ORIGINAL PAPERS

Pterin-lysine photoadduct: a potential candidate for photoallergy
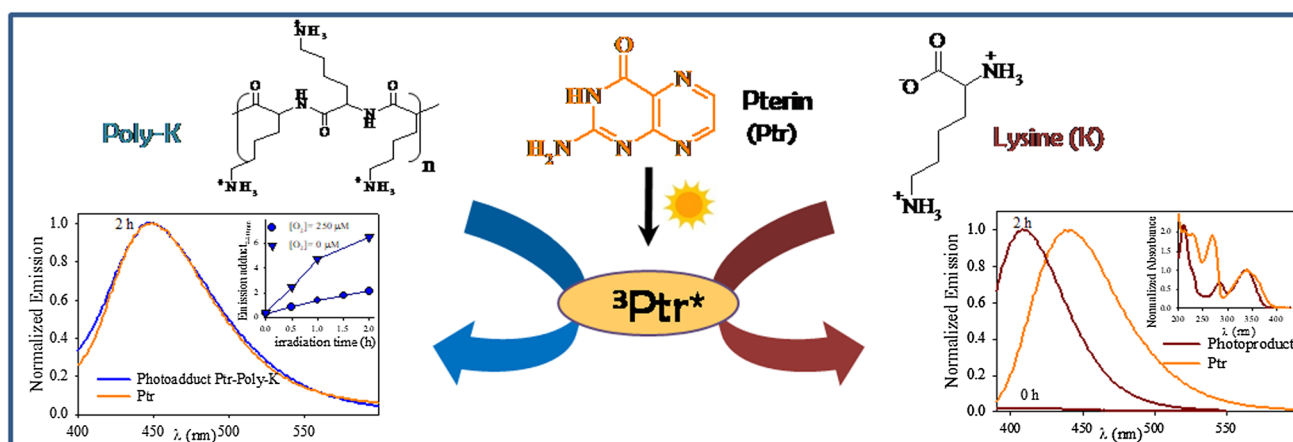
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Abstract

Photoallergy is a photosensitivity disorder associated with a modified ability of the skin to react to the combined effect of drugs and sunlight. It has been attributed to the covalent conjugation of proteins with a photosensitizer, yielding modified macromolecules that can act as antigen provoking the immune system response. The potential role of some endogenous compounds as photoallergens has not been fully established. It has been previously proposed that pterins, which are endogenous photosensitizers present in human skin under pathological conditions, are able to covalently bind to proteins. Here, we evaluated the capability of pterin (Ptr) to form photoadducts with free Lysine (Lys) and poly-L-lysine (poly-Lys). The findings obtained using chromatographic and spectroscopic tools, confirm the formation of photoadducts of Ptr with Lys residues. With poly-Lys the resulting adduct retains the spectroscopic properties of the photosensitizer, suggesting that the aromatic Ptr structure is conserved. On the other hand, the photoproduct formed with free Lys does not behave like Ptr, which suggests that if this product is a photoadduct, a chemical modification may have occurred during the photochemical reaction that alters the pterin moiety.

Graphical abstract

Keywords Pterin · Lysine · Poly-lysine · Photobinding · Photoallergy · UV-A radiation

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This publication is dedicated to Prof. Silvia E. Braslavsky, a pioneer in photobiology and photobiophysics, on the occasion of her 80th birthday.

Extended author information available on the last page of the article

1 Introduction

The combined action of drugs and sunlight on patients can produce both desired and undesired effects. Within the latter group drug-induced photoallergy is one of the most important [1]. From the mechanistic point of view, photoallergy involves covalent drug–protein photobinding (haptenization)

leading to the formation of a photoantigen, which may trigger a hypersensitivity reaction of the immune system. Besides, the photosensitized modifications of proteins may also produce extensive structural changes associated with loss of biological function [2]. Determination of the structures of drug–protein photoadducts is of great relevance to establish the molecular basis of photoallergy and to understand cross-immuno reactivity. Chemicals may potentially react with many different skin proteins at diverse amino acid sites, but, in general, protein molecules are rich in nucleophiles while sensitizing chemicals are reactive electrophiles. One can theorize about the potential mechanisms that lead to skin sensitization [3], but very few of them have been proven experimentally, and when research is carried out on specific chemicals, it is often the case that experimental results show much more mechanistic complexity than it was originally theorized about. It is, therefore, important to fully understand the links between protein haptenization and the ability of a chemical to cause sensitization. A large number of exogenous photoallergenic agents have been investigated [4–7]. However, so far the potential role of some endogenous compounds as photoallergens has not been fully established.

Pterins, a family of heterocyclic compounds, are present in biological systems in multiple forms, and play different roles ranging from pigments to enzymatic cofactors for numerous redox and one-carbon transfer reactions [8, 9]. The most common pterin derivatives are six-substituted compounds (Fig. 1). According to the molecular weight and the functional groups of this substituent, pterins can be divided into two groups: (i) unconjugated pterins, containing substituents with one carbon atom or a short hydrocarbon chain, and (ii) conjugated pterins, with larger substituents containing a p-aminobenzoic acid (PABA) moiety. Pterins can exist in living systems in different redox states and may be classified into three groups according to this property: fully oxidized (or aromatic) pterins, dihydro and tetrahydro derivatives (Fig. 1). These derivatives are present in human epidermis as 5,6,7,8-tetrahydrobiopterin (H_4Bip), which is an essential cofactor for aromatic amino acid hydroxylases [10] and participates in the regulation of melanin biosynthesis [11]. They are of particular importance in vitiligo, a skin disorder characterized by a defective protection against UV radiation due to the acquired loss of constitutional pigmentation [12]. In this disease, the H_4Bip metabolism is altered [13] and dihydro and oxidized pterin derivatives accumulate in the affected tissues at higher concentrations than those reported for healthy cells [14]. Moreover, it can be assumed that pterins can reach any cellular compartment, since they can freely cross phospholipid membranes [15].

