Biochemical and Biophysical Research Communications 424 (2012) 568–572

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inactivation of tyrosinase photoinduced by pterin

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ARTICLE INFO

Article history:
Received 21 June 2012
Available online 7 July 2012

Keywords:
Tyrosinase
Pterins
Photosensitization
Vitiligo

ABSTRACT

Tyrosinase catalyzes in mammals the first and rate-limiting step in the biosynthesis of the melanin, the main pigment of the skin. Pterins, heterocyclic compounds able to photoinduce oxidation of DNA and its components, accumulate in the skin of patients suffering from vitiligo, a chronic depigmentation disorder in which the protection against UV radiation fails due to the lack of melanin. Aqueous solutions of tyrosinase were exposed to UV-A irradiation (350 nm) in the presence of pterin, the parent compound of oxidized pterins, under different experimental conditions. The enzyme activity in the irradiated solutions was determined by spectrophotometry and HPLC. In this work, we present data that demonstrate unequivocally that the enzyme is photoinactivated by pterin. The mechanism of the photosensitized process involves an electron transfer from tyrosinase to the triplet excited state of pterin, formed after UV-A excitation of pterin. The biological implications of the results are discussed.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing glycoprotein that catalyses the orthohydroxylation of monophenols and the subsequent oxidation of the *o*-diphenolic products to the resulting *o*-quinones [1]. In vegetables, fruits, and mushrooms, tyrosinase is a key enzyme in the browning that occurs upon bruising or long term storage. In mammals, tyrosinase catalyzes the first and rate-limiting step in melanin biosynthesis, the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (DOPA) and the subsequent oxidation of DOPA to L-dopaquinone [2]. This compound, in turn, undergoes fast oxidation and rearrangement to yield L-dopachrome. The active site structure, containing two copper atoms coordinated by histidine residues, and the catalytic mechanism of tyrosinase have been described [3,4]. This enzyme is quite significant in the fields of medicine, agriculture, and, in the cosmetic industry, the development and screening of potent inhibitors of tyrosinase are especially attractive.

Pterins are a family of heterocyclic compounds widespread in living systems. In particular, 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 [5]. The importance of this cofactor in the human epidermis and its participation in the regulation of melanin biosynthesis are well recognized [6]. Several dihydro and tetrahydropterins are involved in the metabolism of H_4Bip and, hence, also present in human skin [7].

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 [8]. In this disease the H_4Bip metabolism is altered [9] and oxidized pterins accumulate in the affected tissue [6], where the protection against UV radiation fails due to the lack of melanin, the main pigment of skin. Therefore, the photochemistry of pterins is of particular interest for the study of this disease. Moreover, it has been demonstrated that photooxidation of pterins occurs *in vivo* under pathological conditions [10].

Solar radiation induces modifications to different biomolecules and is implicated in the generation of human skin cancers [11]. In particular, UV-A radiation (320–400 nm) can induce damage to DNA and other macromolecules through photosensitized reactions [12]. This indirect action may be mediated by endogenous or exogenous photosensitizers and can take place through different mechanisms: energy transfer from the triplet state of the photosensitizer to the substrate and photosensitized oxidations, which can involve the generation of radicals (type I mechanism), e.g., via electron transfer or hydrogen abstraction, and/ or the production of singlet oxygen ($^1O_2$) (type II mechanism) [13]. Although

Abbreviations: DOPA, 3,4-dihydroxy-L-phenylalanine; H_4Bip, 5,6,7,8-tetrahydrobiopterin; $^1O_2$, singlet oxygen; I^-, iodide; $O_2^{\cdot-}$, superoxide anion; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; $^3Ptr^*$, pterin triplet excited state; $Ptr^{\cdot-}$, pterin radical anion; $TYR^{\cdot+}$, tirosinase radical cation; HPLC, high-performance liquid chromatography; TYR, Tyrosinase; Ptr, pterin; V, reaction rates; t_i, irradiation time; ESI, electronic supplementary information.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.06.155

the photosensitized damage to DNA is well characterized, much less is known about proteins and very little has been studied on inactivation of enzymes caused by photosensitized processes. Therefore the photochemical behavior, and, in particular, the photosensitizing properties of compounds present in the skin are very important from a biomedical point of view.

