions. Dissolved O_2 does not deactivate singlet excited states of pterins,[29,30] but it efficiently quenches the pterin triplet state.[31,45]

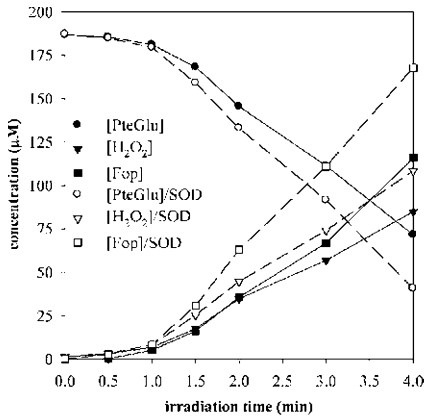


Fig. 3 Time evolution of PteGlu, Fop and H_2O_2 concentrations in air-equilibrated aqueous solutions of PteGlu under UV-A irradiation. $[PteGlu]_0$ = 185 μM, pH = 5.5. Experiments performed in the absence (black symbols) and in the presence of SOD (300 U/ml) (white symbols). Errors on an individual data point are ~±4 μM.

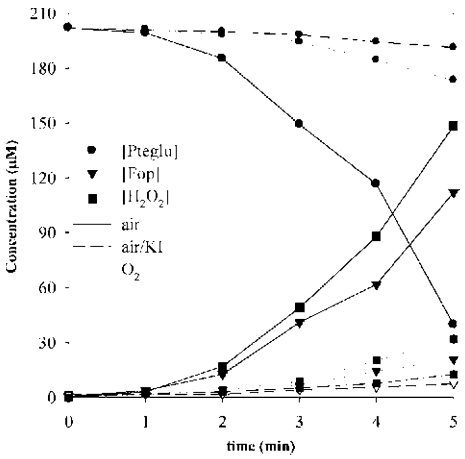


Fig. 4 Time evolution of PteGlu, Fop and H_2O_2 concentrations in aqueous solutions of PteGlu under UV-A irradiation. $[PteGlu]_0$ = 200 μM, pH = 5.5. Experiments were performed in air-equilibrated solutions in the absence (solid lines) and the presence of KI (300 μM) (dashed lines), and in O_2-saturated solutions (dotted lines). Errors on an individual data point are ~±4 μM.

Therefore the inhibition of the photooxidation of PteGlu at high O_2 concentrations suggests that the pterin triplet excited state is involved in the reactions leading to the formation of Fop and Cap.

To further investigate the possible participation of the pterin triplet state, experiments in the presence of iodide (I^-) were performed. This anion enhances the non-radiative decay of the lowest triplet state of pterins[46] and has been used as a selective quencher to investigate the role of pterin triplet states in different photochemical processes.[47–49] It has been demonstrated for several pterin derivatives that I^-, over the concentration range of 100 to 500 μM, does not quench the pterin singlet excited state.[48,49] Therefore, air-equilibrated solutions of PteGlu were irradiated in the presence of I^-. Under these conditions, a striking inhibition of the photooxidation of PteGlu was likewise observed (Fig. 4). In a series of controls performed in the dark to discard interferences, no reactions between I^- and the reactants and products of the studied processes (PteGlu, Fop and H_2O_2) were observed. These results are in agreement with experiments performed in O_2-saturated solutions, and strongly suggest the participation of the pterin triplet state in the mechanism of PteGlu photooxidation.

Therefore, taking into account the results presented so far along with data from previous studies (see Introduction), two processes can be considered for the photochemical degradation of PteGlu: (i) photooxidation of PteGlu initiated by excited states of PteGlu itself; (ii) oxidation of PteGlu photosensitized by Fop and/or Cap. Clearly, the first process must play a role because Fop and Cap are not present at the beginning of the photolysis. However, the latter can quickly dominate when photoproducts accumulate in the solution and absorb a portion of the incident radiation. The experiments shown in this section suggest that the photosensitized oxidation involves an electron transfer reaction in which triplet states of the unconjugated pterins participate. The oxidation of PteGlu photosensitized by unconjugated pterins is analyzed in detail in the next sections.