The behaviour of oxidized pterins as endogenous photosensitizers is relevant to understand the harmful effects of radiation on skin, and is of particular interest in depigmentation disorders. Depending on the target molecule, pterin-photosensitized oxidations may be purely type I (electron transfer) or type II (singlet oxygen, $^1O_2$), or a combination of both mechanisms [16–19]. In the former case, pterin (Ptr, Fig. 1), the parent and unsubstituted compound of oxidized pterins, under UV-A radiation is able to photoinduce damage in proteins present in the skin [17]. Studies performed with human serum albumin (HSA) revealed that Ptr photosensitizes the oxidation and the oligomerization of HSA [20]. Several amino acids are oxidized in these processes and the oligomerization is mediated by the formation of tyrosine dimers (Tyr_2). Recently, we have demonstrated for the first time, that Ptr is able to covalently bind to Ubiquitin (Ub), a small globular protein present in all eukaryotic cells, yielding a Ptr-Ub adduct that retains the spectroscopic properties of the free photosensitizer. This reaction takes place in the presence and absence of molecular oxygen (O_2), being more efficient under anaerobic conditions. It was suggested that the covalent bond takes place, at least, in two specific sites of the Ub sequence: Lysine (Lys) 6 and Histidine (His) 68 [21].

With this background, the goal of the present work is to obtain more information about covalent photobinding of Ptr to protein, using Lysine (Lys) and poly-L-lysine (15,000–30,000 Da) as target molecules, and to gain a deeper insight into the molecular basis of skin photosensitivity mediated by endogenous photosensitizers. For this purpose, Ptr was exposed to UV-A radiation in the presence of free Lys and Lys-polypeptide under aerated and deaerated conditions. The resulting photoproducts were analyzed by UV/visible spectrophotometry, fluorescence spectroscopy, high performance liquid (HPLC) and size exclusion (SEC)
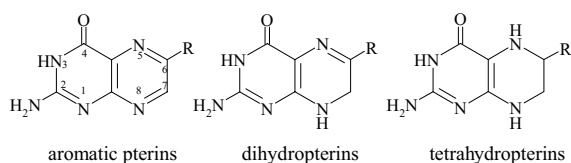
R	Compound
Unconjugated pterins	
-H	pterin (Ptr)
- CH_3	6-methylpterin (Mep)
-CHO	6-formylpterin (Fop)
-COOH	6-carboxypterin (Cap)
-$(CHOH)_2$-CH_3	biopterin (Bip)
Conjugated pterins	
	Folic acid (PteGlu)

Fig. 1 Molecular structures of pterin derivatives

chromatographies, UPLC–mass spectrometry (UPLC–MS) and laser flash photolysis.

2 Experimental section

2.1 General

2.1.1 Chemicals

Pterin (Ptr, purity > 99%, Schircks Laboratories, Switzerland), Lysine hydrochloride (Lys, Sigma-Aldrich, St. Louis, MO) and poly-L-Lysine hydrochloride (15,000–30,000 Da, Sigma-Aldrich, St. Louis, MO) were used without further purification after checking for impurities by HPLC. L-Glutamine (Gln), L-Arginine hydrochloride (Arg), L-Leucine (Leu) and other chemicals were from Sigma Chemical Co. Solutions were prepared using deionized water further purified in a Milli-Q® purification system (Millipore Corporation, USA).

2.1.2 Measurements of pH

The pH measurements were performed using a pH-meter sensION + pH31 GLP combined with a pH electrode 5010T (Hach) or microelectrode XC161 (Radiometer Analytical). The pH of the aqueous solutions was adjusted by adding drops of HCl and NaOH solutions from a micropipette. The concentration of the acid and the base used for this purpose ranged from 0.1 to 2 M.

2.2 Steady-state irradiation

The continuous irradiation of the solutions containing Ptr and the target molecule were carried out in quartz cells (0.4 cm optical path length) at room temperature, using two Rayonet RPR 3500 lamps (Southern N.E. Ultraviolet Co.) with emission centered at 350 nm (bandwidth (fwhm) ~20 nm, power 8 W, emission spectrum is given in Fig. S1, Supplementary material). The irradiation was performed at a distance of 3 mm between the sample and each lamp. The experiments were performed in the presence and absence of dissolved O_2. Experiments with air-equilibrated solutions were carried out in open quartz cells without bubbling but stirring during irradiation time. Argon saturated solutions were obtained by bubbling for 20 min with this gas, previously water saturated (Linde, purity > 99.998%).

Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurement of the incident photon flux density ($q_{n,p}^{0,V}$) at the excitation wavelength, which is the amount of incident photons per time interval ($q_{n,p}^0$) and

divided by the volume of the sample [22]. The method for the determination of $q_{n,p}^{0,V}$ has been described in detail elsewhere [23]. The value of $q_{n,p}^{0,V}$ measured for the radiation source was 5.0 (± 0.4) $\times 10^{-5}$ Einstein L^{-1} s^{-1}. Taking into account that the lamp emits quasi-monochromatic radiation, $q_{n,p}^{0,V}$ value was converted into the UV irradiance of the lamp (E_{UV}^L) with the following equation:

$$E_{UV}^L = q_{n,p}^{0,V} N_A h\nu \frac{V}{S} \tag{1}$$

where $N_A h\nu$ is the energy of a mol of photons emitted by the lamp and V and S are, respectively, the volume and the area exposed to irradiation of the cell used. A value of 71 (± 6) W m^{-2} was obtained for E_{UV}^L.

2.3 Analysis of irradiated solution

2.3.1 UV/Vis analysis

Electronic absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer. Measurements were made using quartz cells of 0.4 or 1 cm optical pathlength. The absorption spectra of the solutions were recorded at regular intervals of irradiation time. Water was used as blank for these measurements.

