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Oxidation of Tyrosine Photoinduced by Pterin in Aqueous Solution†

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ABSTRACT

Pterins, heterocyclic compounds widespread in biological systems, accumulate in the skin of patients suffering from vitiligo, a chronic depigmentation disorder. Pterins have been previously identified as good photosensitizers under UV-A irradiation. In this work, we have investigated the ability of pterin (Ptr), the parent compound of oxidized pterins, to photosensitize the oxidation of tyrosine (Tyr) in aqueous solutions. Tyr is an important target in the study of the photodynamic effects of UV-A radiation because it is oxidized by singlet oxygen ($^1O_2$) and plays a key role in polymerization and cross-linking of proteins. Steady UV-A irradiation of solutions containing Ptr and Tyr led to the consumption of Tyr and dissolved O_2, whereas the Ptr concentration remained unchanged. Concomitantly, hydrogen peroxide (H_2O_2) was produced. By combining different analytical techniques, we could establish that the mechanism of the photosensitized process involves an electron transfer from Tyr to the triplet excited state of Ptr. Mass spectrometry, chromatography and fluorescence were used to analyze the photoproducts. In particular, oxygenated and dimeric compounds were identified.

INTRODUCTION

Pterins are a family of heterocyclic compounds widespread in living systems that can exist in different redox states and may be classified, according to this property, into oxidized (or aromatic) and reduced pterins (one-electron transfer [ET] products, dihydro- and tetrahydropterins). Unconjugated oxidized pterins are a family of compounds that includes 2-aminopteridin-4(3H)-one or pterin (Ptr) and its derivatives bearing a substituent at position 6 with one carbon atom or a short hydrocarbon chain (Fig. 1). These compounds are photochemically reactive in aqueous solution and, upon UV-A excitation (320–400 nm), can fluoresce, undergo photooxidation to produce different photoproducts, generate reactive oxygen species, such as singlet molecular oxygen ($^1O_2$), and photosensitize the oxidation of biomolecules (1–5).

5,6,7,8-Tetrahydrobiopterin (H_4Bip) is an essential cofactor in the hydroxylation of the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan (6). The importance of this cofactor in the human epidermis and its participation in the regulation of melanin biosynthesis are well recognized (7). Several dihydro and tetrahydropterins are involved in the metabolism of H_4Bip and, hence, also present in human skin (8).

Under physiological conditions, unconjugated oxidized pterins are not present in mammalians, but they accumulate in the skin of human beings suffering from vitiligo, a depigmentation disorder (9). Moreover, these patients express a characteristic fluorescence in their white skin patches upon Wood's light examination that is due to the presence of oxidized pterins (10). Under pathological conditions, the oxidation of 7,8-dihydrobiopterin (H_2Bip), likely *via* a photochemical process (11), leads to the accumulation of biopterin (Bip) (Fig. 1) in the skin. In addition, 6-carboxypterin (Fig. 1), a product of Bip photolysis, has been isolated from the affected tissues (10), which indicates that excited states of pterins are photogenerated *in vivo*. In vitiligo, deactivation of enzymes of the melanin biosynthesis takes place and the protection of the skin against UV radiation fails because of the lack of melanin. Therefore, the photochemistry of pterins is of particular interest for the study of this disease.

Due to their relatively high abundance, their ability to bind chromophoric compounds and the reactivity of particular amino acid residues, proteins are one of the preferential targets of the photosensitized damaging effects of UV radiation on biological systems (12). However, to the best of our knowledge, the only studies on processes photosensitized by pterins that affect proteins have been reported very recently in two publications: one concerns the inactivation of tyrosinase, enzyme that catalyzes the first step in the biosynthesis of the melanin, and the other the damage to bovine serum albumin photoinduced by Ptr (13,14).

In another recent work, we have demonstrated that Ptr is able to photosensitize the oxidation of tryptophan (Trp) in aqueous solution (15). Currently, it is accepted that the photosensitization of proteins occurs mainly through the reactions of $^1O_2$ (type II mechanism) with Trp, tyrosine (Tyr), histidine, methionine and cysteine side-chains (16). However, our study on photooxidation of Trp suggested that the main mechanism involves an ET process (type I mechanism), instead of an oxidation *via* $^1O_2$. Previous studies, carried out using purine nucleotides as oxidizable targets, indicated that, although pterins are relatively good $^1O_2$ photosensitizers, they can also photoinduce the oxidation of biomolecules through ET-initiated processes (17).

In the context of our investigations on the photosensitizing properties of pterins, we herein report a study of the oxidation of Tyr photoinduced by Ptr, the parent Ptr moiety, under UV-A

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irradiation in aqueous solutions. Tyr is an important target in the study of the photodynamic effects of UV-A radiation in living systems, not only due to its reactivity towards $^1O_2$ (18,19), but also because this amino acid plays a key role in polymerization and cross-linking of proteins (20,21) *via* reactions initiated by Tyr radicals (22). We have evaluated the role of molecular oxygen (O_2), investigated the participation of the different excited states of Ptr, and analyzed the products. Mechanistic aspects and the biological implications of the results are discussed.

MATERIALS AND METHODS

Chemicals. Pterin (purity > 99%; Schircks Laboratories, Switzerland) and tyrosine (purity > 99%; Sigma Chemical Co.) were used without further purification after checking for impurities by HPLC. Methanol (MeOH) was purchased from J. T. Baker. Other chemicals were from Sigma Chemical Co. Solutions were prepared by dissolving Ptr and Tyr in water. The final pH of the solutions was adjusted by adding drops of HCl or NaOH solutions (0.1–0.2 M) with a micropipette. The ionic strength was *ca* 10^{-3} M in all experiments.

