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Original Contribution

Tryptophan oxidation photosensitized by pterin

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ABSTRACT

Pterins are normal components of cells and they have been previously identified as good photosensitizers under UV-A irradiation, inducing DNA damage and oxidation of nucleotides. In this work, we have investigated the ability of pterin (Ptr), the parent compound of oxidized pterins, to photosensitize the oxidation of another class of biomolecules, amino acids, using tryptophan (Trp) as a model compound. Irradiation of Ptr in the UV-A spectral range (350 nm) in aerated aqueous solutions containing Trp led to the consumption of the latter, whereas the Ptr concentration remained unchanged. Concomitantly, hydrogen peroxide (H_2O_2) was produced. Although Ptr is a singlet oxygen ($^1O_2$) sensitizer, the degradation of Trp was inhibited in O_2-saturated solutions, indicating that a $^1O_2$-mediated process (type II oxidation) was not an important pathway leading to Trp oxidation. By combining different analytical techniques, we could establish that a type I photooxidation was the prevailing mechanism, initiated by an electron transfer from the Trp molecule to the Ptr triplet excited state, yielding the corresponding radical ions ($Trp^{\bullet+}$/$Trp(-H)^{\bullet}$ and $Ptr^{\bullet-}$). The Trp reaction products that could be identified by UPLC-mass spectrometry are in agreement with this conclusion.

Introduction

Proteins have been shown to represent important targets for photodamage under UV and visible irradiation [1]. Among the amino acids, tryptophan (Trp) is particularly susceptible to a variety of oxidizing agents. UV-A (320–400 nm) and visible radiation induce oxidation reactions through photosensitized processes. These processes involve excitation of the photosensitizers to yield singlet and triplet excited states. The latter having longer lifetimes may undergo bimolecular reactions more efficiently and, in particular, may transfer energy to molecular O_2, forming the reactive singlet oxygen ($^1O_2(^1\Delta_g)$ denoted as $^1O_2$) responsible for type II photooxidation reactions [2]. Electron transfer involving excited triplet states and different substrates may also initiate oxidation reactions (type I photooxidation).

Pterins, heterocyclic compounds widespread in biological systems, have been identified as photosensitizers. Under UV-A excitation (320–400 nm), pterins can fluoresce, undergo photooxidation, and generate reactive oxygen species (ROS) [3]. In the presence of oxygen, pterin (Ptr), the parent and unsubstituted compound of oxidized pterins, acts as a photosensitizer through both type I [4] and type II mechanisms [5]. Moreover, pterin photoinduces DNA damage [6,7] and the oxidation of nucleotides, such as 2′-deoxyguanosine 5′-monophosphate [8] and 2′-deoxyadenine 5′-monophosphate [9].

Pterins are present in human epidermis. In particular, 5,6,7,8-tetrahydrobiopterin (H_4Bip) is an essential cofactor in the hydroxylation of the aromatic amino acids [10] and participates in the regulation of melanin biosynthesis [11]. Several dihydro and tetrahydropterins are involved in the metabolism of H_4Bip and, hence, also present in human skin [12]. Vitiligo is a skin disorder that affects an estimated 1% of the world population and is characterized by the acquired loss of constitutional pigmentation manifesting as white macules and patches [13]. In this disease, the H_4Bip metabolism is altered [14] and the protection against UV radiation fails due to the lack of melanin, the main pigment of skin. Patients suffering from vitiligo express a characteristic fluorescence in their white skin patches upon Wood's light examination due to the presence of oxidized pterins [11]. Several studies have reported that the concentration of pterins in diseased skin cells is more than one order of magnitude higher than in healthy cells. In

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; PBN, α-(4-pyridyl-1-oxide) *N*-*t*-butylnitrone; NH_4OAc, ammonium acetate Ptr, pterin; ROS, reactive oxygen species; SOD, superoxide dismutase.

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the affected tissues micromolar concentrations of pterins have been determined; e.g., in human keratinocytes and cell cultures from suction blister roofs the concentration of total biopterin was determined to be in the range 4–93 μM (41–950 pmol/mg of protein), depending on the cell type [11]; concentrations higher than 35 μM (360 pmol/mg of protein) were determined for 6-carboxypterin [15]. In addition, fluorescence studies have determined that the concentration of pterins is not homogeneous inside the cells. Therefore the local concentrations could be even much higher than those noted above.

In a recent publication, it has been suggested that in the skin of patients affected by vitiligo, Trp is oxidized, since its concentration is depleted and known oxidation products are present in high concentrations [16]. However, tryptophan hydroxylase activity is undetectable, indicating that, in this case, the oxidation of Trp is a nonenzymatic process. The Trp oxidation may imply different mechanistic pathways such as direct UV-B excitation or photosensitization processes.