2.4 Chromatographic analysis

2.4.1 High-performance liquid chromatography (HPLC)

A Prominence equipment from Shimadzu (solvent delivery module LC-20AT, online degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, column oven CTO-10AS VP, photodiode array (PDA) detector SPD-M20A and fluorescence (FL) 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 product separation and to analyze the progress of the photochemical processes when Lys is used as target molecule. 100% aqueous solution of ammonium acetate (NH$_4$Ac, 1 mM, pH 6) was used as mobile phase. The same column and mobile phase were used in the experiments performed with Arg, Leu and Gln. When poly-Lys was used as substrate, a PolySep-GFC-4000 M LC column (300×7.8 mm, Phenomenex) and 100% aqueous solution of NH$_4$Ac (10 mM) and NaCl (100 mM) (pH 6) as mobile phase were used. In some cases, for further analysis, the products were isolated from HPLC runs (preparative HPLC), by collecting the mobile phase after passing through the PDA.

2.4.2 Preparative chromatography

Deaerated solutions of Ptr (100 µM) and poly-Lys (75 µM) were UV-A irradiated for 60 and 120 min. The polypeptide fraction was separated from free Ptr by size exclusion chromatography using water as eluent and disposable Sephadex G-25 columns (PD-10, GE Healthcare, Spain). Experiments in the presence of Guanidine Chloride (3 M) were performed. Photobinding was monitored by fluorescence spectroscopy of the eluate, exciting at 350 nm.

2.4.3 Mass spectrometry analysis

The liquid chromatography equipment coupled to mass spectrometry (LC/MS) system was equipped with an UPLC chromatograph (ACQUITY UPLC from Waters) coupled to a quadrupole time-of-flight mass spectrometer (XevoQTof-MS from Waters) (UPLC–QTof–MS), equipped with an electrospray ionization source (ESI). UPLC analyses were performed using a Zorbax Eclipse Plus C18 (4.6×100 mm, 3.5 µm, Agilent) column. The mobile phase was a mixture of water with 0.1% formic acid (A) and ACN with 0.1% formic acid (B) and a flow rate of 0.3 mL/min. The elution profile was as follows: 0 min, 97% A, 3% B; 10 min, 97% A, 3% B; 20 min, 0% A, 100% B; 22 min, 0% A, 100% B; 25 min, 97% A, 3% B. The injection volume was 10 µL. The ESI source was operated in positive ionization mode with a capillary voltage of 1 kV. The source and desolvation temperatures were set at 120 and 400 °C, respectively. The cone and desolvation gas flows were 10 and 800 L h^{-1}, respectively. All data were collected in centroid mode. Leucine–enkephalin was used as the lock mass, generating an $[M + H]^+$ ion (m/z 556.2771) at a concentration of 250 pg mL^{-1} and a flow rate of 50 mL min^{-1} to ensure accuracy during the MS analysis. Area of Lys peak was obtained from their Selected Ion Monitoring (SIM) chromatogram at m/z 147.1134.

2.5 Fluorescence spectroscopy

Fluorescence measurements were performed using a Single-Photon-Counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon). The equipment has been previously described in detail [24].

2.5.1 Steady-state experiments

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 350 nm were recorded in the range 370–550 nm. The excitation spectra were recorded between 240 and 400 nm, monitoring the fluorescence intensity at 440 nm.

2.5.2 Time-resolved experiments

NanoLED source (maximum at 341 nm) was used for excitation. The emitted photons, after passing through the iHR320 monochromator, were detected by a TBX-04 detector connected to a TBX-PS power supply and counted by a Fluoro-Hub-B module, controlled using the Data Station measurement control software application. The selected counting time window for the measurements reported in this study was 0–200 ns.

2.6 Laser flash photolysis

A pulsed Nd:YAG laser (Lotis TII) was used for the excitation at 355 nm. The single pulses were of ca. 10 ns duration and the energy was 21 mJ/pulse. A pulsed Xenon lamp was employed as detecting light source. The laser flash photolysis apparatus consisted of the pulsed laser, the Xe lamp, a monochromator, and a photomultiplier made up of a tube, housing, and power supply. The output signal from the oscilloscope was transferred to a personal computer. All transient spectra were recorded using 1×1 cm^2 quartz cells with 4 mL capacity, and solutions were bubbled for 20 min with N$_2$ before acquisition. Aqueous solutions of 75 µM Ptr ($A_{355} = 0.4$) were prepared in acidic water at pH = 5. For quenching experiments, stock solutions of Lys were prepared so that it was only necessary to add microliter volumes to the sample cell to obtain appropriate concentrations of the quencher.