Steady-state irradiation. Irradiation set-up. The continuous irradiation of aqueous solutions containing Ptr and Tyr (pH = 5.5–6.0) was carried out in quartz cells (1 cm optical path length) at room temperature. A Rayonet RPR lamp emitting at 350 nm (bandwidth ≈ 20 nm) (Southern N.E. Ultraviolet Co.) was used as an irradiation source. The experiments were performed in the presence and in the absence of dissolved O_2. Experiments with air-equilibrated solutions were carried out in open quartz cells without bubbling, whereas argon and oxygen-saturated solutions were obtained by bubbling for 20 min with these gases, previously water saturated (Linde, purity > 99.998%).

Actinometry. Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurements of the incident photon flux ($q_{p,0}$) at the excitation wavelength. The method for the determination of $q_{p,0}$ has been described in detail elsewhere (23,24). Values of the photon flux absorbed ($q_{p,a}$) were calculated from $q_{p,0}$ according to the Lambert-Beer law ($q_{p,a} = q_{p,0}\ (1–10^{-A})$, where A is the absorbance of the reactant at the excitation wavelength).

Analysis of irradiated solutions. UV/Vis spectrophotometric 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 path length. The absorption spectra of the solutions were recorded at regular time intervals during irradiation.

High-performance liquid chromatography (HPLC). A Prominence equipment from Shimadzu (solvent delivery module LC-20AT, on-line degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, column oven CTO-10AS VP, photodiode array detector SPD-M20A and fluorescence detector RF-20A) was employed for monitoring the photochemical processes. A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping, 150 × 4.6 mm, 4 μm; Phenomenex) was used for separation of the photosensitizer, the substrate and the products. Solution of 10 mM $ACNH_4$ (pH:6.5) was used as mobile phase.

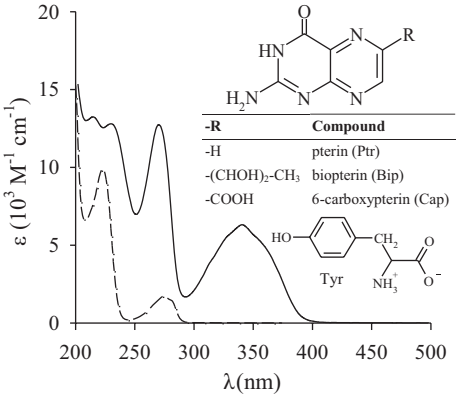

Figure 1. Molecular structure of aromatic pterins and tyrosine (Tyr) and the absorption spectra of Ptr (solid line) and Tyr (dashed line) in air-equilibrated aqueous solutions at pH = 5.5.

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 (25,26). 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 calibration.

Mass spectrometry analysis. The LC/MS system was equipped with an UPLC chromatograph (UHPLC Ultimate 3000 RS Dionex) and a triple quadrupole mass spectrometer (Q TRAP Applied Biosystems). UPLC analyses were performed using an Acquity UPLC®BEH C18 (1.7 μm; 2.1 × 50 mm) column (150 mm, Waters), and isocratic elution with an aqueous solution of formic acid (0.1%) at a flow rate of 0.6 mL min^{-1}. The mass spectrometer was equipped with an electrospray ion source (Turbo Ion Spray [TIS]) and was operated in both positive and negative ion modes. Nitrogen served as auxiliary, collision gas and nebulizer gas. The nitrogen temperature of the TIS source was 450°C and the declustering potential 30 V. The detection was scan mode with a step size of 0.1 atomic mass units (Da) and a scan range of 150–500 Da. Mass chromatograms, *i.e.* representations of mass spectrometry data as chromatograms (the *x*-axis representing time and the *y*-axis signal intensity), were registered using different scan ranges.

Determination of O_2 concentration. The O_2 consumption during irradiation was measured with an O_2-selective electrode (Consort c932). The solutions and the electrode were placed in a closed glass cell of 130 mL.

Fluorescence spectroscopy. Measurements were performed using a single-photon-counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon). The equipment has been previously described in detail (27). Briefly, 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 registered at 90° with respect to the incident beam using a room-temperature R928P detector. Corrected fluorescence spectra obtained by excitation at 300 nm were recorded between 320 and 550 nm. To avoid inner filter effects, the absorbance of the solutions at the excitation wavelength was kept below 0.10.

RESULTS AND DISCUSSION

Capability of Ptr to photoinduce the oxidation of Tyr

The first aim of this work was to find out if Ptr was able to photoinduce the oxidation of Tyr in aqueous solutions upon UV-A irradiation. Therefore air-equilibrated aqueous solutions containing Ptr and Tyr were exposed to UV-A (350 nm) irradiation for different periods of time. The experiments were performed in the pH range 5.5–6.0, where Ptr is present at more than 99% in its acid form (pK_a 7.9 [28]). Under these experimental conditions only Ptr was excited, as it can be inferred from the corresponding absorption spectra (Fig. 1). The samples, irradiated in quartz cells of 1.0 cm optical path length, were analyzed by UV–Vis spectrophotometry, HPLC and the concentration of H_2O_2 produced was measured (see Materials and Methods).

