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Degradation of α-melanocyte-stimulating hormone photosensitized by pterin†

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Oxidized pterins, efficient photosensitizers under UV-A irradiation, accumulate in the skin of patients suffering from vitiligo, a chronic depigmentation disorder. In this work, we have investigated the ability of pterin (Ptr), the parent compound of oxidized pterins, to photosensitize the oxidation of the peptide α-melanocyte-stimulating hormone (α-MSH), which stimulates the production and release of melanin by melanocytes in skin and hair. Our results showed that Ptr is able to photoinduce the degradation of α-MSH upon UV-A irradiation and that the reaction is initiated by an electron transfer from the peptide to the triplet excited state of Ptr. The photosensitized process produces chemical changes in at least two different amino acid residues: tryptophan and tyrosine (Tyr). It was shown that α-MSH undergoes dimerization and oxidation, the former process taking place after the formation of Tyr radicals. The present findings are analyzed in the context of the general behavior of pterins as photosensitizers and the biological implications are discussed.

Introduction

Most of the solar UV energy incident on the Earth surface corresponds to UV-A radiation (320–400 nm), which is not absorbed significantly by DNA and proteins and therefore does not affect directly the biomacromolecules. However, UV-A radiation acts indirectly by photosensitized reactions and is now recognized as a class I carcinogen.[1] Moreover, epidemiological evidence has shown that exposure of humans to artificial UV-A radiation (sun lamps, tanning beds) is a major risk factor for melanoma induction.[2–4]

The chemical changes in biological components resulting from photosensitized reactions can take place through different mechanisms. Currently, it is accepted that the photosensitization of proteins occurs mainly through type II photooxidation, which involves the reaction of singlet oxygen ($^1O_2$) with tryptophan (Trp), tyrosine (Tyr), histidine, methionine and cysteine side-chains.[5] In this mechanism, $^1O_2$ is most often produced by energy transfer from the excited triplet state of a sensitizer to dissolved molecular oxygen (O_2).

In recent studies, we have demonstrated that pterin (Ptr), the parent compound of oxidized pterins, is able to photosensitize the oxidation of Trp and Tyr in aqueous solution.[6,7] However, although pterins are efficient $^1O_2$ photosensitizers,[8,9] the mechanism of degradation of Trp and Tyr photoinduced by Ptr does not involve $^1O_2$ oxidation as the main pathway. In contrast, our studies suggest that the reactions are initiated by an electron transfer from the amino acid to the triplet excited state of Ptr. In particular, the formation of radicals of those amino acids was demonstrated and, in the case of Tyr, evidence of a dimerization process was reported.[6,7] This class of processes (called type I mechanisms) was described for the photosensitization by pterins of the model protein bovine serum albumin[10] and other substrates.[11,12]

Pterins are present in human epidermis because 5,6,7,8-tetrahydrobiopterin (H_4Bip) is an essential cofactor in the hydroxylation of the aromatic amino acids[13] and participates in the regulation of melanin biosynthesis.[14] Several dihydro and tetrahydropterins are involved in the metabolism of H_4Bip and, hence, are also present in human skin.[15] 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.[16] In this disease the protection against UV radiation fails due to the lack of melanin, the main pigment of skin. In addition, the H_4Bip metabolism is altered[17] and unconjugated oxidized

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pterins accumulate in the affected tissues. These compounds are photochemically reactive and, upon UV-A excitation (320–400 nm), can fluoresce, undergo photooxidation to produce different photoproducts, generate reactive oxygen species, such as $^1O_2$, and, as mentioned above, photosensitize the oxidation of biomolecules.[18] Therefore, the photochemistry of pterins and, especially their photosensitizing properties, are of particular interest for the study of this disease.

In the context of our investigations on the photosensitizing properties of pterins, we have recently started the study of reactions that affect biomolecules involved in the pigmentation of the skin. In this way we have demonstrated that Ptr is able to photosensitize the inactivation of tyrosinase,[19] an enzyme that catalyzes the first and rate-limiting step in the biosynthesis of the melanin in mammals. In the present work we report our investigations on the degradation of the α-melanocyte-stimulating hormone (α-MSH), which stimulates the production and release of melanin by melanocytes in skin and hair and has the following amino acid sequence: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val.

The main aims of this work were to evaluate the capability of pterins to photoinduce chemical changes in peptides with biological activity and to find out whether the reactions observed for free amino acids, particularly Trp and Tyr, take place in polypeptide chains. We have performed the experiments in aqueous solutions under UV-A irradiation and used Ptr as a model pterin photosensitizer, which is a relatively good $^1O_2$ sensitizer (Φ_Δ = 0.18(±0.02)[18]). Under these experimental conditions only Ptr was excited, as can be inferred from the corresponding absorption spectra (Fig. 1). 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.

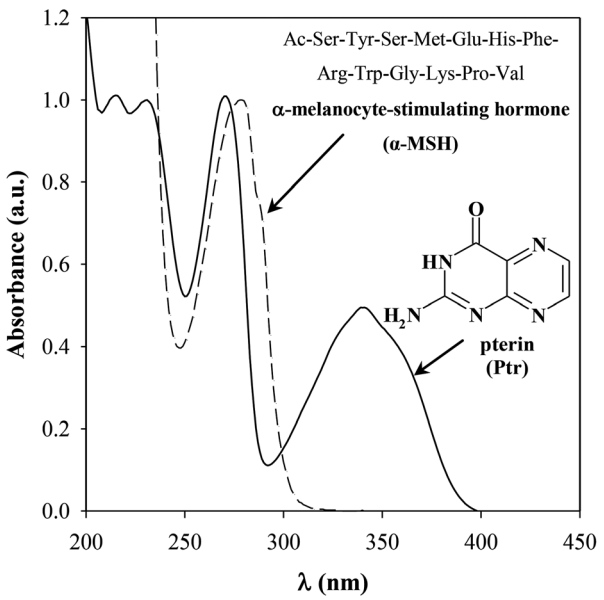

Fig. 1 Molecular structure of Ptr and α-MSH, and their corresponding absorption spectra in air-equilibrated acidic (pH 5.5) aqueous solutions.

Experimental

General

Chemicals. Pterin (Ptr, purity >99%, Schircks Laboratories, Switzerland) and α-melanocyte-stimulating hormone (α-MSH, purity >99%, Sigma Chemical Co.) were used without further purification after checking for impurities by HPLC. Acetonitrile (ACN) was purchased from J. T. Baker. Other chemicals were from Sigma Chemical Co. Solutions were prepared by dissolving Ptr and α-MSH in deionized water further purified in a Milli Q Reagent Water System apparatus. The specific electrical resistance of water measured was ~10 MΩ cm.

Samples. All the experiments were carried out in aqueous solutions. 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. The experiments were performed with air-equilibrated and O_2-saturated solutions, and the latter were obtained by bubbling for 20 min with O_2 (Linde, purity >99.998%), previously water saturated.