3 Results and discussion

3.1 Photodamage of poly-Lysine

To the best of our knowledge, only one work has reported that, under UV-A radiation, Ptr is able to add covalently to a protein, being His and Lys residues the probable binding sites [21]. Otherwise, this kind of reaction has not been observed when free amino acids, such as tryptophan, Tyr, methionine, are used as target molecule and Ptr as sensitizer. Taking into account this background, we evaluated the formation of Ptr-Lys photoadduct using ʟ-Lys homopolymer as a substrate. As can be inferred from the corresponding absorption spectra, under these experimental conditions,
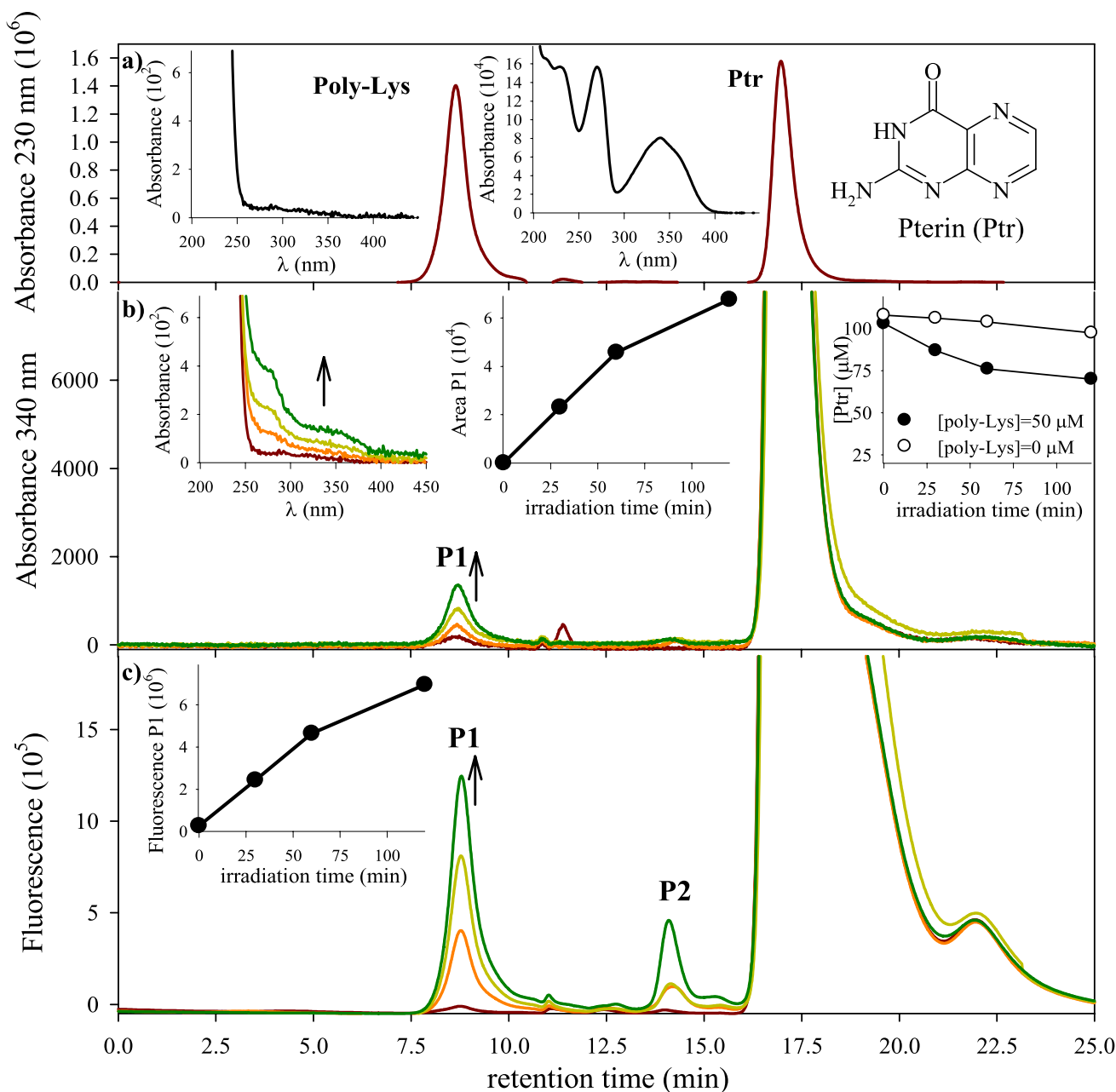
Fig. 2 HPLC analysis of a solution (pH 5.5 ± 0.1) containing poly-Lys (50 µM) and Ptr (100 µM) under anaerobic conditions, before and after 30, 60 and 120 min of irradiation. **a** HPLC–PDA chromatograms registered at 230 nm, absorption spectrum of poly-Lys and Ptr recorded at t_R 8.6 min and 17.0 min, respectively, from the unirradiated sample run. Molecular structure of Ptr. **b** HPLC–PDA chromatograms registered at 340 nm. Insets: time-evolution of Ptr concentration, area and absorption spectrum of P1, recorded at t_R 8.6 min from the irradiated sample run. **c** HPLC–FL chromatograms registered at 440 nm ($\lambda_{exc} = 350$ nm). Inset: time-evolution of the area of the peak of the fluorescent product P1 ($\lambda_{em} = 440$ nm)

Ptr is the only absorbing species, whereas poly-Lys is not excited by UV-A radiation (Inset Fig. 2a). To better mimic the physiological conditions, experiments were performed using Ptr concentrations of the same order of magnitude as those found in human skin affected by vitiligo [14], and a pH of approximately 5.5, which is within the range of skin pH varying between 4.5 and 5.8. It is noteworthy that under

these conditions Ptr is present at more than 99% in its acid form [25].

O_2-free aqueous solutions containing poly-Lys (50 µM) were exposed to UV-A radiation (350 nm) in the presence of Ptr (100 µM) for different periods of time. The experiments were performed in anaerobic conditions as it was reported that the efficiency of formation of Ptr–Ub photoadduct is

much higher in the absence of O_2 than in aerobic condition [21]. The HPLC analysis using the PDA detector, at a condition in which both poly-Lys and Ptr absorb (230 nm), showed two peaks at retention times (t_R) of 8.6 and 17.0 min, corresponding to poly-Lys and Ptr, respectively. Since a size exclusion column was used, poly-Lys eluted at t_R lower than Ptr (Fig. 2a). Chromatograms recorded at 340 nm (Fig. 2b) showed that a new peak (called P1), at the same t_R as poly-Lys, appeared in the irradiated solution. The absorption spectra registered for this peak showed absorption in the UV-A region (Inset Fig. 2b), which is compatible with the absorption features of pterins (Fig. 2a) [25]. This absorption increases with irradiation time (Inset Fig. 2b). While the area corresponding to P1 increases as a function of irradiation time, the concentration of Ptr decreases (Inset Fig. 2b). The consumption of Ptr in the absence of poly-Lys was significantly slower than in its presence (Inset Fig. 2b). The HPLC–FL analysis showed that P1 emitted at 440 nm when excited at 350 nm (Fig. 2c), which is consistent with the fluorescence properties of pterins [25]. The results presented up to now suggest that P1 is a polymer that bears the Ptr moiety. In addition, another fluorescent product P2 was observed (Fig. 2c). The t_R value of P2 indicated that the molecular weight of this photoproduct is higher than that of the Ptr, but lower than the molecular weight of poly-Lys, suggesting that in this product the structure of poly-Lys is not conserved, but a chromophore with spectroscopic properties compatible to Ptr is present.