Significant changes in the absorption spectra of the solutions were registered after irradiation (Fig. 2), showing that products absorbing at wavelengths longer than 300 nm were formed. The concentration profiles of Ptr and Tyr, determined by HPLC, showed a decrease of the Tyr concentration as a function of irradiation time, whereas the Ptr concentration did not change in the analyzed time-window (Fig. 3). Accordingly, compounds other than Ptr and Tyr were detected by HPLC analysis of the irradiated solutions (*vide infra*). H_2O_2 was found to be generated and its concentration increased as a function of irradiation time. However, its initial rate of production was smaller than that corresponding to the initial rate of Tyr consumption (Fig. 3).

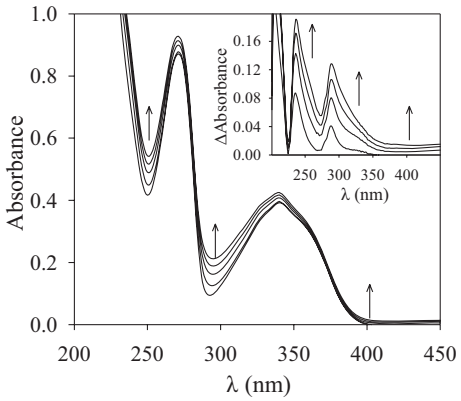

Figure 2. Time evolution of the absorption spectrum of an air-equilibrated solution of Tyr irradiated in the presence of Ptr. Spectra were recorded every 5 min, optical path length = 1.0 cm. Arrows indicate the changes observed at different wavelengths. Inset: Experimental difference spectra. $[Ptr]_0$ = 76 μM, $[Tyr]_0$ = 70 μM, pH = 5.5.

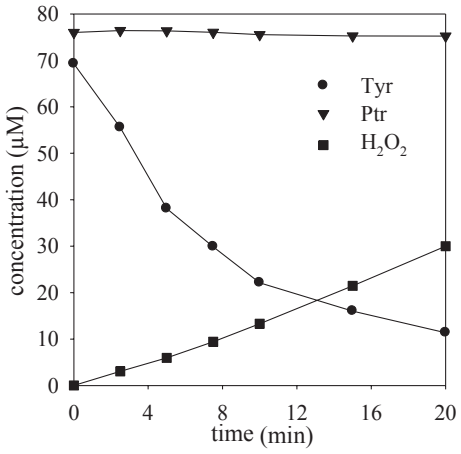

Figure 3. Evolution of the Tyr, Ptr and H_2O_2 concentrations in air-equilibrated aqueous solutions under UV-A irradiation as a function of time. $[Tyr]_0$ = 70 μM, $[Ptr]_0$ = 76 μM, pH = 5.5.

In experiments performed with a different optical geometry (Materials and Methods), an O_2-selective electrode was used to monitor the decrease of the O_2 concentration upon irradiation of air-equilibrated solutions containing Tyr (400 μM) and Ptr (100 μM) at pH = 5.5 (Fig. 4). Control experiments in the absence of Tyr, showed that the consumption of O_2 resulting from the photolysis of Ptr itself (29) was negligible in comparison with that observed in the presence of Tyr (Fig. 4). These results strongly suggest that the process observed in air-equilibrated solutions is a photoinduced oxidation. In several experiments, for determining the relationship between O_2 and Tyr consumptions ($\Delta[O_2]/\Delta[Tyr]$), the solutions were analyzed by HPLC, before and after irradiation, to obtain the initial and final Tyr concentrations, respectively. A value of *ca* 2.3 was obtained for $\Delta[O_2]/\Delta[Tyr]$ calculated for different irradiation times.

To confirm that the reaction observed was a photosensitized oxidation, additional controls were carried out. Tyr degradation was not observed in solutions containing Ptr and Tyr that were kept in the dark, excluding the possibility of thermal

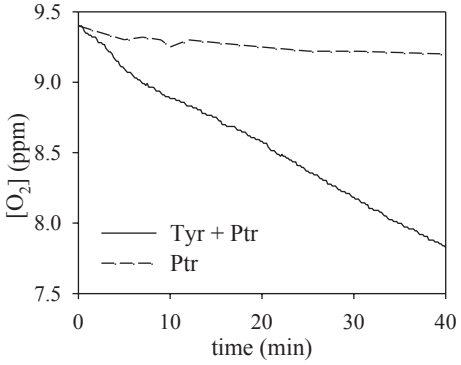

Figure 4. Evolution of the O_2 concentration in irradiated solutions containing Tyr and Ptr as a function of time (solid line). $[Tyr]_0$ = 400 μM, $[Ptr]_0$ = 100 μM, pH = 5.5. Control experiment: photolysis of Ptr (170 μM) at pH = 5.5 in the absence of Tyr (dashed line).

reactions. In another set of control experiments, Tyr (150 μM, pH = 5.5) solutions were irradiated with UV-A in the absence of Ptr and no consumption of the amino acid was detected, thus excluding the possibility of product formation by spurious direct excitation of Tyr. Therefore, the results presented in this section clearly demonstrate that Ptr photosensitizes the oxidation of Tyr under UV-A irradiation in air-equilibrated aqueous solutions.

Mechanistic analysis

To better elucidate the role of O_2 in the photosensitization of Tyr by Ptr, photolysis experiments were carried out in deoxygenated and O_2-saturated solutions and the results were compared with those performed in air-equilibrated solutions (Fig. 5a). HPLC measurements showed that, in the experiments lacking oxygen, the Tyr concentration did not decrease and, accordingly, no photoproducts were detected. On the other hand, the concentration profiles clearly showed that the rate of Tyr disappearance and the rate of H_2O_2 production were much greater in air-equilibrated than in O_2-saturated solutions (Fig. 5a).