The photochemistry of oxidized pterins has been studied [14] and it has been demonstrated that they are able to photoinduced DNA damage [15] and can act as photosensitizer through both type I and type II mechanisms [16]. However, to the best of our knowledge, no study has been reported on processes photosensitized by pterins that affect specific biomolecules of the metabolism of melanin. Therefore, given the important biological and medical ramifications of the photosensitizing properties of pterins, we set out to investigate the inactivation of tyrosinase photoinduced by pterin, the parent and unsubstituted compound of oxidized pterins.

2. Material and methods

Tyrosinase from mushroom (lyophilized powder, ⩾1000 unit/mg solid, Sigma Chemical Co) and pterin (>98%, Schircks Laboratories) were used without further purification. KI and other chemicals were purchased by Sigma Chemical Co. The pH measurements were performed with a pH-meter PHM220 (Radiometer Copenhagen) and a combined pH electrode pHC2011-8 (Radiometer Analytical).

The solutions were irradiated in quartz cells at room temperature using a Rayonet RPR lamp (Southern N. E. Ultraviolet Co.) with emission centered at 350 nm (bandwidth ~ 20 nm). The experiments were performed in aqueous solutions in the presence and absence of O_2. Experiments with air-equilibrated solutions were carried out in open quartz cells without bubbling, whereas oxygen-free and oxygen-saturated solutions were obtained by bubbling for 20 min with Ar gas and O_2, respectively.

The enzyme activity was assayed according to the method of Pomerantz [17]. Briefly, the formation of L-dopachrome was measured spectrophotometrically at 475 nm ($\varepsilon = 3600\ M^{-1}\ cm^{-1}$). The reaction was performed at 37 °C in KH_2PO_4 buffer 20 mM at pH 6.5 using L-tyrosine 0.15 mg/mL (0.83 mM) as the substrate. The conversion of an inactive form of the catalytic site of the enzyme into an active form gives rise to a lag period before the reaction reaches maximal rate, which is a characteristic of the overall activity of the tyrosinase (oxidation of L-tyrosine into L-dopachrome) [18]. Therefore the enzyme activity (rate of formation of L-dopachrome, $nM\ s^{-1}$) was determined in the linear phase by measuring the slope of the curve of absorbance at 475 nm *vs.* time after the addition of the substrate.

A high-performance liquid chromatograph Prominence from Shimadzu equipped with a photodiode array detector SPD-M20A was employed for determining the concentrations of substrates and products. A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping, 150 × 4.6 mm, 4 μm, Phenomenex) was used for product separation. Aqueous solution containing ammonium acetate 10 mM (pH = 7.0) was used as mobile phase.

3. Results and discussion

To ascertain if pterin is able to photoinduce the inactivation of the enzyme, aqueous solutions (1 mM KH_2PO_4, pH 6.5) containing tyrosinase and pterin were exposed to UV-A radiation (350 nm). Under these experimental conditions, pterin was excited, whereas tyrosinase did not absorb radiation. After the irradiation, the enzyme activity was assayed according to the method of Pomerantz (see Section 2).

In control experiments, tyrosinase solutions (*ca* 14 μg/mL) were exposed to UV-A radiation during different periods of time in the absence of pterin. No changes in the enzyme activity were detected, even after more than 30 min of irradiation, thus excluding the possibility that spurious effects of direct light absorption by the enzyme could affect its activity. In another set of control experiments, decrease in the tyrosinase activity was not observed in solutions containing pterin (100 μM) that were kept in the dark for 30 min at 37 °C. This, in turn, excludes the possibility that pterin is an inhibitor, which is relevant because H_4Bip is an allosteric inhibitor of tyrosinase due to a specific binding domain [19].