Estimation of the α-MSH concentration. It was assumed that the absorption of photons by α-MSH at 280 nm is only due to the Trp and Tyr residues, and that the molar absorption coefficient of α-MSH at this wavelength ($\varepsilon^{280}_{\alpha\text{-MSH}}$) is equal to the sum of the corresponding molar absorption coefficients of free Trp and Tyr in aqueous solutions at pH 5.5 ($\varepsilon^{280}_{\alpha\text{-MSH}} = \varepsilon^{280}_{\text{Trp}} + \varepsilon^{280}_{\text{Tyr}}$). This calculation yields an $\varepsilon^{280}_{\alpha\text{-MSH}}$ value of 8.9×10^3 M^{-1} cm^{-1}. Therefore, the α-MSH concentration in each solution was estimated by determining the absorbance at 280 nm (before adding Ptr) and then using the Lambert–Beer law ($A^{280} = \varepsilon^{280}_{\alpha\text{-MSH}}\, l[\alpha\text{-MSH}]$) ($l$ = optical path length).

Steady-state irradiation. Continuous photolysis experiments were carried out in quartz cells (1 cm optical path length) at room temperature. Two radiation sources were employed: (I) Rayonet RPR lamps emitting at 350 nm (bandwidth ≈ 20 nm, Southern N.E. Ultraviolet Co., Branford, CT) and (II) a 450 W Xe short arc lamp (Ushio Inc.) with a monochromator Horiba-Jobin-Yvon FL-1004 (single-grating spectrometer, 330 nm blaze grating), set at 350 nm.

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 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, autosampler 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 Jupiter proteo column (150 × 4.6 mm, 4 μm, Phenomenex) was used for separation of the photosensitizer, the substrate and the products. Solutions containing 75% NH_4Ac (10 mM, pH 7) and 25% of ACN were used as the mobile phase.

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.[20,21] 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.

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.[22] 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 (Hamamatsu). Corrected fluorescence spectra obtained by excitation at 300 nm were recorded between 320 and 550 nm.

Ultra-high performance liquid chromatography (UPLC)-mass spectrometry analysis. The LC/MS system was equipped with an UPLC chromatograph (ACQUITY UPLC from Waters) coupled to a quadrupole time-of-flight mass spectrometer (Xevo G2-QTof from Waters). UPLC analyses were performed using the same column used in HPLC analysis. The mass spectrometer was operated in both positive and negative ion modes. 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.

Results and discussion

Degradation of α-MSH photo-induced by pterin

Air-equilibrated aqueous solutions containing Ptr and α-MSH were exposed to UV-A (350 nm) irradiation (radiation source I, Experimental) for various 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[18]). Under these experimental conditions only Ptr was excited (Fig. 1). The samples, irradiated in quartz cells of 1.0 cm optical path length, were analyzed by UV-vis spectrophotometry and HPLC (see the Experimental section).

Significant changes in the absorption spectra of the solutions were registered after irradiation (Fig. 2), revealing that a photosensitized reaction took place and showing that products absorbing at wavelengths longer than 300 nm were formed. The spectral changes are compatible with those observed for the photosensitization of free Trp and Tyr.[6,7] In these experiments the concentration of Ptr, determined by HPLC, did not decrease. H_2O_2 was found to be generated and its concentration increased as a function of irradiation time (Fig. S1†).

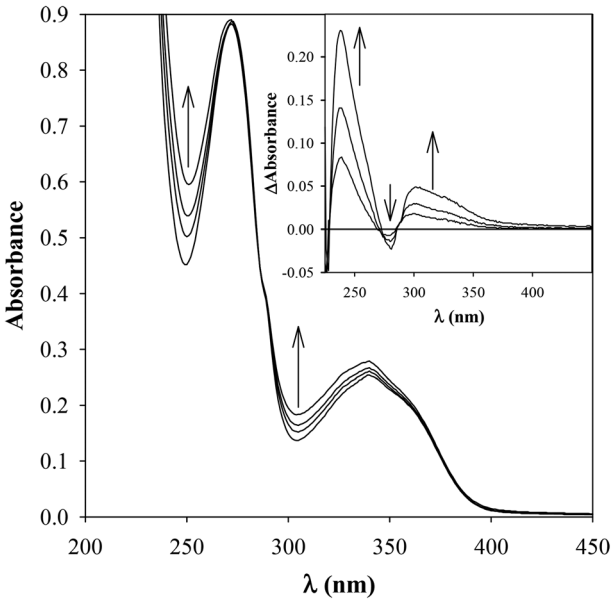

Fig. 2 Time evolution of the absorption spectrum of an air-equilibrated solution of α-MSH irradiated in the presence of Ptr. Spectra were recorded at 0, 4, 8 and 15 min; optical path length = 1.0 cm. Arrows indicate the trends of the changes observed at different wavelengths. Inset: experimental difference spectra. $[Ptr]_0$ = 65 μM, $[\alpha\text{-MSH}]_0$ = 25 μM, pH = 5.5.

On the HPLC chromatograms, the peak corresponding to the hormone was well separated from that of the photosensitizer (Fig. 3). An analysis using the PDA detector revealed that the chromatographic peak corresponding to α-MSH decreased with irradiation time when aqueous solutions were exposed to UV-A irradiation in the presence of Ptr. Concomitantly, new peaks were detected, revealing the formation of several photoproducts with absorption above 300 nm, which is in agreement with the corresponding spectral changes recorded (Fig. 2). On the other hand, the peak corresponding to Ptr did not decrease upon irradiation.

To confirm that the reaction observed was a photosensitized process, additional controls were carried out. Hormone degradation was not observed in solutions containing Ptr and α-MSH at concentrations similar to those used in experiments shown in Fig. 2 and 3, kept in the dark, thus excluding the possibility of thermal reactions. In another set of control experiments, an α-MSH (25 μM) solution was irradiated with UV-A in the absence of Ptr and no spectral change was observed, thus excluding the possibility of product formation by spurious direct excitation of α-MSH.