It has been previously demonstrated that iodide (I^-) at micromolar concentrations is an efficient and selective quencher of triplet excited states of pterins (30,31). Therefore, photosensitization experiments were carried out in air-equilibrated aqueous solutions containing Tyr and Ptr at pH = 5.5 in the presence of KI (500 μM). The results revealed that, under these conditions, the rate of Tyr consumption was slower than in the absence of I^- (Fig. 5b). Accordingly, the rate of H_2O_2 production was also slower in the presence of I^- than in its absence. Thus, taking into account that no quenching of the singlet excited state of Ptr was previously observed at an I^- concentration of 500 μM (30), the inhibition of the photosensitized degradation of Tyr by I^- strongly suggests the participation of the triplet excited state of Ptr (^{3}Ptr*).

Taking into account that (1) the process needs O_2 to take place, (2) the triplet excited of the photosensitizer (^{3}Ptr*) is involved and (3) Ptr produces efficiently $^1O_2$ upon UV-A irradiation (32), it may be assumed that the photooxidation of Tyr is a $^1O_2$–mediated reaction (type II mechanism). However, the fact that the efficiency of the process was greater in air-equilibrated

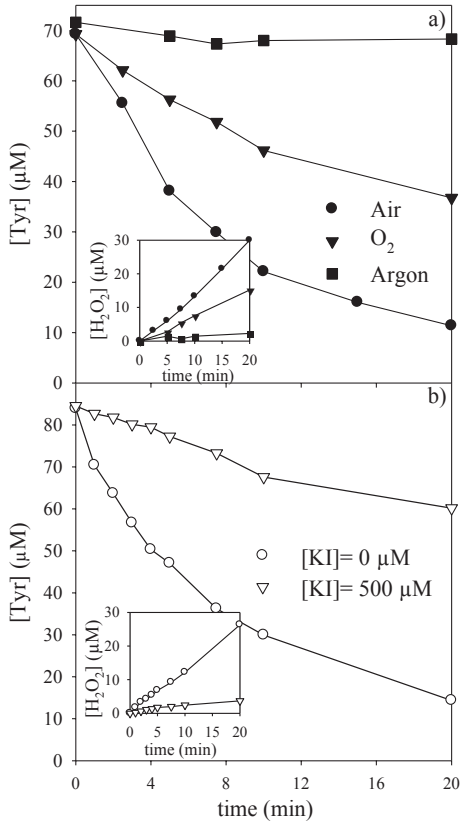

Figure 5. (a) Time-evolution of the Tyr and H_2O_2 (inset) concentrations in air-equilibrated, O_2-saturated and O_2-free aqueous solutions under UV-A irradiation; $[Tyr]_0 = 70$ μM, $[Ptr]_0 = 76$ μM, pH = 5.5. (b) Time-evolution of the Tyr and H_2O_2 (inset) concentrations in air-equilibrated aqueous solutions under UV-A irradiation in the absence and in the presence of KI; $[Tyr]_0 = 85$ μM, $[Ptr]_0 = 76$ μM, pH = 5.5.

solutions than in O_2-saturated solutions (Fig. 5a) is not consistent with a process wherein Tyr would be consumed by $^1O_2$. Furthermore, we have previously demonstrated that pterins act mainly through ET-initiated processes (type I mechanisms), even in cases where the substrate reacts rapidly with $^1O_2$ (15,33).

The oxidation of Tyr and its derivatives by $^1O_2$ has been extensively studied (34) and values for the rate constant of the chemical reaction between $^1O_2$ and Tyr (k_r^{Tyr}) have been reported in several studies ($k_r^{Tyr} < 10^6$ M^{-1} s^{-1}, pD = 7.0 [35]; $k_r^{Tyr} = 8 \times 10^6$ M^{-1} s^{-1}, pD = 8.4 [36]; $k_r^{Tyr} = 1.7 \times 10^8$ M^{-1} s^{-1}, pD = 12 [18]). It is evident from the reported values that k_r^{Tyr} depends on the pH/pD conditions, its value increasing at higher pH. Under our experimental conditions k_r^{Tyr} should be lower than 10^6 M^{-1} s^{-1}.

The contribution of $^1O_2$ in the photosensitized oxidation of Tyr was estimated considering a k_r^{Tyr} of 10^6 M^{-1} s^{-1} to calculate the initial rate of the chemical reaction between $^1O_2$ and Tyr $(d[Tyr]/dt)_0$, Eq. (1):

$$\left(\frac{d[Tyr]}{dt}\right)_0 = -k_r^{Tyr}[^1O_2]_{SS}[Tyr]_0 \quad (1)$$

where $[^1O_2]_{SS}$ is the steady-state concentration of $^1O_2$ during irradiation of a solution containing Ptr and Tyr and can be calculated using Eq. (2),

$$[^1O_2]_{SS} = \frac{q_{p,a}\Phi_\Delta}{(k_d + k_t^{Ptr}[Ptr] + k_t^{Tyr}[Tyr])} \quad (2)$$