On the other hand, when aqueous solutions of tyrosinase (*ca* 14 μg/mL) were exposed to UV-A radiation in the presence of the photosensitizer (95 μM), a fast inactivation of the enzyme was registered (Fig. 1), leading to a complete loss of activity in 15 min of irradiation. In another set of experiments air-equilibrated solutions with the same concentration of tyrosinase and different concentrations of pterin were prepared. The samples were irradiated for the same period of time (12 min) and the activity of the enzyme was determined. Results showed a correlation between the pterin concentration and the photoinactivation (Supplemental Figure S1). These results provide evidence, for the first time, suggesting that processes photosensitized by pterins might affect the synthesis of melanin and, in consequence, play a key role in pigmentation disorders.

To investigate the role of O_2 in the mechanism of the photoinactivation of tyrosinase, photolysis experiments were carried out in deoxygenated and O_2-saturated solutions and the results were compared with those performed in air-equilibrated solutions. Deoxygenated and O_2-saturated solutions were obtained by bubbling for 20 min with Ar and O_2 gas, respectively. After irradiation, these solutions were bubbled with air before determining the enzyme activity. The values of the enzyme activity clearly showed that the efficiency of the photoinactivation strongly depends on the O_2 concentration during the irradiation: for the same irradiation time and pterin concentration, the higher the O_2 concentration, the higher the remaining activity (Table 1). Although pterin

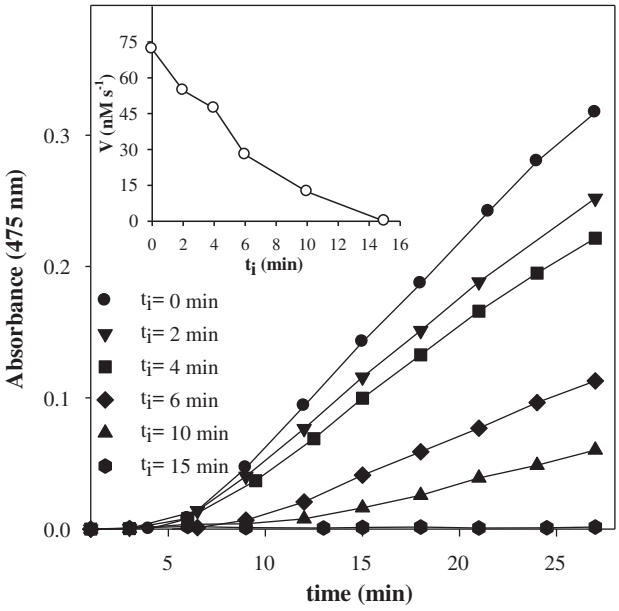


Fig. 1. Determination of the tyrosinase activity in solutions (1 mM KH_2PO_4, pH 6.5) irradiated at room temperature in the presence of pterin (95 μM). For each sample the formation of L-dopachrome was followed measuring the absorbance at 475 nm as a function of the time elapsed after adding L-tyrosine as the substrate (37 °C). Inset: reaction rates (V) determined in the linear phase as a function of irradiation time (t_i).

Table 1
Tyrosinase activity determined in deoxygenated (Ar), air-equilibrated and O_2-saturated solutions after different irradiation times (t_i) in the presence of pterin (95 μM, pH 6.5). Control experiments correspond to solutions irradiated in air-equilibrated solutions in the absence of pterin.

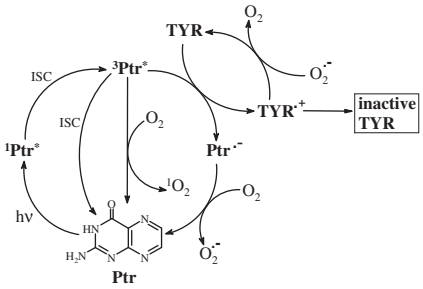
t_i/min	Activity/nM s^{-1}			
	Control	Ar	Air	O_2
3.5	65 ± 5	32 ± 3	45 ± 3	55 ± 5
8	63 ± 4	0.8 ± 0.4	9 ± 3	41 ± 4
12	68 ± 4	0	3.4 ± 0.6	35 ± 3

is an efficient $^1O_2$ photosensitizer [14], this "protective effect" of oxygen rules out the participation of that reactive oxygen species in the mechanism of the reaction. Taking into account the electron-transfer processes induced by the triplet excited state of pterins recently described [20], the behavior observed could be due to the deactivation of the triplet state by dissolved O_2, thus avoiding the electron transfer from the enzyme.