Therefore, the results presented in this section showed that Ptr is able to photoinduce chemical changes in α-MSH upon UV-A irradiation. In the photochemical process many products were formed. The absorption spectrum of α-MSH is dominated by the spectral features of Trp, the main chromophore in proteins. Therefore, the changes observed in the absorption

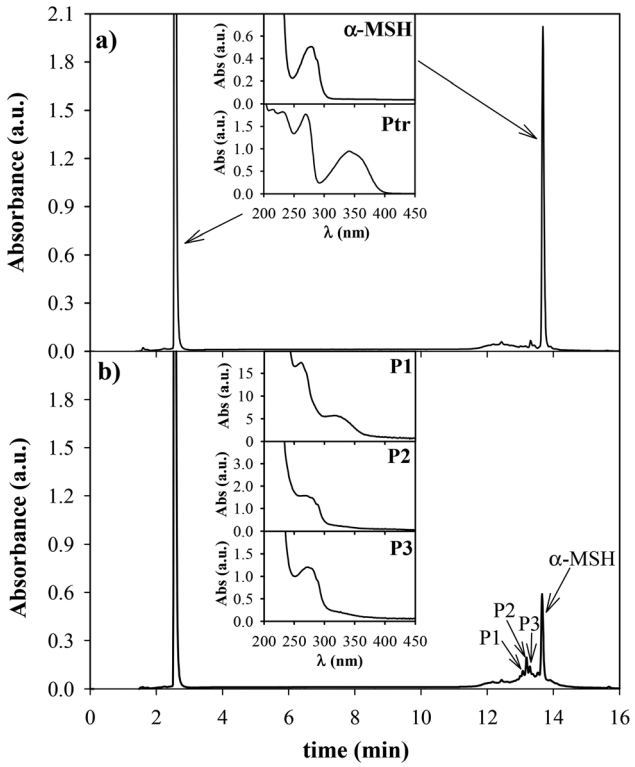

Fig. 3 Chromatograms obtained in HPLC-UV analysis at 280 nm; (a) before irradiation; (b) after 10 min of irradiation. Insets: absorption spectra of the reactants and the main photoproducts. $[Ptr]_0$ = 70 μM, $[\alpha\text{-MSH}]_0$ = 32 μM, pH = 5.5.

spectrum of the solution and in the chromatogram at 280 nm suggested that the Trp residues of α-MSH were affected.

Effect on the tryptophan residue

Taking into account that the photosensitization of Trp by Ptr has previously been reported[6] and that Trp is particularly susceptible to a variety of oxidizing agents, it is expected that the Trp residue of α-MSH should be affected by the photosensitized process. Trp has particular emission features; *e.g.* it is the only amino acid residue that absorbs at 295 nm and presents an intense emission band centered at 355 nm. Therefore, the effect of the photochemical process on the Trp residue was studied by means of fluorescence measurements.

Solutions containing Ptr and α-MSH were exposed to UV-A radiation (350 nm, radiation source II, Experimental) and the emission spectra under excitation at 295 nm were registered at various irradiation times. At this excitation wavelength, Ptr absorbs and fluoresces with a maximum at 439 nm, so that the emission of the photosensitizer is partially superimposed on that of the Trp (Fig. 4). However, under our experimental conditions, the Ptr concentration remains constant and the typical emission band of Trp could be adequately registered. The band intensity decreased as a function of irradiation time (Fig. 4). It is worth mentioning that an increase in the emission above 390 nm was observed, which can be assigned to the formation of dimers of Tyr (*vide infra*).

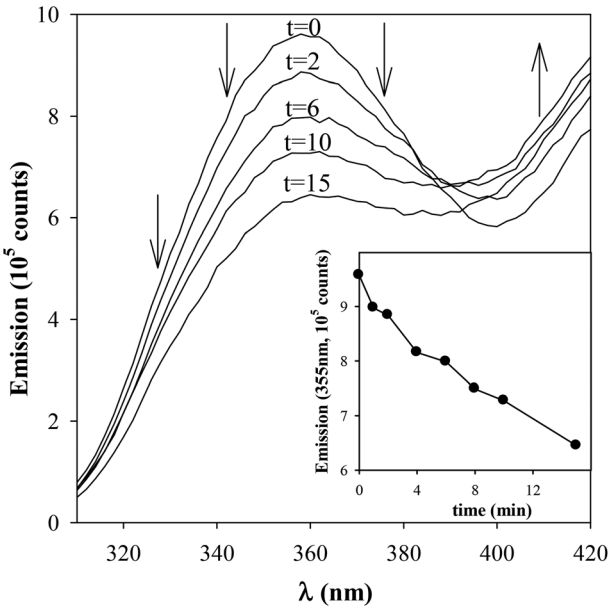

Fig. 4 Corrected fluorescence spectra (λ_{exc} = 295 nm) of an aqueous solution (pH = 5.5) of α-MSH (27 μM) and Ptr (15 μM) irradiated at 350 nm (radiation source II). The irradiation time (min) appears above each spectrum. Inset: decrease of the fluorescence intensity at 355 nm as a function of irradiation time.

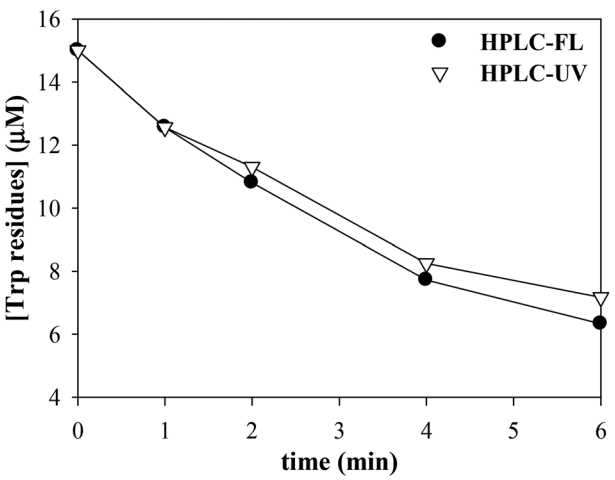

Fig. 5 Time evolution of the concentration of Trp residues estimated by HPLC-FL (λ_{exc} = 295 nm, λ_{em} = 355 nm) and HPLC-UV (at 280 nm) in irradiated (350 nm, radiation source I) aqueous solutions of α-MSH (10 μM) and Ptr (80 μM).

Trp residues were also investigated by chromatography coupled to a fluorescence detector (HPLC-FL, see the Experimental section). Fluorescence chromatograms of solutions containing Ptr and α-MSH (excitation at 295 nm and emission at 355 nm) showed a main peak whose retention time matched that of the peak of the hormone in chromatograms obtained in HPLC-UV at 280 nm. Therefore solutions containing Ptr and α-MSH were exposed to UV-A radiation (350 nm, radiation source I, Experimental) and HPLC-UV and HPLC-FL analyses were carried out as a function of irradiation time. Fig. 5 shows

the concentration of α-MSH estimated by both analyses as a function of irradiation time. These results confirm that Trp residues were consumed during α-MSH photosensitization by Ptr.