Control experiments were performed to confirm that P1 is only produced when poly-Lys, Ptr and radiation are present. P1 was not observed when a solution of poly-Lys was exposed to UV-A radiation in the absence of Ptr or when a solution of Ptr was exposed to UV-A radiation in the absence of poly-Lys. As a control, the solution of Ptr and poly-Lys was kept in the dark for 2 h, no production of P1 was observed in this sample (Figure S2, supplementary material).

To get more information about the photophysical properties of P1, the irradiated samples were purified by gel-filtration chromatography to eliminate the free Ptr (Experimental Section). The fluorescence spectra of the poly-Lys fractions isolated from samples before and after irradiation were recorded by excitation at 350 nm. Figure 3a shows that, in treated samples, the emission increased with the irradiation time, indicating that the photochemical process generates a fluorescent polymer. The emission band presented a maximum at 445 nm, which is very close to that of free Ptr (440 nm) (Inset Fig. 3a) [25]. In addition, the fluorescence excitation spectrum of the poly-Lys fractions isolated from treated solutions was also similar to that corresponding to free Ptr (Fig. 3b) [25]. The results presented so far clearly demonstrate that UV-A irradiation induces the
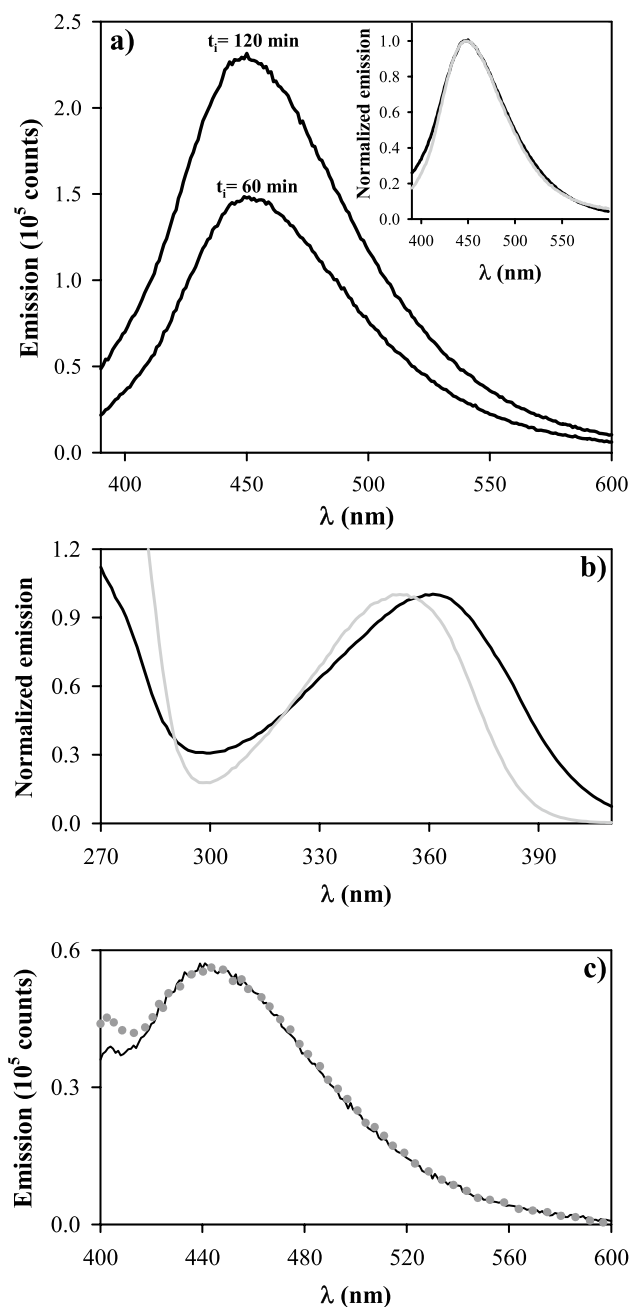

Fig. 3 Fluorescence analysis of irradiated oxygen-free solutions (pH = 5.5 ± 0.1) of poly-Lys (75 μM) and Ptr (100 μM) after isolation by gel-filtration chromatography. **a** Emission spectra obtained upon excitation at 350 nm after 60 and 120 min of irradiation. Inset: Normalized emission spectra of isolated product P1 (black line) and Ptr (gray line). **b** Normalized excitation spectra (λ_{em} = 440 nm) of isolated product P1 (black line) and Ptr (gray line). **c** Emission spectra obtained upon excitation at 350 nm of solutions irradiated 60 min and incubated in the absence (black solid line) and the presence of Guanidinum Chloride (gray dot line), before separation

association of Ptr with poly-Lys. To investigate the nature of the interaction, the irradiated sample was divided into two fractions: one of them was added to an aqueous solution of

Guanidinum Chloride, a strong chaotropic agent that weakens van der Waals interactions but does not affect covalent bonds, and the other one was diluted in the same proportion with water. After incubation for 1 h, the polymer was purified by gel-filtration chromatography and the eluted samples were examined by fluorescence spectroscopy. The emission spectra of both samples were equal, within the experimental error, confirming that Ptr binds to poly-Lys by a covalent bond (Fig. 3c).

It has been reported that in free Lys the photoaddition could involve the α or ε-amino group [26–28]. However, in poly-Lys the α-amino group is not available, because it is involved in the peptide bonds. Taking into account this and the experimental results showed above, we can conclude that: (i) under anaerobic conditions a Ptr-poly-Lys adduct is formed, (ii) the pterin moiety bound to the homopolymer retains the spectroscopic properties of the free photosensitizer and (iii) the photoaddition occurs through nucleophilic attack of the free amino group of the Lys side chain to the pterin moiety.