The role of singlet oxygen in the photosensitized oxidation of PteGlu

Given that the behavior of this photosystem is sensitive to the presence of oxygen, it is incumbent upon us to ascertain if singlet oxygen ($^1O_2$) is involved in these reactions. Although PteGlu itself does not sensitize the production of $^1O_2$ (*vide supra*), $^1O_2$ can be formed by energy transfer from the triplet states of unconjugated pterins.[31] If $^1O_2$, produced by a triplet state pterin, was an intermediate in the photosensitized oxidation of PteGlu, one would expect that the rate of PteGlu consumption in an O_2-saturated solution should be at least equal to that in an air-saturated solution; *i.e.*, the amount of $^1O_2$ produced upon quenching of the triplet state pterin by O_2 in an O_2-saturated solution should be at least equal that in an air-saturated solution.[50] However, the observation that the rate of PteGlu disappearance decreases with an increase in the O_2 concentration clearly points away from this scenario.

In a recent study in which $^1O_2$ was independently produced, the reaction between PteGlu and $^1O_2$ was studied in detail.[51] Fop and PABA-Glu were identified as products, but only 27% of the PteGlu consumed was transformed into Fop. In addition to these compounds, other products, resulting from the oxidation of the pterin moiety, were also formed. In the current study, solutions containing PteGlu and independently-added Fop were irradiated and the corresponding concentration profiles were obtained by HPLC analysis. As expected, Fop and Cap were formed, and PteGlu was consumed. In another set of experiments, solutions containing PteGlu and a different unconjugated pterin were irradiated. 6-Methylpterin (Mep) was chosen for this latter study because it is photostable (*i.e.*, quantum yields of consumption under UV-A irradiation ~2.4×10^{-4}) and it also generates $^1O_2$ ($\Phi_\Delta = 0.10$).[52] In contrast to the results obtained in the study of the reaction between independently-produced $^1O_2$ and PteGlu,[51] the fraction of PteGlu converted to Fop and Cap (*i.e.*, Δ([Fop] + [Cap])/Δ[PteGlu]) in these latter PteGlu/Fop and PteGlu/Mep experiments was very close to 1 (*vide infra*).

Thus, the analysis presented in this section clearly indicates that, upon irradiation of PteGlu, oxidation by $^1O_2$ does not play a significant role in the degradation of PteGlu. This conclusion

is also consistent with that of Moan *et al.*,[28] which was obtained on the basis of competitive kinetic experiments performed in H_2O and D_2O (because the lifetime of $^1O_2$ is longer in D_2O than H_2O,[53] one would expect more pronounced PteGlu degradation in D_2O if $^1O_2$ was involved).

Photolysis of PteGlu in the presence of Mep

In order to focus our attention on the photosensitized oxidation of PteGlu by unconjugated pterins, a series of experiments was performed in the presence of Mep, whose photophysical properties, such as absorption and fluorescence spectra, fluorescence quantum yields, fluorescence lifetimes, are comparable to those of Fop.[29,30] In these experiments, the initial concentration of Mep was higher than that of PteGlu. Under these conditions, the direct photolysis of PteGlu does not contribute to its consumption; rather, PteGlu disappearance is a result of photosensitization by Mep. In addition, in contrast to Fop, Mep is photostable (*vide supra*); as such, its photochemistry does not interfere with the analysis of PteGlu disappearance. Thus, the results obtained from this experiment complement the data obtained upon direct photolysis of PteGlu and yield more information about the mechanism involved in PteGlu oxidation.