To evaluate the capability of Ptr to photosensitize the degradation of Trp, aqueous solutions containing both compounds were exposed to UV-A irradiation (320–400 nm) under different experimental conditions. In this spectral region, Ptr absorbs radiation, whereas Trp does not (Fig. 1). Most of the experiments were performed in the pH range 5.5–6.0, so that more than 99% of the pterin ($pK_a = 7.9$) [17] was in the acid form, the predominant form at physiological pH. The photochemical reactions were followed by UV/visible spectrophotometry, HPLC, and an enzymatic method for H_2O_2 determination. Radical intermediates were investigated by electronic paramagnetic resonance (EPR) and reaction products were characterized by UPLC-mass spectrometry.

Experimental

General

Pterin (purity > 99%, Schircks Laboratories, Switzerland and Sigma-Aldrich) was used without further purification after checking for impurities by HPLC. Tryptophan and ammonium acetate (NH_4OAc) (Sigma Chemical Co.) were of the highest purity available (> 98%) and were used without further purification. Methanol (MeOH) and KI were purchased from J. T. Baker and Sigma, respectively. Other chemicals were from Sigma Chemical Co. Solutions were prepared dissolving Ptr and Trp 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. Concentration ranges used for the experiments were 50–100 and 50–300 μM for Ptr and Trp, respectively.

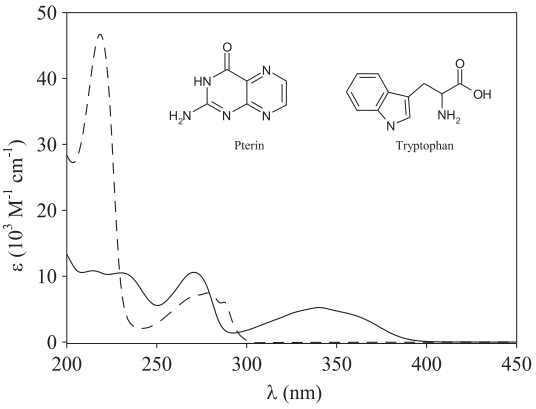

Fig. 1. Molecular structure of Ptr and Trp, and the corresponding absorption spectra in air-equilibrated aqueous solutions; solid line, acid form of Ptr (pH 5.5); dashed–dotted lines, Trp.

Steady-state irradiation

Irradiation setup

Aqueous solutions containing Ptr and Trp (pH 5.5) were irradiated in 1 cm path length quartz cells at room temperature with Rayonet RPR3500 lamps with emission centered at 350 nm (Southern N.E. Ultraviolet Co.). The spectral discrimination was achieved using filters with bandwidths (fwhm) of ~20 nm. The experiments were performed in the presence and in the absence of O_2. Oxygen-free solutions were obtained by bubbling with Ar for 20 min. The measurements were carried out under conditions of reduced environmental light.

Actinometry

Aberchrome 540 (Aberchromics Ltd.), the anhydride form of the (*E*)-*R*-(2,5-dimethyl-3-furylethylidene)(isopropylidene)-succinic acid, was used as an actinometer for the measurements of the incident photon flux (P_0) at the excitation wavelength. The method for the determination of P_0 has been described in detail elsewhere [18,19]. Values of the photon flux absorbed (P_a) were calculated from P_0 ($P_0^{350} = 5.12 \times 10^{-4}$ einstein L^{-1} min^{-1}) according to the Lambert–Beer law ($P_a = P_0\ (1-10^{-A})$, where A is the absorbance of the sensitizer at the excitation wavelength).

UV/visible analysis

UV-visible absorption spectra were registered on a Shimadzu UV-1800 spectrophotometer. Measurements were made in quartz cells of 0.4 and 1 cm optical path length.

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, oven CTO-10AS VP, and photodiode array detector SPD-M20A) was used to monitor and quantify the reactants and the photoproducts. Separation was performed on a Sinergy Polar-RP column (150 × 4.6 mm, 5 μm; Phenomenex) using as mobile phase solutions containing 10 mM NH_4OAc aqueous solution (pH 6.8). HPLC runs were monitored by UV/vis spectroscopy at different wavelengths.

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 [20,21]. 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 was added to 600 μl of reagent. The absorbance of the resulting mixture at 505 nm was measured after 30 min at room temperature, under conditions of reduced environmental light, 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 specifically the decomposition of H_2O_2 to H_2O and O_2, were also 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.