where $q_{p,a}$ and Φ_Δ are the photon flux absorbed by Ptr (9.1×10^{-6} einstein L^{-1} s^{-1}) and the quantum yield of $^1O_2$ production ($\Phi_\Delta = 0.18$ at pH = 5.5 [32]), respectively; k_d is the rate constant for the unimolecular deactivation of $^1O_2$ which is solvent dependent (37) ($k_d = \tau_\Delta^{-1}$, τ_Δ: $^1O_2$ lifetime in the absence of quencher; k_d has a value of approx. 2.6×10^5 s^{-1} in H_2O); k_t^{Ptr} and k_t^{Tyr} are the overall rate constants of $^1O_2$ quenching by Ptr and Tyr, respectively ($k_t = k_r + k_q$, where k_q is the rate constant of $^1O_2$ physical quenching by the substrate). Considering $k_t^{Ptr} \approx 10^6$ M^{-1} s^{-1} as previously determined (32) the quenching of $^1O_2$ by Ptr is negligible under our experimental conditions (*i.e.* $k_t^{Ptr}[Ptr] \ll k_d$). On the other hand, taking into account values of k_t^{Tyr} reported in the literature ($k_t^{Tyr} = 2.7 \times 10^7$ M^{-1} s^{-1}, pH = 7.0 [38]), the quenching of $^1O_2$ by Tyr is also negligible (*i.e.* $k_t^{Tyr}[Tyr] \ll k_d$). Therefore, combining Eqs. (1) and (2), we can provide an estimate for $(d[Tyr]/dt)_0$ for a given initial steady-state experiment [Eq. (3)].

$$\left(\frac{d[Tyr]}{dt}\right)_0 = -k_r^{Tyr}q_{p,a}\Phi_\Delta\tau_\Delta[Tyr]_0 \quad (3)$$

Under our experimental conditions, the values of $(d[Tyr]/dt)_0$ calculated from Eq. (3) were much lower than the initial rate of Tyr consumption experimentally determined by HPLC analysis, $(d[Tyr]/dt)_{0,exp}$. For instance, for the experiment shown in Fig. 3, $(d[Tyr]/dt)_0$ calculated from Eq. (3) was 0.11 ($\pm$0.02) μM min^{-1}, whereas $(d[Tyr]/dt)_{0,exp}$ was 5.4 ($\pm$0.5) μM min^{-1}. By itself, this result suggests that, in agreement with the reduced rate of Tyr consumption observed in O_2-saturated solutions, the chemical reaction between Tyr and $^1O_2$ does not appear to contribute significantly to the photosensitized oxidation of Tyr.

In order to confirm the hypotheses proposed in the previous paragraphs, comparative photolysis experiments were performed in H_2O and D_2O. Given that the $^1O_2$ lifetime in D_2O is longer than that in H_2O (*i.e.* $k_d(H_2O) > k_d(D_2O)$) by a factor of *ca* 15 (37,39) the photosensitized oxidation of Tyr should be faster in the deuterated solvent if $^1O_2$ contributed significantly to the process. Air-equilibrated solutions containing Ptr (80 μM) and Tyr (71 μM) in H_2O and D_2O at pH/pD 5.5 were irradiated under otherwise identical conditions. The evolutions of the absorption spectra and of the concentrations of Ptr and Tyr as a function of the irradiation time (Fig. 6a) showed that, in agreement with the kinetic analysis presented in the previous paragraphs, the studied process was not significantly faster in D_2O than in H_2O, within experimental error.

Therefore, if the contribution of $^1O_2$ may be discarded or is negligible, a mechanism initiated by an ET from the amino acid to the triplet excited stated of Ptr should be considered. It is well established that, in a typical type I process, ground state O_2 will readily quench an organic radical anion to produce the superoxide anion ($O_2^{\bullet-}$) (40,41). The detected H_2O_2 (*vide supra*) can then be the product of the spontaneous disproportionation of $O_2^{\bullet-}$ in aqueous solution (42). Therefore, we have investigated the participation of $O_2^{\bullet-}$ in the mechanism, by performing experiments at pH 5.5 in the presence of superoxide dismutase (SOD), an enzyme that catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2 and O_2 (43). The data showed a significant increase in the rate of Tyr consumption when SOD was present in the solution

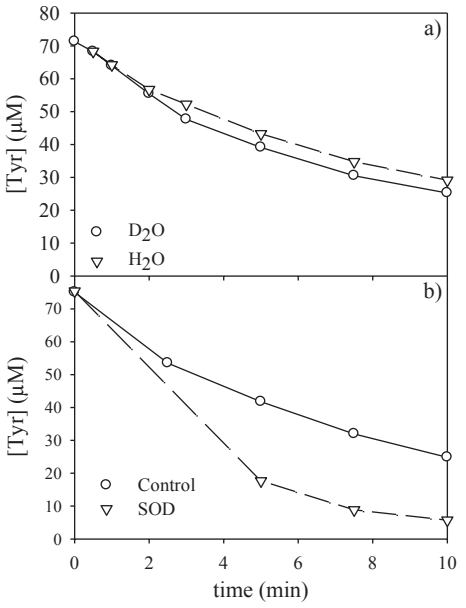

Figure 6. Evolution of the Tyr concentration in solutions containing Ptr and Tyr as a function of irradiation time. a) Experiments carried out in D_2O (○) and H_2O (▽); $[Ptr]_0$ = 80 μM, $[Tyr]_0$ = 71 μM, pH(pD) = 5.5. b) Experiments performed in H_2O in the absence (○) and in the presence (△) of SOD; $[Ptr]_0$ = 80 μM, $[Tyr]_0$ = 75 μM, [SOD] = 340 U/mL, pH (pD) = 5.5.

(Fig. 6b). These results indicate that $O_2^{\bullet-}$ is involved in the photosensitized process and provide further evidence for the existence of an ET reaction. In addition, this result suggests that trapping of $O_2^{\bullet-}$ by SOD represents a pathway competing with a step that prevents the photoinduced oxidation of Tyr.