To investigate if Ptr-poly-Lys adduct is formed in the presence of O_2, air-equilibrated aqueous solutions of poly-Lys were exposed to UV-A radiation in the presence of Ptr. The HPLC–FL analysis of the treated solutions showed a product, with a t_R value close to that of the poly-Lys, which emits at 440 nm upon excitation at 350 nm (Fig. 4). The polymer fraction of the irradiated samples, isolated from the HPLC runs, showed an emission spectrum equal to that obtained under anaerobic condition (Figure S3, supplementary material). This result confirmed that the product observed under aerobic condition is the same as that generated under anaerobic condition. The comparison of the area of the peaks showed that the P1 formation was faster in the absence of molecular O_2 (Inset Fig. 4). Other products, with molecular weights higher than Ptr and smaller than poly-Lys were observed, suggesting that in the presence of O_2 the homopolymer undergoes other chemical reactions that produce changes in its molecular weight.

The results presented up to now clearly demonstrated that under UV-A radiation Ptr reacts with poly-Lys to form a Ptr-poly-Lys adduct that retains the spectroscopic properties of the free photosensitizer and in which the Ptr moiety becomes covalently bound to the polymer. This fact confirms the hypothesis proposed in the previous study on Ptr-photosensitization of Ub, that is, the ε-amino group of Lys is a site where Ptr covalently binds to proteins upon UV-A irradiation. This reaction is more efficient in the absence of oxygen, which is logical, because O_2 quenches the triplet excited state of Ptr (^{3}Ptr*), the excited state responsible for the photosensitizing properties of oxidized pterins, and thus avoids the formation of the photoadduct.

3.2 Photolysis of Lys in the presence of Ptr

Taking into account the results obtained with poly-Lys, we evaluated if Ptr is able to form a photoadduct with free Lys. To achieve this, anaerobic acid aqueous solutions of Lys (200 μM) were exposed to UV-A radiation in the presence of Ptr (100 μM). HPLC–PDA chromatograms, registered at 340 nm, showed the formation of only one photoproduct (P) upon irradiation (Fig. 5a). The absorption spectrum of P (Inset Fig. 5a) showed three absorption bands; the low-energy band is in the same region of the typical band corresponds to the transition $S_0 \rightarrow S_1$ of Ptr (Inset Fig. 2a), nevertheless, a significant difference was observed in the absorption band corresponding to the transition $S_0 \rightarrow S_2$. Furthermore, this product emits at 440 nm when excited at 350 nm (see chromatogram, Fig. 5a) and the corresponding area increases with irradiation time (Fig. 5b). Generation of P was not detected in control experiments in which deaerated aqueous solutions of both reactants were independently exposed to UV-A radiation. In addition, the decrease in the Ptr concentration was faster in the presence than in the absence of Lys (Inset Fig. 5a). Taking into account the spectroscopic properties of Lys, its concentration could not be determined by HPLC with UV and fluorescence detection. The mass chromatograms of irradiated solutions were

Fig. 4 HPLC–FL chromatograms registered at 440 nm ($\lambda_{exc} = 350$ nm) of a solution (pH 5.5 ± 0.1) containing poly-Lys (50 μM) and Ptr (100 μM) under aerobic conditions, before and after 30, 60 and 120 min of irradiation. Inset: comparison of the time-evolution of the area of the peak of the fluorescent product P1 ($\lambda_{em} = 440$ nm) obtained under anaerobic (black circle) and aerobic condition (black triangle)
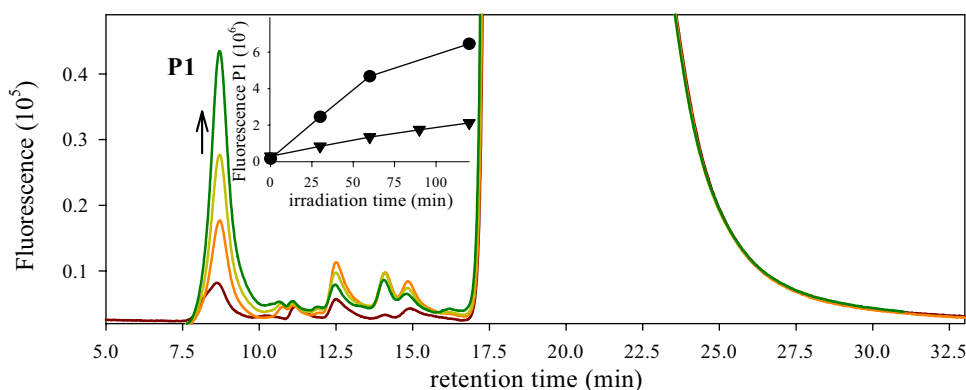
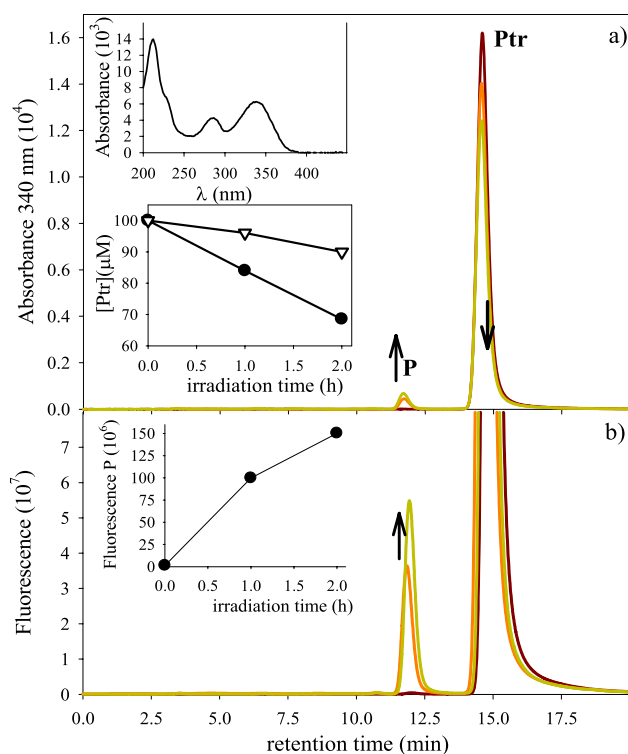**Fig. 5** HPLC analysis of a solution (pH 5.5 ± 0.1) containing Lys (200 μM) and Ptr (100 μM) under anaerobic conditions, before and after 60 and 120 min of irradiation. **a** HPLC–PDA chromatograms registered at 340 nm. Inset: absorption spectrum of P, recorded at t_R 11.2 min from the irradiated sample run and time-evolution of the Ptr concentration in the absence (empty triangle) and in the presence of Lys (black circle). **b** HPLC–FL chromatograms registered at 440 nm ($\lambda_{exc} = 350$ nm). Inset: time-evolution of the area of the peak of the fluorescent product P ($\lambda_{em} = 440$ nm)