Concentration profiles obtained by HPLC analysis revealed that, upon irradiation of air-equilibrated solutions containing PteGlu and Mep, PteGlu was rapidly oxidized into Fop and PABA-Glu, whereas the concentration of Mep did not significantly vary (Fig. 5). Upon prolonged irradiation, Cap was produced as a result of the photooxidation of Fop. In addition, H_2O_2 was formed in a 1 : 1 stoichiometry (for each time, $[H_2O_2] = [Fop] + 2[Cap]$) (Fig. 5). These results indeed indicate that the photosensitized oxidation of PteGlu takes place using Mep instead of Fop as a photosensitizer. Control experiments were carried out under the same conditions but in the absence of Mep (inset of Fig. 5). The data clearly indicate that the process initiated by direct excitation of PteGlu is negligible compared to that sensitized by Mep. In addition, no consumption of PteGlu was detected in a control experiment performed by irradiating an O_2-free solution containing PteGlu and Mep.

Complementary experiments were carried out irradiating solutions containing PteGlu (42 μM) and Mep (135 μM) in the presence of SOD and I^-, and in O_2-saturated solutions (Fig. 6). In all experiments, within the time window analyzed, the Mep concentration was constant and the H_2O_2 concentration was *hν* equal, within the experimental error, to [Fop] + 2[Cap]. Therefore, to simplify the information shown, plots of [Mep] and [Fop] + 2[Cap] *vs.* irradiation time were omitted in Fig. 6. In agreement with the data shown in Fig. 3, a significant increase in the rates of PteGlu consumption and H_2O_2 formation was observed when SOD was present in the solution. This behavior, which will be discussed in detail in the next section, implicates that SOD, by removing $O_2^{\bullet-}$, prevents the oxidation of PteGlu. Likewise, in agreement with the results found for solutions containing initially only PteGlu, the oxidation of PteGlu was practically negligible in the presence of I^- or in O_2-saturated solutions, again suggesting the participation of triplet excited states.

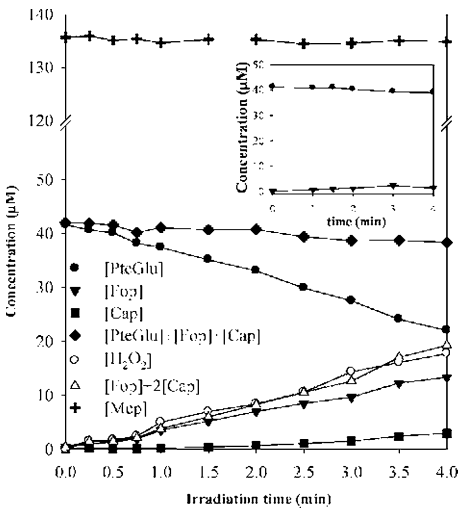


Fig. 5 Time evolution of concentrations of substrate (PteGlu), photosensitizer (Mep) and products (Fop, Cap and H_2O_2) in air-equilibrated aqueous solutions initially containing PteGlu and Mep, under UV-A exposure. $[PteGlu]_0$ = 42 μM, $[Mep]_0$ = 135 μM, pH = 5.5. Inset: Time evolution of concentrations of PteGlu and Fop in an experiment performed under similar conditions, but in the absence of Mep.

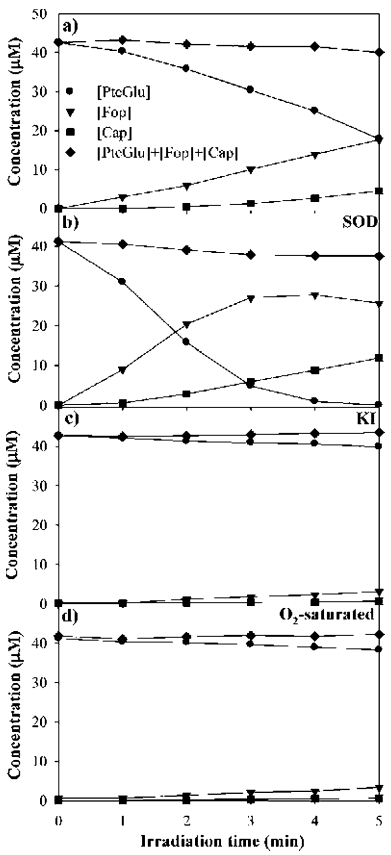


Fig. 6 Time evolution of concentrations of PteGlu, Fop and Cap in air-equilibrated aqueous solutions containing initially PteGlu and Mep, under UV-A exposure. $[PteGlu]_0$ = 42 μM, $[Mep]_0$ = 135 μM, pH = 5.5. (a) Control in air-equilibrated solutions; (b) air-equilibrated solutions, [SOD] = 50 U ml^{-1}; (c) air-equilibrated solutions, [KI] = 500 μM; (d) O_2-saturated solutions.