Taking into account the results presented thus far, the following mechanism can be proposed (Reactions 4–13):

$$\mathrm{Ptr} \xrightarrow{h\nu} {}^1\mathrm{Ptr}^* \quad (4)$$

$${}^1\mathrm{Ptr}^* \xrightarrow{\mathrm{ISC}} {}^3\mathrm{Ptr}^* \quad (5)$$

$${}^3\mathrm{Ptr}^* \longrightarrow \mathrm{Ptr} \quad (6)$$

$${}^3\mathrm{Ptr}^* + {}^3\mathrm{O}_2 \longrightarrow \mathrm{Ptr} + {}^1\mathrm{O}_2 \quad (7)$$

$${}^3\mathrm{Ptr}^* + \mathrm{Tyr} \xrightarrow{\mathrm{ET}} \mathrm{Ptr}^{\bullet-} + \mathrm{Tyr}^{\bullet+} \quad (8)$$

$$\mathrm{Tyr}^{\bullet+} + \rightleftharpoons \mathrm{Tyr}\ (\text{-H})^{\bullet} + \mathrm{H}^+ \quad (9)$$

$$\mathrm{Ptr}^{\bullet-} + \mathrm{Tyr}^{\bullet+}/\mathrm{Tyr}(\text{-H})^{\bullet} \longrightarrow \mathrm{Ptr} + \mathrm{Tyr}\ (+\mathrm{H})^+ \quad (10)$$

$$\mathrm{Ptr}^{\bullet-} + \mathrm{O}_2 \longrightarrow \mathrm{Ptr} + \mathrm{O}_2^{\bullet-} \quad (11)$$

$$2\mathrm{H}^+ + 2\mathrm{O}_2^{\bullet-} \longrightarrow \mathrm{H}_2\mathrm{O}_2 + \mathrm{O}_2 \quad (12)$$

$$\mathrm{Tyr}^{\bullet+} + \mathrm{O}_2^{\bullet-} \longrightarrow \mathrm{Tyr} + \mathrm{O}_2 \quad (13)$$

$$\mathrm{Tyr}^{\bullet+}/\mathrm{Tyr}(\text{-H})^{\bullet} + \mathrm{O}_2 \xrightarrow{\mathrm{H_2O}} \mathrm{Tyr(ox)} \quad (14)$$

$$2\mathrm{Tyr}(\text{-H})^{\bullet} \longrightarrow \mathrm{Tyr}_2 \quad (15)$$

After excitation of Ptr and formation of its triplet excited state, ^{3}Ptr* (Reactions 4 and 5), three reaction pathways compete for the deactivation of the latter: intersystem crossing to singlet ground state (Reaction 6), energy transfer to molecular oxygen leading to the regeneration of Ptr and the formation of $^1O_2$ (Reaction 7), and ET from Tyr to ^{3}Ptr* to form the Tyr radical cation ($Tyr^{\bullet+}$) (Reaction 8), that may deprotonate to the tyrosyl radical ($Tyr(\text{-H})^{\bullet}$) (Reaction 9). In experiments performed in air-equilibrated solutions, the rate of Reaction 8 is significant and an important consumption of Tyr is observed. In contrast, in oxygen-saturated solutions, Reaction 7 competes more efficiently with Reaction 8, the proportion of ^{3}Ptr* reacting with Tyr is much lower and, consequently, the rate of Tyr consumption is also much slower (Fig. 5a). In the following step, the radical ions may recombine (Reaction 10), which explains the absence of Tyr consumption under anaerobic conditions. Alternatively, the ET from $Ptr^{\bullet-}$ to O_2 regenerates Ptr and forms $O_2^{\bullet-}$ (Reaction 11). This radical may disproportionate with its conjugated acid $HO_2^{\bullet}$ to form H_2O_2 (summarized by Reaction 12) or react with the $Tyr^{\bullet+}$ to regenerate Tyr (Reaction 13). SOD accelerates the former reaction and, therefore faster elimination of $O_2^{\bullet-}$ through this pathway hinders regeneration of Tyr by Reaction 13. As a consequence, in the presence of SOD, enhancement of the photosensitized oxidation of the amino acid is observed experimentally (Fig. 6b). Finally a group of processes, represented schematically by Reaction 14 and that might include the reactions of $Tyr^{\bullet+}/Tyr(\text{-H})^{\bullet}$ with O_2 (and eventually H_2O), leads to the oxidation of Tyr and consumption of O_2. Alternatively, radical dimerization might contribute to Tyr consumption (Reaction 15) (*vide infra*).

Analysis of photoproducts

The study of photoproducts was divided into two parts: (1) investigation of oxygenated compounds and, (2) investigation of dimers and oligomers. The analysis was performed using UPLC coupled to ESI mass spectrometry (UPLC-MS), HPLC coupled to UV–Vis and fluorescence detection, and steady-state fluorescence measurements. Solutions containing Ptr and Tyr were analyzed with the above mentioned techniques before and after irradiation.

As expected, in solutions analyzed by UPLC-MS before irradiation, the signals corresponding to the intact molecular ions of Tyr and Ptr as $[M–H]^-$ species at *m/z* 180 and 162 Da, respectively, were observed in ESI^- mode. In addition, in ESI^+ mode the intact molecular ion as $[M + H]^+$ and the adduct $[M + Na]^+$ were detected respectively at *m/z* 182 and 204 Da for Tyr, and 164 and 186 Da for Ptr.