Effect on the tyrosine residue

Tyrosine 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 $^1O_2$,[23,24] but also because this amino acid plays a key role in polymerization and crosslinking of proteins[25,26] *via* reactions initiated by Tyr radicals.[27] In a recent study performed with free Tyr in aqueous solutions, we have demonstrated that the excitation of Ptr is followed by an electron transfer from the amino acid to the Ptr triplet excited state, leading to the formation of the corresponding ion radicals, $Ptr^{\bullet-}$ and $Tyr^{\bullet+}$.[7] The latter undergoes deprotonation to the neutral radical of tyrosine (Tyr(-H)$^\bullet$) that may be trapped by O_2 to yield oxidation products. Dimerization of Tyr(-H)$^\bullet$ to form the so-called dimers of Tyr (denoted Tyr_2), which are involved in crosslinking of proteins, was also observed. To investigate whether the photosensitization of α-MSH by Ptr leads to the generation of Tyr_2, fluorescence experiments were carried out, taking advantage of their particular emission features. The absorption and emission spectra of dimers of Tyr_2 are red shifted with respect to those of Tyr.[28]

Therefore, solutions containing Ptr and α-MSH were exposed to UV-A radiation (350 nm, radiation source II) and the emission spectra under excitation at 310 nm were registered at various irradiation times. For a given irradiation time, the spectrum of the solution before irradiation, *i.e.* the fluorescence of Ptr, was subtracted from the spectrum registered. Results showed that an emission band with a maximum coinciding with that expected for Tyr_2 [28] was present in the irradiated solutions and that the intensity increased as a function of irradiation time (Fig. 6), which is strong evidence in favor of the photodimerization of Tyr residues of α-MSH induced by Ptr.

It is known since the 1960s that 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.[29] Dimers of Tyr(-H)$^\bullet$ were also investigated by HPLC-FL. Fluorescence chromatograms (excitation at 310 nm and emission at 400 nm) of irradiated solutions (350 nm, radiation source I, Experimental) showed several fluorescent products. Under our experimental conditions, the area of the major peak increased with irradiation time. These results are in agreement with those presented in the previous paragraph. However, it is obvious from the structure of Tyr(-H)$^\bullet$ that dimerization can lead to several products, as reported.[30] This fact explains why several dimeric products were found by HPLC-FL analyses.

The detection of Tyr_2, which forms in highly oxidative environments,[31,32] is important because it suggests that the hormone might undergo crosslinking. This point is discussed in more detail in the next section. In addition, this finding supports the hypothesis that an electron transfer process takes place during the irradiation, since it has been reported that dimers are not formed by oxidation with reactive oxygen species such as $^1O_2$, $O_2^{\bullet-}$ and H_2O_2.[33,34]

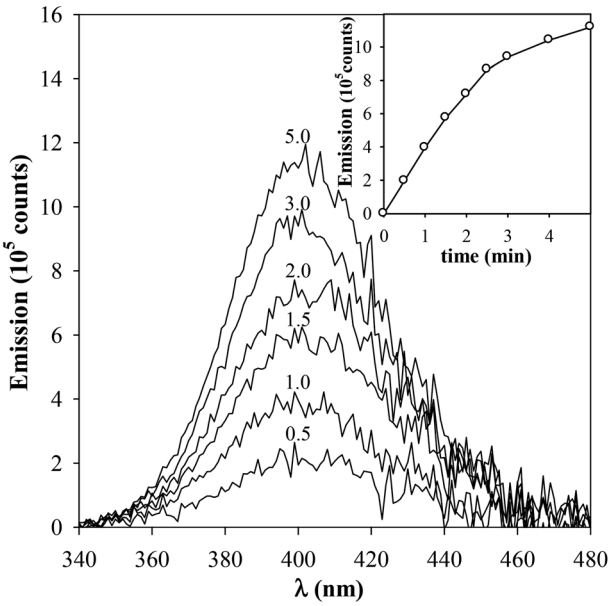

Fig. 6 Corrected fluorescence spectra (λ_{exc} = 310 nm) of an aqueous solution (pH = 5.5) of α-MSH (25 μM) and Ptr (15 μM) irradiated at 350 nm (radiation source II). The irradiation time (min) appears above each spectrum. For each time, the spectrum of the solution before irradiation was subtracted. Inset: the increase of the fluorescence intensity at 405 nm as a function of irradiation time.

Table 1 Wavelengths of the absorption (λ_{abs}) and emission (λ_{em}) maxima of the compounds studied in this work, in aqueous solution and the corresponding wavelengths of excitation (λ_{exc}) and emission analysis (λ_{an}) used for the fluorescence measurements

Compound	λ_{abs} (nm)	λ_{em} (nm)	λ_{exc} (nm)	λ_{an} (nm)
Ptr	340	439		
Trp	280	355	295	355
Tyr	274	305	275	300
Tyr_2	315	400	310	400

It is not easy to monitor the concentration of the Tyr residue during the photochemical process by means of spectrophotometric measurements because the absorption spectrum of Tyr is completely superimposed on that of the Trp residue (Table 1) and, in addition, the values of the molar absorption coefficient of the former are much lower than those of the latter. However, the Tyr emission spectrum is blue-shifted and more narrowly distributed in the wavelength range of interest in comparison to the Trp spectrum.[35] Therefore, the emission at 300 nm of α-MSH upon excitation at 275 nm can be attributed to Tyr residues with a negligible contribution of Trp emission. Taking into account these fluorescence features, Tyr residues were investigated by HPLC-FL and fluorescence chromatograms (excitation at 275 nm and emission at 300 nm) of irradiated solutions (350 nm, radiation

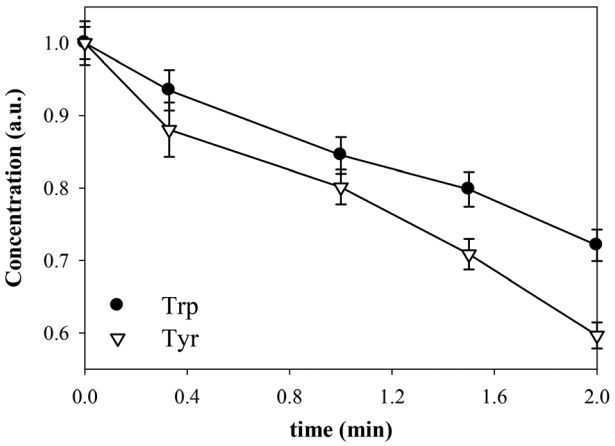

Fig. 7 Time evolution of the concentration of Trp and Tyr residues estimated by HPLC-FL (λ_{exc} = 295 nm, λ_{em} = 355 nm and λ_{exc} = 275 nm, λ_{em} = 300 nm, respectively) in irradiated (350 nm, radiation source I) aqueous solutions of α-MSH (10 μM) and Ptr (80 μM) at pH 5.5.

source I) were registered. The evolution of the area of the corresponding peak was compared to that assigned to Trp (excitation at 280 nm, emission at 355 nm, *vide supra*). This analysis indicated that the Tyr residue reacted faster than the Trp residue (Fig. 7). Taking into account that Trp is considered more reactive than Tyr due to the values of parameters related to the reactivity (*e.g.*, Trp has a lower ionization energy[36] and a higher rate constant of the reaction of oxidation by $^1O_2$), the behaviour shown in Fig. 7 (faster reaction of Tyr residues in this case) is surprising.