Fig. 6 Fluorescence analysis of irradiated oxygen-free solutions (pH $= 6.0 \pm 0.1$) of an isolated aqueous solution of P. **a** Emission spectra obtained upon excitation at 350 nm before and after 2 h of irradiation (black line) and emission spectrum of Ptr (gray line). Inset: Emission decay recorded at 440 nm ($\lambda_{exc} = 341$ nm). **b** Normalized excitation spectra ($\lambda_{em} = 440$ nm) of isolated product P (black line) and Ptr (gray line)

registered for the specific ion mass of Lys (*m/z* 147.1134) and the peak was integrated (Figure S4, supplementary material). Data showed that the concentrations of Lys decreased as a function of irradiation (Inset Figure S4, supplementary material). On another note, in contrast to what was observed with poly-Lys, experiments performed in air-equilibrated solutions revealed that P is not formed at all in the presence of O_2 (Figure S5, Supplementary material).

To study the photophysical properties of P, this compound was isolated from HPLC runs and the fluorescence spectra were recorded by excitation at 350 nm (Fig. 6). Whereas the emission was negligible in non-irradiated solutions, the emission intensity increased with irradiation time, indicating that the photochemical process generates a fluorescent product. The emission band presented a maximum at 410 nm, which is 30 nm blue shifted with respect to the maximum emission registered for free Ptr (440 nm) (Fig. 6a). As it was observed for the absorption spectrum, the fluorescence excitation spectrum of P is similar to the Ptr spectrum (Fig. 6b) in the wavelength region corresponding to the low energy
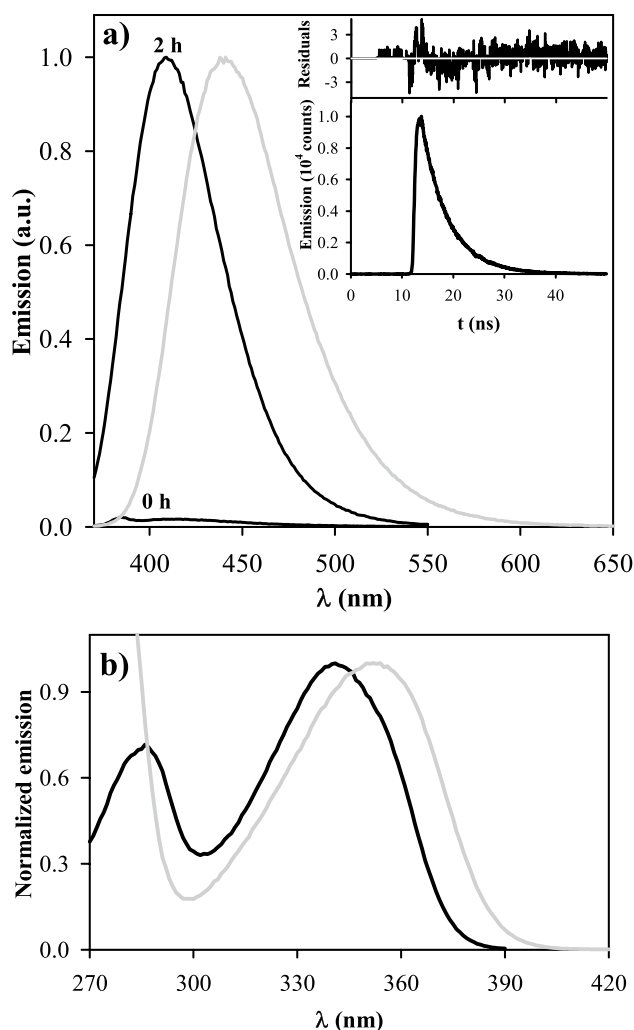
band. Nevertheless, a significant difference was observed in the excitation spectrum, it corresponds to the transition $S_2 \rightarrow S_0$. The fluorescence emission decay of P was registered (Inset Fig. 6a). A first-order rate law was observed for this decay, being the obtained lifetime of ca. 5.7 (± 0.5) ns.

The results showed up to now clearly indicate that a fluorescent product is formed during the anaerobic irradiation of an acidic aqueous solution of Lys and Ptr. Nevertheless, its spectroscopic properties are not exactly the same as those of Ptr suggesting that if P is a product that contains the pterin ring, a chemical modification has taken place that alters the pterin moiety. Next, to get more insight in the chemical structure of P and in the involvement of the α-amino and/ or ε-amino groups of Lys in its formation, new experiments

were performed using amino acids, with different side chain. First, L-leucine (Leu), an amino acid containing an aliphatic side chain and only one amino in position α was used to evaluate the reactivity of this latter. Anaerobic acid aqueous solutions of Leu (200 μM) were exposed to UV-A radiation in the presence of Ptr (100 μM) for different periods of time. No Ptr consumption or product formation was observed when the irradiated samples were analyzed by HPLC. With this result, it can be indirectly concluded that the α-amino group of Lys does not participate in the photogeneration of P. Moreover, experiments using L-arginine (Arg) and L-glutamine (Gln) were performed. These amino acids contain in their side chain, a primary amine linked to different functional group providing them different reactivity, in comparison with the Lys side chain. Like in the case of Leu, no consumption of Ptr or product formation was observed by HPLC analysis for degassed acid aqueous solution of Arg or Gln (200 μM) exposed to UV-A radiation in the presence of Ptr (100 μM). Therefore, the results presented up to now, strongly suggest that the photoproduct P is only formed in the presence of Lys. Interestingly, it has been reported, that under UV radiation the ε-amino group of Lys is able to link covalently to C-2 of Thymine yielding an intermediate which by an intramolecular cyclization gives rise to a stable Thymine-Lys photoadduct [27, 29]. This adduct is not generated when Thymine is exposed to UV radiation in the presence of Arg [27]. Taking into account, that Ptr molecular structure is composed by a pyrimidine and a pyrazine ring (Fig. 1), it can be proposed that P could arise from the nucleophilic attack of the ε-amino of Lys to the C-4 of the pyrimidine moiety of Ptr (Scheme 1), followed by cyclization with elimination of NH_3.