Mechanistic analysis of PteGlu photooxidation

Based on the results obtained for the photolysis of PteGlu in the presence and absence of Mep, we propose the mechanism described below for the photosensitized oxidation of PteGlu (reactions (2)–(9)).

$$\text{Sens} \xrightarrow{h\nu} {}^1\text{Sens}^* \xrightarrow{\text{ISC}} {}^3\text{Sens}^* \quad (2)$$

$${}^3\text{Sens}^* + {}^3\text{O}_2 \rightarrow \text{Sens} + {}^1\text{O}_2/{}^3\text{O}_2 \quad (3)$$

$${}^3\text{Sens}^* + \text{PteGlu} \rightarrow \text{Sens}^{\bullet-} + \text{PteGlu}^{\bullet+} \quad (4)$$

$$\text{Sens}^{\bullet-} + \text{PteGlu}^{\bullet+} \rightarrow \text{Sens} + \text{PteGlu} \quad (5)$$

$$\text{Sens}^{\bullet-} + \text{O}_2 \rightarrow \text{Sens} + \text{O}_2^{\bullet-} \quad (6)$$

$$2\text{H}^+ + 2\text{O}_2^{\bullet-} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (7)$$

$$\text{PteGlu}^{\bullet+} + \text{O}_2^{\bullet-} \rightarrow \text{PteGlu} + \text{O}_2 \quad (8)$$

$$\text{PteGlu}^{\bullet+} + \text{O}_2 \rightarrow \text{PteGlu(ox)} \quad (9)$$

In this mechanism the photosensitizer (Sens) is Fop formed by the photooxidation of PteGlu itself or Mep initially present in the sample. After excitation of the photosensitizer, three reaction pathways compete for the deactivation of the triplet excited state thus formed: intersystem crossing to singlet ground state, quenching by dissolved molecular oxygen (reaction (3)), and electron transfer between PteGlu and ^{3}Sens* to form corresponding radical ions, $\text{Sens}^{\bullet-}$ and $\text{PteGlu}^{\bullet+}$ (reaction (4)). Using available rate constants for these respective processes, along with the pertinent concentrations of PteGlu and O_2, it is readily demonstrated that (1) in an air-saturated system, ~50% of the ^{3}Sens* produced is quenched by PteGlu, whereas (2) in an O_2-saturated system, ~20% of the ^{3}Sens* is quenched by PteGlu. In light of the data presented in Fig. 2–6, these O_2 concentration dependent changes in the fraction of ^{3}Sens* quenched are consistent with a process wherein PteGlu degradation derives from reaction (4); *i.e.*, electron transfer between PteGlu and ^{3}Sens*. Specifically, as outlined below, a consistent picture develops with the assumption that PteGlu degradation occurs as a result of the trapping of $\text{PteGlu}^{\bullet+}$ by O_2 (reaction (9)).

The PteGlu and Sens radical ions formed in reaction (4) may recombine (reaction (5)), which explains the absence of PteGlu consumption under anaerobic conditions. Alternatively, the electron transfer from $\text{Sens}^{\bullet-}$ to O_2 regenerates Sens and forms $\text{O}_2^{\bullet-}$ (reaction (6)). The superoxide radical may disproportionate with its conjugate acid $\text{HO}_2^{\bullet}$ to form H_2O_2 (summarized by reaction (7)) or react with the $\text{PteGlu}^{\bullet+}$ to regenerate PteGlu (reaction (8)). SOD accelerates the former reaction and, therefore, fast elimination of $\text{O}_2^{\bullet-}$ through this pathway prevents reaction (8). In consequence, in the presence of SOD enhancement of the photosensitized oxidation of PteGlu is observed experimentally (Fig. 3 and 6).