Analysis of the photoproducts by mass spectrometry

A qualitative analysis of the photoproducts was carried out by means of UPLC coupled to mass spectrometry (see the Experimental section). Solutions containing α-MSH and Ptr at pH 5.5 were analyzed in both positive and negative ion modes (ESI^+ and ESI^-, respectively). As expected, the signals corresponding to the intact molecular ion of Ptr as $[M + H]^+$ and $[M - H]^-$ species at *m*/*z* 164 Da and 162 Da, respectively, were observed. However, in the case of α-MSH, the resolution was much better in ESI^+ than in ESI^- mode. Therefore all the results presented in this section correspond to mass spectrometry analysis carried out in ESI^+ mode. The molecular formula of α-MSH is $C_{77}H_{109}N_{21}O_{19}S$ and its molecular weight is 1664.88 Da. This value is the average of different weights due to the naturally occurring isotopes of carbon (natural abundance, ^{12}C: 98.93%, ^{13}C: 1.07%), although isotopes of nitrogen and oxygen also contribute. Therefore the registered mass spectrum of α-MSH consisted of groups of signals for the mono-charged (*m*/*z* ≈ 1666 Da), di-charged (*m*/*z* ≈ 833 Da) and tri-charged ions (*m*/*z* ≈ 556 Da), the group of signals corresponding to the di-charged ion being much more intense than that corresponding to the mono-charged ion.

UV exposed solutions (irradiation source I) containing α-MSH and Ptr were analyzed without previous separation with the UPLC chromatograph and the corresponding mass spectra were compared to those obtained before irradiation. Analysis of mass spectra of treated solutions showed new peaks with *m*/*z* values higher than those of α-MSH and corresponding to oxygenated products. In particular, *m*/*z* values indicated the incorporation of oxygen atoms ([M + O], [M + 2O], [M + 3O], *etc.*). For instance, for the di-charged ions, species at *m*/*z* ≈ 841 Da, 849 Da and 857 Da were detected (Fig. 8). After normalization with respect to the intensity of the peaks corresponding to the intact α-MSH, the intensity of the peaks of the photoproducts increased as a function of irradiation time. This behaviour was also observed for the mono- and di-charged ions.

In agreement with the HPLC analysis described above (Fig. 3), the UPLC-UV analysis showed many peaks superimposed on that corresponding to the intact peptide. However, the mass chromatograms of irradiated solutions registered for the specific ion masses corresponding to the oxygenated products were different, *i.e.* the retention times (t_r) of the maxima of the peaks were different. For instance, for a solution similar to that used in Fig. 8 and irradiated for 10 min, t_r values of 12.8, 12.7 and 11.7 min were observed for the intact peptide, the monooxygenated product and the dioxygenated product, respectively.

These results indicate that upon irradiation in the presence of Ptr, α-MSH undergoes photooxidation. Taking into account that consumption of Trp and Tyr residues has been demonstrated (*vide supra*), these amino acids very likely are involved in the photosensitized oxidation. Moreover, for free Tyr, the incorporation of at least one oxygen atom photoinduced by Ptr has already been reported.[7] Likewise, photosensitization of free Trp by Ptr yields, among other products, hydroxy-tryptophan and *N*-formylkynurenine, which correspond to the incorporation of one and two oxygen atoms, respectively. It is clear that the oxidation of α-MSH might also involve other residues.

In the previous section, evidence in favor of the photosensitized formation of Tyr_2 was provided. If such a hypothesis is correct, the peptide should undergo crosslinking and products with molecular weights about twice the molecular weight of α-MSH should be formed. In particular, the binding of two molecules of the peptide by coupling of two Tyr(-H)$^\bullet$ would lead to a compound with the molecular weight of [2α-MSH − 2H]. In the solutions before irradiation no dimeric-type products could be detected.

The separation of the photoproducts with the UPLC chromatograph revealed the presence of at least one compound with a t_r value (13.5 min) higher than that of 2α-MSH, which suggests the formation of compounds larger than the intact peptide. In mass spectrometry analysis of this compound, mono-charged ions with molecular weights about twice the molecular weight of α-MSH could not be detected, which is logical considering that the group of signals corresponding to the mono-charged ion of the intact peptide is not intense (*vide supra*). However, in the region of about *m*/*z* ~ 830 Da, which corresponds to two charges per molecule of peptide, ions compatible with a molecular weight of [2α-MSH − 2H] were found (Fig. 9); *i.e.* a peak of *m*/*z* = 832.4 Da was recorded.