UV-A irradiation of Ptr produces a singlet excited state ($^1Ptr^*$) [30] and a long-lived triplet excited state ($^3Ptr^*$) [31]. However, the latter has been shown to be the only responsible for the photosensitized reactions using nucleotides, amino acids and proteins as targets [19]. Thus, interaction between $^3Ptr^*$ and the Lys was investigated by laser flash photolysis experiments, under anaerobic conditions. The
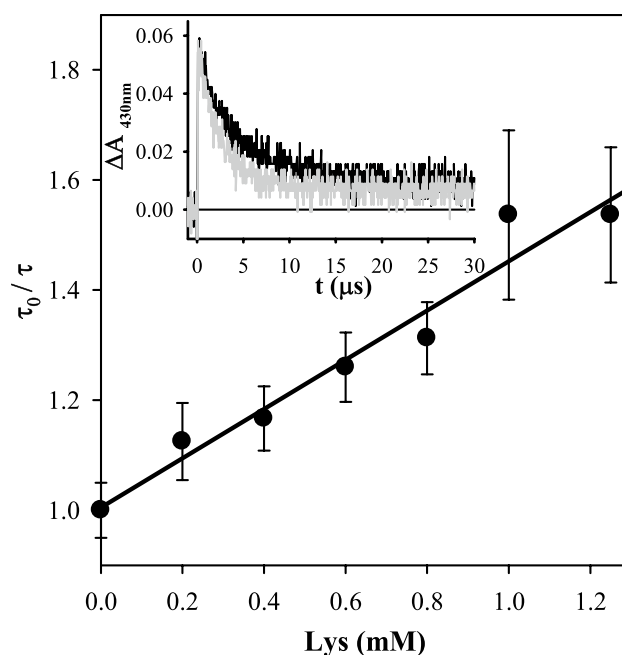
Fig. 7 Stern–Volmer plot of the quenching of the $^3Ptr^*$ by Lys. τ values were calculated analyzing the transient absorbance ΔA vs. t; excitation wavelength 355 nm, analysis wavelength 430 nm, [Ptr] = 75 μM. Inset: Time dependence of the absorbance at 430 nm at 0 (black line) and 0.6 mM (gray line) of Lys

rate constant of the quenching of the $^3Ptr^*$ by Lys (k_q^{Lys}) was calculated using the Stern–Volmer equation [Eq. (2)]:

$$\tau_0/\tau = 1 + k_q^{Lys}\tau_0[Lys] \tag{2}$$

where τ_0 and τ are the triplet lifetimes (in s) in the absence and in the presence of Lys, respectively, and [Lys] the concentration of the amino acid in M. The τ values were calculated from the corresponding decays (Fig. 7) for different Lys concentrations ($\tau_0 = (5.4 \pm 0.8)$ μs), the Stern–Volmer plot obtained (Fig. 7) was linear and a value of (8 ± 2) 10^7 M^{-1} s^{-1} was determined for k_q^{Lys}. Hence, these results

Scheme 1 Proposed structure of P based on the mechanism reported for pyrimidines-Lys photoadducts [27, 29]
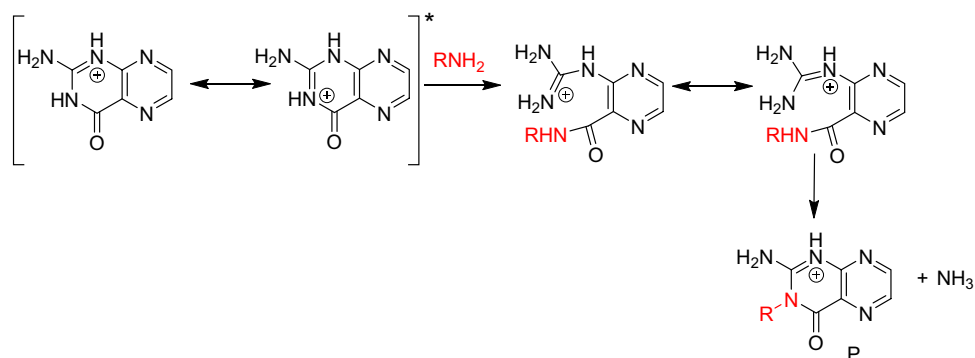
confirm that the ^{3}Ptr* reacts with Lys, but less efficiently, that it was observed with other amino acids [32].

4 Conclusions

We have investigated the photobinding of Ptr to Lys using the free amino acid and poly-Lys as target compounds. Overall, the findings confirm the formation of photoadducts of Ptr with Lys residues. Considering comparatively the results obtained with both substrates, it is clear that Lys and Ptr react upon UV-A excitation and that the photochemical processes lead to the formation of different products indicating that the reactions are not exactly the same. With poly-Lys a photoadduct is formed and this product retains the spectroscopic properties of the photosensitizer, suggesting that the aromatic Ptr structure is conserved. On the other hand, the photoproduct formed with free Lys does not behave like Ptr, which suggests that if this product is a photoadduct, a chemical modification may have occurred during the photochemical reaction that alters the pterin moiety. The results obtained in this work allowed us to conclude that Lys residues of protein are able to covalently bind to Ptr, an endogenous photosensitizer, yielding a photoadduct that may acts as potential photoallergen.

Declarations

Conflict of interest The authors declare no competing financial interests.

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