It is worth mentioning that, like many organic radical cations, $\text{PteGlu}^{\bullet+}$ might undergo deprotonation to yield a neutral radical ($\text{PteGlu}(-\text{H})^{\bullet}$), which, in turn, might react with $\text{O}_2^{\bullet-}$. Taking into account our experimental data, if reaction between $\text{PteGlu}(-\text{H})^{\bullet}$ and $\text{O}_2^{\bullet-}$ led to consumption of PteGlu, this process should be a very minor pathway. In addition, $\text{PteGlu}(-\text{H})^{\bullet}$ could undergo oxidation in a process similar to that proposed for $\text{PteGlu}^{\bullet+}$ (reaction (9)), contributing to the overall consumption of PteGlu.

The oxidation of biomolecules photoinduced by pterins *via* an electron transfer-mediated process has been reported.[54] Moreover, mechanisms similar to that described by reactions (2) to (9) have been proposed for other photosensitized processes, in which a given oxidizable substrate, such as purine nucleotides,[55,56] dihidropterins[48] and ethylenediaminetetraacetic acid (EDTA),[49] transfers an electron to a pterin molecule in its triplet excited state. On the other hand, to the best of our knowledge, this is the first time that the photosensitized oxidation of PteGlu is described as proceeding through an electron transfer process. However, the acceleration of photoinitiated PteGlu degradation by riboflavin (Rf) has been reported.[57] Although no mechanistic analysis was made in that work, Rf is a well-known photosensitizer, able to act *via* electron transfer. Therefore, the mechanism proposed in this section could explain the results found in the PteGlu-Rf system.

Considering the mechanism proposed (reactions (2) to (9)), it is interesting to analyze which portion of the PteGlu molecule is the reactive one. For nucleotides, the nucleobase is the fragment that acts as an electron donor.[58,59] In the case of PteGlu, it is unlikely that the amino acid will be the electron donor because it is not easily oxidized,[60] so that the oxidation should take place in either the pterin or PABA moieties (Fig. 1). In principle, the pterin moiety should be stable, since photooxidation quantum yields of pterin derivatives such as Mep or pterin itself have been reported to be very low ($<10^{-3}$).[41,52] Therefore, it is most likely that the key electron transfer step should occur from the PABA ring. This expectation is in agreement with the explanation to account for the low quantum yields of PteGlu fluorescence and sensitized singlet oxygen generation: *i.e.*, internal electron transfer from the PABA to the pterin moiety in the PteGlu molecule (see Introduction).[32–34]

Photolysis of PABA and PABA-Glu in the presence of Mep

To ascertain if unconjugated pterins are indeed able to react with the PABA moiety upon UV-A exposure, solutions containing PABA or PABA-Glu as a substrate and Mep as a photosensitizer were irradiated and analyzed as a function of time. In these experiments, only Mep was excited; PABA and PABA-Glu do not absorb in the UV-A region. Assuming Mep is photostable under the conditions used (*vide supra*), any change in the composition of the solutions irradiated would correspond to a photosensitized process. As controls, solutions containing substrates and Mep were kept in the dark for several hours, and PABA and PABA-Glu solutions were irradiated in the absence of Mep. As expected, spectral and HPLC analysis of these control solutions showed no evidence of chemical reaction.

Upon UV-A irradiation, solutions containing PABA-Glu and Mep became colored and the spectral changes recorded revealed a broad absorption band in the range 400–600 nm whose intensity

increased as a function of irradiation time (Fig. 7). This behavior was particularly evident in experiments carried out at relatively high PABA-Glu concentrations. In the time window analyzed, HPLC analysis showed consumption of PABA-Glu, no variation in Mep concentration and the formation of two products with absorption bands above 400 nm (Fig. 7). In addition, H_2O_2 was also detected and its concentration also increased during the experiment (Fig. 7). As with PteGlu, addition of SOD likewise caused an increase in the rate of PABA-Glu disappearance (Fig. 7). Similar results were observed in experiments performed using PABA as substrate.