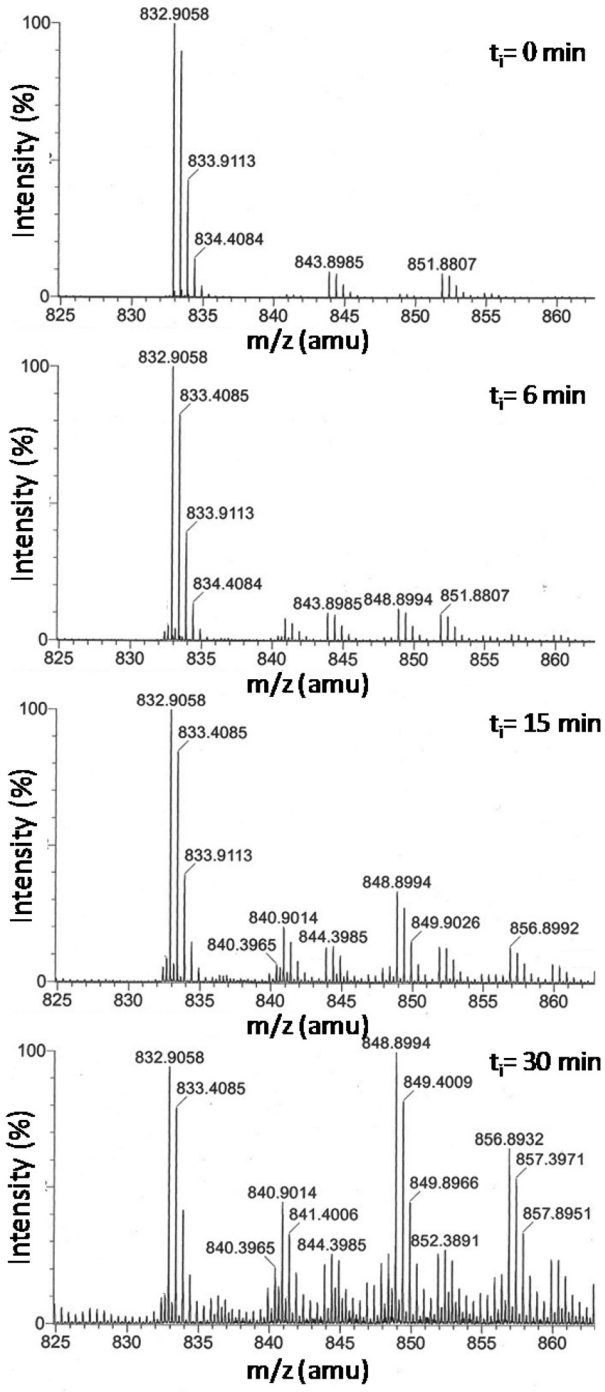

Fig. 8 Mass spectra of the di-charged ions corresponding to the intact α-MSH molecule ($m/z \approx 833$ Da) and its oxygenated products ($m/z \approx 841$ Da, 849 Da and 857 Da). Analysis of the untreated sample and irradiated for 6, 15 and 30 min, carried out in positive mode, voltage = 50 V. $[\alpha\text{-MSH}]_0$ = 43 μM, $[\text{Ptr}]_0$ = 50 μM, pH 5.5.

Mechanistic analysis

In the presence of oxygen, Ptr and pterin derivatives act as photosensitizers through both type I and type II mechanisms (see Introduction). To investigate the mechanism involved in the photosensitization of α-MSH, steady-state photolyses were carried out using the irradiation source I under different experimental conditions and the evolution of the concentrations of α-MSH and its photoproducts was determined as a function of irradiation time by HPLC-FL. In particular, for each experiment the consumption of Trp residues (λ_{exc} = 295 nm, λ_{em} = 355 nm), the consumption of Tyr residues (λ_{exc} = 275 nm, λ_{em} = 300 nm) and the production of Tyr_2 (λ_{exc} = 310 nm, λ_{em} = 400 nm) were evaluated. In almost all cases, the same behaviour was observed for the three parameters investigated. Therefore, for the sake of brevity, we show only the results corresponding to the consumption of Trp residues, unless a significant difference in the behavior of the three parameters was observed.

Photolysis experiments were carried out in O_2-saturated solutions and the results were compared with those performed in air-equilibrated solutions. HPLC-FL measurements showed that the rate of α-MSH chemical modification was much greater in air-equilibrated than in O_2-saturated solutions (Fig. 10). This behavior is not consistent with a process wherein the hormone would be consumed by $^1O_2$.

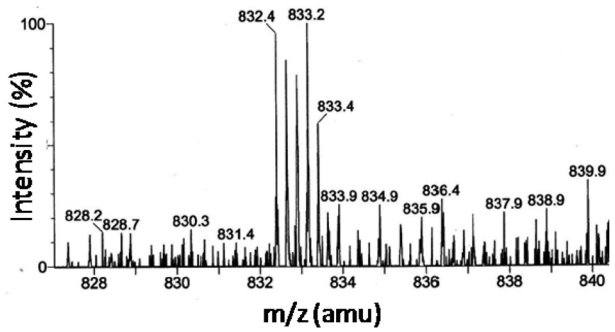

Fig. 9 Mass spectra of a dimeric product with the mass of [2α-MSH – 2H]. The sample after 15 min of irradiation was separated using a UPLC equipment before mass analysis. Analysis carried out in positive mode, voltage = 50 V. $[\alpha\text{-MSH}]_0$ = 43 μM, $[\text{Ptr}]_0$ = 50 μM, pH 5.5.

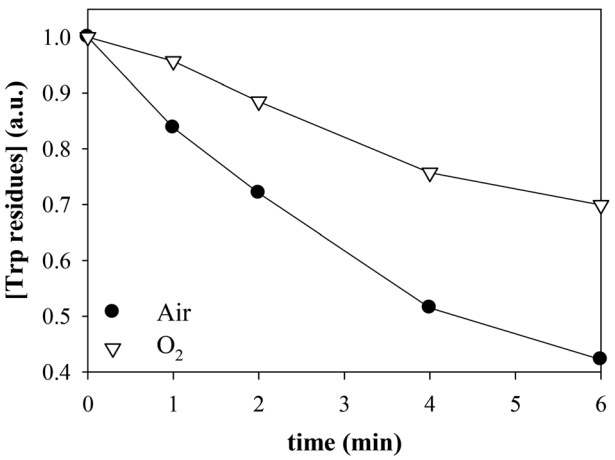

Fig. 10 Time evolution of the concentration of Trp residues estimated by HPLC-FL (λ_{exc} = 295 nm, λ_{em} = 355 nm) in irradiated (350 nm, radiation source I) aqueous solutions of α-MSH (30 μM) and Ptr (100 μM).

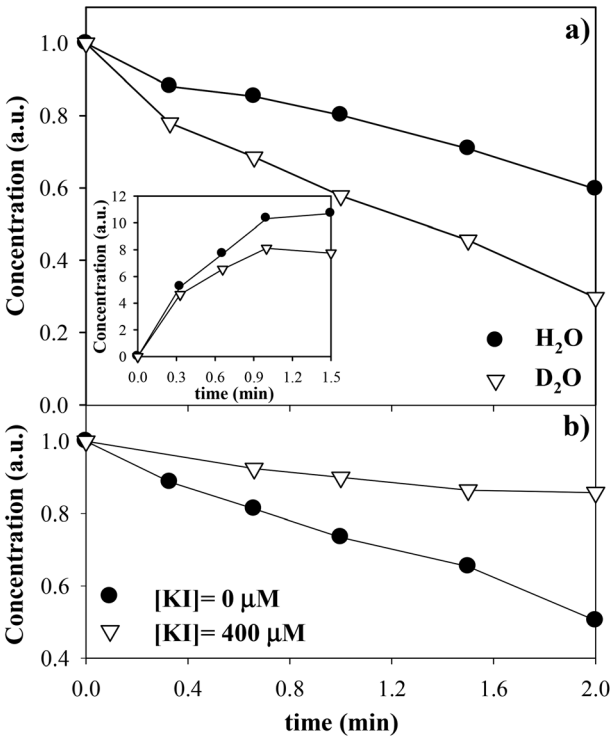

Fig. 11 Time evolution of the concentration of Trp residues estimated by HPLC-FL (λ_{exc} = 295 nm, λ_{em} = 355 nm) in irradiated (350 nm, radiation source I) solutions of α-MSH (10 μM) and Ptr (100 μM). (a) Experiments carried out in H_2O (●) and D_2O (▽); pH (pD) = 5.5. Inset: time evolution of the concentration of Tyr_2 estimated by HPLC-FL (λ_{exc} = 310 nm, λ_{em} = 400 nm). (b) Experiments carried out in H_2O in the absence (●) and presence (▽) of KI (400 μM), pH = 5.5.