In irradiated solutions, a species at *m/z* 196 Da was detected in ESI^- mode, which may be attributed to a compound resulting from the incorporation of an oxygen atom to the Tyr moiety ($[Tyr + O–H]^-$, *e.g.* hydroxylation of Tyr on the aromatic ring). Moreover, the molecular weight 197 Da corresponds to 3,4-dihydroxy-L-phenylalanine (DOPA), product of the enzymatic oxidation of Tyr *in vivo*. Using the corresponding standard, this compound was investigated by HPLC-UV in irradiated solutions. However, chromatograms registered at wavelength shorter than 280 nm revealed the presence of many photoproducts in the irradiated solutions, most of them with similar retention times (t_r) (Fig. 7a). Therefore, although chromatograms showed photoproducts with t_r and spectra compatible with L-DOPA, its presence in the irradiated solutions could not be confirmed by HPLC-UV.

L-dopachrome is another product of Tyr oxidation *in vivo* with particular spectral features, *i.e.* its spectrum in water has a broad absorption band of low intensity centered at 475 nm. In contrast

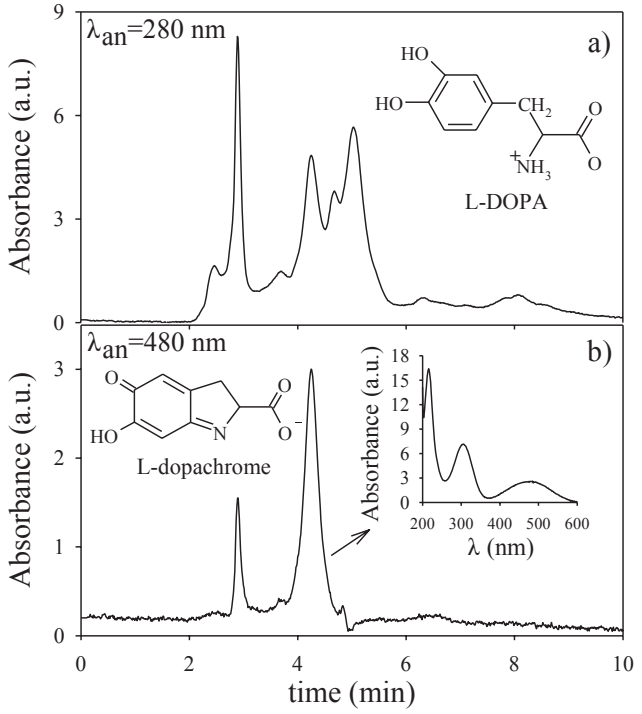

Figure 7. Chromatograms obtained in HPLC-UV analysis of irradiated solutions. (a) Analysis at 280 nm; (b) analysis at 480 nm, irradiation time: 10 min, inset: spectrum of the peak corresponding to L-dopachrome. $[Ptr]_0$ = 80 μM, $[Tyr]_0$ = 70 μM, pH = 5.5.

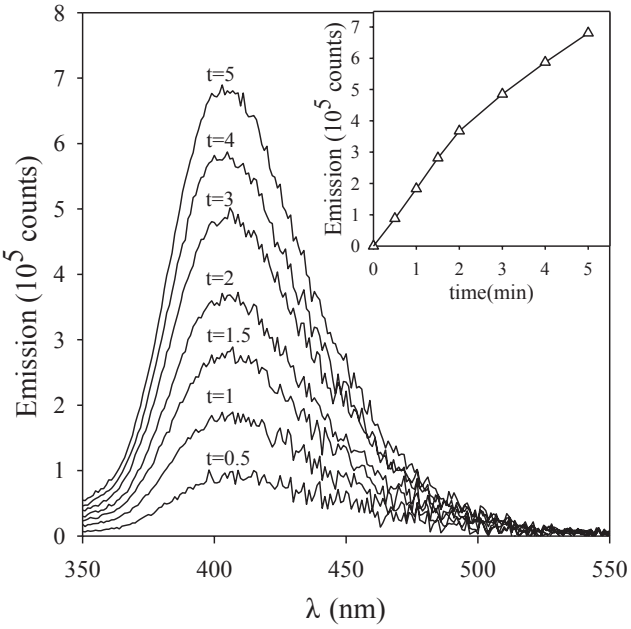

Figure 8. Corrected fluorescence spectra (λ_{exc} = 300 nm) of an aqueous solution (pH = 5.5) of Tyr (70 μM) and Ptr (37 μM) irradiated at 350 nm. The irradiation time (min) appears above each spectrum. For each time, the spectrum of the solution before irradiation was subtracted. Inset: increase of the fluorescence intensity at 405 nm as a function of irradiation time.

to chromatograms registered below 280 nm, chromatograms at wavelength higher than 400 nm showed very few peaks (Fig. 7b). L-dopachrome was generated, for comparative purposes, from the oxidation of Tyr catalyzed by the enzyme tyrosinase as described elsewhere (13). The standard enzymatically prepared was analyzed by HPLC and showed the same t_r value and the same spectrum as the product corresponding to the main peak observed in the chromatograms registered at 480 nm (Figure 7b). This result strongly suggests that L-dopachrome is formed in the studied photosensitized process.

The detection of so-called dimers of Tyr is important from a biological point of view because it implies that pterins might photoinduce the oligomerization and cross-linking of proteins *in vivo* (see Introduction). Therefore, species at *m/z* higher than 350 Da were searched. Whereas in the solutions before irradiation no dimeric products could be detected, in irradiated solutions a compound with a molecular weight of 360 Da [2Tyr-2H] was detected in, at least, two chromatographic peaks in both ESI^+ and ESI^- modes (ions at *m/z* 361 and 359 Da, respectively). This molecular weight corresponds to the structure of the well-characterized compound called dityrosine (denoted Tyr_2), which forms in highly oxidative environments (44,45).