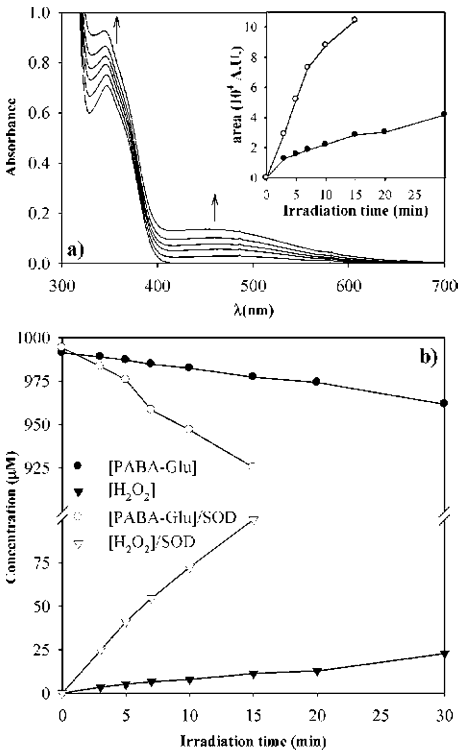


Fig. 7 Irradiation of air-equilibrated solutions (pH = 5.5) containing PABA-Glu (1 mM) and Mep (120 μM). Experiments performed in the absence (black symbols) and in the presence of SOD (200 U ml^{-1}) (white symbols). (a) Time evolution of the absorption spectra. Experiment in the presence of SOD. Spectra were recorded at 0, 3, 5, 7, 10 and 15 min. Optical path length = 10 mm. Arrows indicate the changes observed at different wavelengths. Inset: Time evolution of the area of the chromatographic peaks registered at 450 nm. (b) Time evolution of PABA-Glu and H_2O_2 concentrations.

The photosensitized oxidations of PABA and PABA-Glu, although slower than those observed for PteGlu, are nevertheless compatible with the mechanism proposed in reactions (2)–(9). Therefore these results are consistent with the expectation that the PABA unit is the oxidizable portion of PteGlu.

Quenching of Mep triplet state by PABA and PABA-Glu

Results shown so far suggest the triplet states of Mep and Fop play a key role in the oxidation of PteGlu, PABA, and PABA-Glu (*vide supra*). However, direct evidence for the interaction between the excited states of Mep, for example, and the ground state of a given substrate has yet to be presented. With this in mind, quenching studies by means of laser flash photolysis were performed. To avoid excitation of both Mep and the substrate, the experiments were carried out using PABA and PABA-Glu; the latter do not absorb in the UV-A region.

Laser flash excitation at 355 nm of deaerated solutions of Mep showed strong transient absorption in the 400–600 nm spectral region. The transient decay followed first-order kinetics, yielding a lifetime of 6.6 ± 0.6 μs (Fig. 8). This transient signal could be assigned to the triplet state of Mep based on the following results: (i) increase in its decay rate in the presence of O_2, (ii) spectrum and lifetime (τ) comparable to those reported for the triplet states of the similar compounds pterin,[61] biopterin (Bip)[62] and Cap.[33]

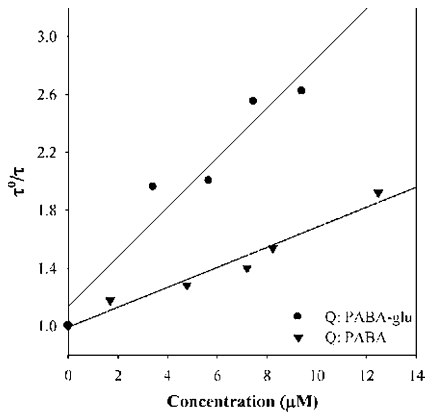


Fig. 8 Laser flash photolysis experiments in O_2-free solutions. Stern–Volmer plots of the quenching of the Mep triplet state by PABA and PABA-Glu. [Mep] = 145 μM, pH = 5.0, excitation wavelength 355 nm, analysis wavelength 420 nm.