In order to further investigate the participation of $^1O_2$, 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 approx. 15[37,38] the photosensitized oxidation of α-MSH should be much faster in the deuterated solvent if $^1O_2$ contributed significantly to the process. Air-equilibrated solutions containing Ptr (100 μM) and α-MSH (12 μM) in H_2O and D_2O at pH/pD 5.5 were irradiated under otherwise identical conditions. HPLC-FL measurements showed that the rate of α-MSH chemical modification was slightly faster in D_2O than in H_2O (Fig. 11a), but not to the extent expected from the differences in $^1O_2$ lifetimes in the two solvents. These results indicate that the reaction with $^1O_2$ is present in our reaction system, but its contribution in the consumption of Trp and Tyr residues is not significant in air-equilibrated solutions. On the other hand, it is also worth mentioning that the production of Tyr_2 was slightly faster in H_2O than in D_2O (inset of Fig. 11a). Thus, as expected, $^1O_2$ does not contribute to the formation of Tyr_2, even in D_2O.

It has been previously demonstrated that iodide (I^-) at micromolar concentrations is an efficient and selective quencher of triplet excited states of pterins.[12,39] Therefore, photosensitization experiments were carried out in air-equilibrated aqueous solutions containing α-MSH and Ptr at pH 5.5 in the presence of KI (400 μM). The results revealed that the rate of α-MSH consumption was slower than in the absence of I^- (Fig. 11b). Thus, the inhibition of the photosensitized degradation of α-MSH by I^- strongly suggests that the process takes place *via* a purely dynamic mechanism initiated by the triplet excited state of Ptr, which is in agreement with a decreasing reaction rate when the O_2 concentration increases.

Considering the results presented in this work and the former studies reported for the photosensitization of proteins and free amino acids by pterins, a mechanism initiated by an electron transfer from the peptide to the triplet excited state of Ptr can be proposed. The formation of radicals can be summarized as follows (reactions 1–8): after excitation of Ptr and formation of its triplet excited state, ^{3}Ptr* (reactions 1 and 2), three reaction pathways compete for the deactivation of the latter: intersystem crossing to the ground state (reaction 3), energy transfer to molecular oxygen leading to the regeneration of Ptr and the formation of $^1O_2$ (reaction 4), and electron transfer from α-MSH to ^{3}Ptr* to form the peptide radical cation (α-MSH$^{\bullet+}$) (reaction 5), which very likely undergoes deprotonation to the neutral radical (α-MSH(-H)$^\bullet$) (reaction 6). In experiments performed in air-equilibrated solutions, the rate of reaction 5 is significant and an important consumption of α-MSH is observed. In contrast, in oxygen-saturated solutions, reaction 4 competes more efficiently with reaction 5, the proportion of ^{3}Ptr* reacting with α-MSH decreases and, consequently, the rate of α-MSH consumption also decreases (Fig. 10). The electron transfer from Ptr$^{\bullet-}$ to O_2 regenerates Ptr and forms $O_2^{\bullet-}$ (reaction 7). This radical may disproportionate with its conjugated acid $HO_2^\bullet$ to form H_2O_2 (summarized by reaction 8).

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

$${}^1\text{Ptr}^* \xrightarrow{\text{ISC}} {}^3\text{Ptr}^* \quad (2)$$

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

$${}^3\text{Ptr}^* + {}^3\text{O}_2 \rightarrow \text{Ptr} + {}^1\text{O}_2 \quad (4)$$

$${}^3\text{Ptr}^* + \alpha\text{-MSH} \rightarrow \text{Ptr}^{\bullet-} + \alpha\text{-MSH}^{\bullet+} \quad (5)$$

$$\alpha\text{-MSH}^{\bullet+} \rightleftarrows \alpha\text{-MSH(-H)}^\bullet + \text{H}^+ \quad (6)$$

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

$$2\text{H}^+ + 2\text{O}_2^{\bullet-} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (8)$$

The free radical may be generated in different amino acid residues, but in this work we have demonstrated that it is located at least in Trp and Tyr residues (Trp(-H)$^\bullet$ and Tyr(-H)$^\bullet$, respectively). It is worth mentioning that although Trp has a lower ionization energy (*vide supra*), there is no hole transfer from the Tyr(-H)$^\bullet$ to the Trp(-H)$^\bullet$ radical, which would protect Tyr, but increase the amount of Trp damaged. In contrast, this kind of process has been reported in double-stranded DNA from adenine radical to guanine.[40] In the case of α-MSH, the

size of the peptide suggests that in aqueous solution the molecule is unfolded and therefore the distance between the two involved amino acids is too large for the hole transfer to efficiently occur. Subsequently, both amino acids (Trp and Tyr) form radicals and both radicals lead to products. Two chemical photosensitized processes were observed: oxygenation and dimerization. Indeed, Trp(-H)$^{\bullet}$ may undergo oxygenation (Reaction 9) and Tyr(-H)$^{\bullet}$ dimerization (Reaction 10), and very likely also oxidation (Reaction 11).

$$\mathrm{Trp(-H)^{\bullet} + O_2 \rightarrow Trp(ox)} \quad (9)$$

$$\mathrm{2Tyr(-H)^{\bullet} \rightarrow Tyr_2} \quad (10)$$

$$\mathrm{Tyr(-H)^{\bullet} + O_2 \rightarrow Tyr(ox)} \quad (11)$$

Conclusions

The oxidation of the α-melanocyte-stimulating hormone (α-MSH) photosensitized by pterin (Ptr), the parent compound of oxidized pterins, in aqueous solution under UV-A irradiation was investigated. When aerated solutions containing α-MSH and Ptr (pH = 5.5) were exposed to UV-A radiation, the peptide was consumed, H_2O_2 was generated, but the photosensitizer concentration did not change significantly.

The photosensitized process leads to the oxidation of the peptide and to the degradation of at least tryptophan (Trp) and tyrosine (Tyr) residues. Taking into account that the Ptr photosensitized oxidation of these two amino acids in their free form has been previously proven, it can be inferred that the incorporation of oxygen atoms takes place in Trp and Tyr residues of α-MSH. In addition, the dimerization of the peptide was also observed and dimers of Tyr radical were detected in the treated peptide, which indicates that Ptr may photoinduce crosslinking of peptides.

The photosensitized reactions are initiated by an electron transfer from α-MSH to the triplet excited state of Ptr to form the peptide radical cation (α-MSH$^{\bullet+}$) and the Ptr radical anion (Ptr$^{\bullet-}$). The electron transfer from Ptr$^{\bullet-}$ to O_2 regenerates Ptr and forms $O_2^{\bullet-}$, which, in turn, may disproportionate with its conjugated acid $HO_2^{\bullet}$ to form H_2O_2. The radical in the peptide is located at least on the Trp and Tyr residues (Trp(-H)$^{\bullet}$ and Tyr(-H)$^{\bullet}$, respectively). Trp(-H)$^{\bullet}$ undergoes oxidation and Tyr(-H)$^{\bullet}$ dimerization, and very likely also oxidation. On the other hand, singlet oxygen which is produced by Ptr does not play a significant role in the reaction mechanism.