To obtain more evidence about the formation of dimers of Tyr, fluorescence experiments were carried out, taking advantage of their particular emission features. The absorption and emission spectra of dimers of Tyr are red shifted with respect to those of Tyr (46). Therefore solutions containing Ptr and Tyr were exposed to UV-A radiation (350 nm) and the emission spectra under excitation at 300 nm were registered at various irradiation times. At that excitation wavelength, Ptr absorbs and fluoresces with a maximum at 439 nm, so that the emission of the photosensitizer is partially superimposed to that of the dimers of Tyr. Consequently, the spectrum of the solution before irradiation, that means the fluorescence of Ptr, was subtracted from the spectrum registered for a given irradiation time. Results show that an emission band with a maximum coinciding with that expected for the dimers of Tyr (46) was present in the irradiated solutions and that the intensity increased as a function of irradiation time (Fig. 8). Therefore, these results provide more evidence in favor of the photodimerization of Tyr induced by Ptr.

Dimers of Tyr were also investigated by chromatography coupled to a fluorescence detector (HPLC-FL, see Materials and Methods). Fluorescence chromatograms of irradiated solutions (excitation at 300 nm and emission at 400 nm) showed a main fluorescent product and smaller peaks (Fig. 9). Under our experimental conditions, the area of the major peak increased with irradiation time reaching a maximum at about 10 min and then decreasing slowly.

It is known since the 1960s that the one-electron oxidation of Tyr generates the long-lived tyrosyl radical ($Tyr(-H)^\bullet$) and that when two $Tyr(-H)^\bullet$ react the dimer o,o′-dityrosine is formed as the main product (47,48). However, it is obvious from the structure of $Tyr(-H)^\bullet$ that the dimerization can lead to other products, as reported (49). This fact explains why several dimeric products were found by UPLC-MS and HPLC-FL analyses. Finally, the finding of dimers is in agreement with the mechanistic hypotheses proposed above, since it has been reported that dimers are not formed by oxidation with reactive oxygen species such as $^1O_2$, $O_2^{\cdot-}$ and H_2O_2 (50,51).

CONCLUSIONS

The oxidation of Tyr photosensitized by Ptr, the parent compound of oxidized pterins, in aqueous solution (pH = 5.5) under

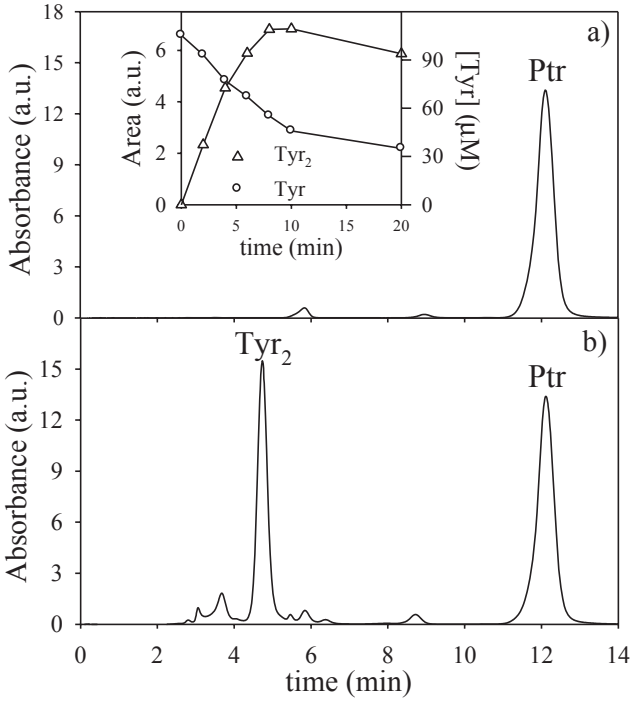

Figure 9. Chromatograms obtained in HPLC-FL analysis of irradiated solutions; excitation at 300 nm, emission at 400 nm, $[Ptr]_0$ = 100 μM, $[Tyr]_0$ = 105 μM, pH = 5.5; (a) before irradiation, (b) irradiation time: 10 min. Inset: time evolution of the Tyr concentration and of the area of the main peak corresponding to the dimer of Tyr (Tyr_2).

UV-A irradiation was investigated. When aerated solutions containing Tyr and Ptr (pH = 5.5) were exposed to UV-A radiation the amino acid was consumed, whereas the photosensitizer (Ptr) concentration did not change significantly. During this process, dissolved O_2 was consumed and H_2O_2 was generated. In contrast, the consumption of Tyr was negligible in the absence of O_2.

Mechanistic analysis indicates that the Ptr-sensitized oxygenation/oxidation of Tyr does not involve $^1O_2$ as an intermediate, but proceeds through an ET-initiated process. In this mechanism, the excitation of Ptr is followed by an ET from the amino acid to the Ptr triplet excited state, leading to the formation of the corresponding ion radicals ($Ptr^{\bullet -}$ and $Tyr^{\bullet +}$). In the following step, the ET from $Ptr^{\bullet -}$ to O_2 regenerates Ptr and forms the superoxide anion ($O_2^{\bullet -}$), which in turn may disproportionate with its conjugated acid ($HO_2^{\bullet}$) to form H_2O_2 or react with $Tyr^{\bullet +}$ to regenerate Tyr. Analysis of photo-products confirmed the presence of oxidized compounds (DOPA and dopachrome), and dimeric products such as dityrosine which is involved in cross-linking of proteins.

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