Experiments performed in the presence of PABA and, independently, PABA-Glu showed that both compounds quench the triplet state of Mep, *i.e.*, the rate of transient signal decay increased with an increase in the quencher concentration. The rate equation for the decrease in ^{3}Mep* concentration is given by eqn (10):

$$-\mathrm{d}[^3\mathrm{Mep}^*]/\mathrm{d}t = k_q[^3\mathrm{Mep}^*][\mathrm{Q}] + \sum k[^3\mathrm{Mep}^*] \quad (10)$$

where k_q is the bimolecular quenching rate constant, [Q] is the quencher (PABA or PABA-Glu) concentration and $\sum k[^3\mathrm{Mep}^*]$ represents the sum of other deactivation pathways existing (*e.g.*, radiative and nonradiative energy losses). Therefore quenching of the Mep triplet state may be evaluated by a Stern–Volmer analysis (eqn (11)):

$$\tau^0/\tau = 1 + k_q\tau^0[\mathrm{Q}] \quad (11)$$

where τ^0 and τ are the Mep triplet lifetimes in the absence and in the presence of quencher, respectively. Values of k_q obtained from the slopes of the Stern–Volmer plots (Fig. 8) are $1.0\,(\pm0.2)\times10^{10}$ M^{-1} s^{-1} and $2.6\,(\pm0.6)\times10^{10}$ M^{-1} s^{-1} for PABA and PABA-Glu, respectively. The data indicate that PABA and PABA-Glu deactivate ^{3}Mep* with a rate constant characteristic of the diffusion-controlled limit.

The results obtained in these flash photolysis experiments provide direct evidence for the reaction between a subunit of PteGlu and the triplet state of Mep. Moreover, the values of the quenching rate constants obtained indicate a very efficient interaction. Most importantly, the data support the assumption

that triplet states of oxidized pterins participate in the mechanism of the photosensitized oxidation of PteGlu.

Conclusions

Folic acid, or pteroyl-L-glutamic acid (PteGlu), is photostable in the absence of oxygen. Upon UV-A excitation in air-equilibrated aqueous solutions, PteGlu undergoes photooxidation to yield 6-formylpterin (Fop) and *p*-aminobenzoyl-L-glutamic acid (PABA-Glu). The rate of this process increases with irradiation time. The data point to an "auto-photo-catalytic" effect. The latter involves a photosensitized process wherein Fop photoinduces the oxidation of PteGlu. This process, in which no excitation of PteGlu is needed, also takes place with other pterins as photosensitizers (Sens), thus revealing a general mechanism. After excitation of the Sens, three reaction pathways compete for the deactivation of the triplet excited state (^{3}Sens*) thus formed: intersystem crossing to singlet ground state, quenching by dissolved molecular oxygen, and electron transfer between PteGlu and ^{3}Sens*. The latter reaction involves an electron transfer from the PABA unit of PteGlu to ^{3}Sens* to form the corresponding radical ions, $Sens^{\bullet-}$ and $PteGlu^{\bullet+}$. These radical ions may recombine, which explains the absence of PteGlu consumption under anaerobic conditions. Alternatively, the electron transfer from $Sens^{\bullet-}$ to O_2 regenerates Sens and forms superoxide anion ($O_2^{\bullet-}$), which may disproportionate with its conjugate acid $HO_2^{\bullet}$ to form H_2O_2 or react with the $PteGlu^{\bullet+}$ to regenerate PteGlu. Finally, PteGlu degradation occurs as a result of the trapping of $PteGlu^{\bullet+}$ by O_2.

The results presented have important implications because the autocatalytic photochemical process described in this work could contribute significantly to the photodegradation of PteGlu in a plethora of relevant biological systems. Moreover, many endogenous or exogenous photosensitizers might cause the degradation of PteGlu, upon UV, or even visible, irradiation. Through an understanding of the mechanism of PteGlu photodegradation one can begin to consider methods by which folate disappearance can be controlled which, in turn, could be implemented in the development of more effective drugs for a range of pathologies.

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