Taking into account that α-MSH stimulates the production and release of melanin by melanocytes in skin and hair and that there is accumulation of pterin derivatives in some pathological skin diseases such as vitiligo, the damage of this hormone photoinduced by Ptr is relevant from a biomedical point of view and can have important ramifications.

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References

1 F. El Ghissassi, R. Baan and K. Straif, *Lancet Oncol.*, 2009, **10**, 51–52.
2 T. B. H. Buckel, A. M. Goldstein, M. C. Fraser, B. Rogers and M. A. Tucker, *Arch. Dermatol.*, 2006, **142**, 485–488.
3 W. Ting, K. Schultz, N. N. Cac, M. Peterson and H. W. Walling, *Int. J. Dermatol.*, 2007, **46**, 1253–1257.
4 J. Moan, A. C. Porojnicu and A. Dahiback, *Adv. Exp. Med. Biol.*, 2008, **624**, 104–116.
5 D. I. Pattison, A. S. Rahmantoa and M. J. Davies, *Photochem. Photobiol. Sci.*, 2012, **11**, 38–53.
6 A. H. Thomas, M. P. Serrano, V. Rahal, P. Vicendo, C. Claparols, E. Oliveros and C. Lorente, *Free Radicals Biol. Med.*, 2013, **63**, 467–475.
7 C. Castaño, M. L. Dántola, E. Oliveros, A. H. Thomas and C. Lorente, *Photochem. Photobiol.*, 2013, **89**, 1448–1455.
8 K. V. Neverov, E. A. Mironov, T. A. Lyudnikova, A. A. Krasnovsky Jr. and M. S. Kritsky, *Biochemistry*, 1996, **61**, 1149–1155.
9 S. Y. Egorov, A. A. Krasnovsky Jr., M. Y. Bashtanov, E. A. Mironov, T. A. Ludnikova and M. S. Kritsky, *Biochemistry*, 1999, **64**, 1117–1121.
10 A. H. Thomas, C. Lorente, K. Roitman, M. M. Morales and M. L. Dántola, *J. Photochem. Photobiol., B*, 2013, **120**, 52–58.
11 M. P. Serrano, C. Lorente, F. E. Morán Vieyra, C. D. Borsarelli and A. H. Thomas, *Phys. Chem. Chem. Phys.*, 2012, **14**, 11657–11665.
12 M. L. Dántola, M. Vignoni, C. González, C. Lorente, P. Vicendo, E. Oliveros and A. H. Thomas, *Free Radicals Biol. Med.*, 2010, **49**, 1014–1022.
13 I. Ziegler, *Med. Res. Rev.*, 1990, **10**, 95–114.
14 K. U. Schallreuter, J. M. Wood, M. R. Pittelkow, M. Gütlich, K. R. Lemke, W. Rödl, N. N. Swanson, K. Hitzemann and I. Ziegler, *Science*, 1994, **263**, 1444–1446.
15 C. A. Nichol, G. K. Smith and D. S. Duch, *Annu. Rev. Biochem.*, 1985, **54**, 729–764.
16 S. J. Glassman, *Clin. Sci.*, 2011, **120**, 99–120.
17 K. U. Schallreuter, J. Moore, J. M. Wood, W. D. Beazley, E. M. Peters, L. K. Marles, S. C. Behrens-Williams,

R. Dummer, N. Blau and B. Thöny, *J. Invest. Dermatol.*, 2001, **116**, 167–174.
18 C. Lorente and A. H. Thomas, *Acc. Chem. Res.*, 2006, **39**, 395–402.
19 M. L. Dántola, A. D. Gojanovich and A. H. Thomas, *Biochem. Biophys. Res. Commun.*, 2012, **424**, 568–572.
20 C. C. Allain, L. S. Poon, C. S. G. Chan, W. Richmond and P. C. Fu, *Clin. Chem.*, 1974, **20**, 470–475.
21 H. M. Flegg, *Ann. Clin. Biochem.*, 1973, **10**, 79–84.
22 M. P. Serrano, M. Vignoni, M. L. Dántola, E. Oliveros, C. Lorente and A. H. Thomas, *Phys. Chem. Chem. Phys.*, 2011, **13**, 7419–7425.
23 S. Criado, A. T. Soltermann, J. M. Marioli and N. A. García, *Photochem. Photobiol.*, 1998, **68**, 453–458.
24 A. Wright, W. A. Bubb, C. L. Hawkins and M. J. Davies, *Photochem. Photobiol.*, 2002, **76**, 35–46.
25 N. Rabgaoui, A. Slaoui-Hasnaoui and J. Torreilles, *Free Radicals Biol. Med.*, 1998, **14**, 519–529.
26 D. A. Malencik and S. R. Anderson, *Biochemistry*, 1996, **35**, 4375–4386.
27 R. Amadò, R. Aeschbach and H. Neukom, *Methods Enzymol.*, 1984, **107**, 377–388.
28 G. S. Harms, S. W. Pauls, J. F. Hedstrom and C. K. Johnson, *J. Fluoresc.*, 1997, **7**, 283–292.
29 H. I. Joschek and S. I. Miller, *J. Am. Chem. Soc.*, 1966, **88**, 3273–3281.
30 J. W. Heinecke, W. Li, H. L. Daehnke III and J. A. Goldstein, *J. Biol. Chem.*, 1993, **268**, 4069–4077.
31 T. G. Huggins, M. C. Wells-Knecht, N. A. Detorie, J. W. Baynes and S. R. Thorpe, *J. Biol. Chem.*, 1993, **268**, 12341–12347.
32 J. W. Heinecke, W. Li, G. A. Francis and J. A. Goldstein, *J. Clin. Invest.*, 1993, **91**, 2866–2872.
33 D. A. Malencik and S. R. Anderson, *Amino Acids*, 2003, **25**, 233–247.
34 J. D. Spikes, H. R. Shen, P. Kopeckova and J. Kopecek, *Photochem. Photobiol.*, 1993, **70**, 130–137.
35 J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Springer, New York, 2006, ch. 3.
36 D. M. Close, *J. Phys. Chem. A*, 2011, **115**, 2900–2912.
37 F. Wilkinson, H. P. Helman and A. B. Ross, *J. Phys. Chem.*, 1995, **24**, 663–677.
38 P. R. Ogilby and C. S. Foote, *J. Am. Chem. Soc.*, 1983, **105**, 3423–3430.
39 M. S. Kritsky, T. A. Lyudnikova, E. A. Mironov and I. V. Moskaleva, *J. Photochem. Photobiol., B*, 1997, **39**, 43–48.
40 B. Giese, *Acc. Chem. Res.*, 2000, **33**, 631–636.