To explore with more detail this hypothesis, photolysis experiments in the presence of iodide (I^-) were performed. This anion, under certain conditions of concentration (100–300 μM), is a selective quencher of the triplet excited states of pterins [20]. Therefore solutions containing tyrosinase and pterin (100 μM) were irradiated in the absence and in the presence of I^- (200 μM) at room temperature and pH 6.5. The experiments were performed under anaerobic conditions to prevent possible reactions between I^- and reactive oxygen species generated by pterin. For an irradiation time of 3.5 min, activities of 25 (±3) nM s^{-1} and 60 (±4) nM s^{-1} were determined in the absence and in the presence of I^-, respectively. The latter value was very close to that obtained in a control experiment carried out irradiating the enzyme in the absence of both pterin and I^- (63 (±4) nM s^{-1}). Similar results were obtained in other experiments performed at different pterin concentrations. Therefore results of photolysis in the presence of I^- are in agreement with experiments performed in O_2-saturated solutions and support our assumption that the triplet excited state of pterin is involved in the mechanism of the photosensitized inactivation of tyrosinase.

According to the evidence shown thus far, the photosensitized inactivation should start with an electron transfer from the enzyme to the triplet excited state of pterin. It is well established that ground state O_2 quenches organic radical anions to produce the superoxide anion ($O_2^{\cdot-}$) [20]. Therefore, to investigate the participation of $O_2^{\cdot-}$ in the mechanism taking place under aerobic conditions, the effect of superoxide dismutase (SOD), an enzyme that catalyzes the conversion of $O_2^{\cdot-}$ into hydrogen peroxide (H_2O_2) and O_2, was investigated. Surprisingly, in the presence of SOD the photoinactivation of tyrosinase by pterin was much more efficient. For instance, air-equilibrated solutions containing tyrosinase and pterin (100 μM) were irradiated in the absence and in the presence of SOD (50 U mL^{-1}) at room temperature and pH 7.0 and, for an irradiation time of 3.5 min, activities of 43 (±3) nM s^{-1} and 17 (±4) nM s^{-1} were determined, respectively. In control experiments performed in the absence of pterin the same activity of tyrosinase was determined for solutions irradiated in the presence and in the absence of SOD, thus excluding the possibility that SOD directly affects the activity of tyrosinase. These results indicate that $O_2^{\cdot-}$ is involved in the photosensitized process in the presence of O_2, provide further evidence for the existence of an electron transfer reaction and suggests that elimination of $O_2^{\cdot-}$ inhibits a step that prevents the photoinduced inactivation of tyrosinase.

Based on the results shown so far and on the general photochemical behavior of pterins described previously, the mechanism summarized in Scheme 1 can be proposed. After UV-A excitation of pterin and formation of its triplet excited state (^{3}Ptr*), three



Scheme 1. Mechanism of the inactivation of tyrosinase (TYR) photosensitized by pterin (Ptr).

reaction pathways compete for the deactivation of the latter: intersystem crossing to singlet ground state, energy transfer to O_2 leading to the regeneration of pterin and the production of $^1O_2$, and electron transfer from tyrosinase to ^{3}Ptr* yielding the corresponding pair of radical ions (Ptr$^{\cdot-}$ and TYR$^{\cdot+}$). In the following step, the electron transfer from Ptr$^{\cdot-}$ to O_2 regenerates pterin and forms $O_2^{\cdot-}$. The reaction of the latter with TYR$^{\cdot+}$ regenerates partially tyrosinase and O_2. Finally, processes that may include reactions of TYR$^{\cdot+}$ with H_2O and O_2 lead to the irreversible inactivation of the enzyme.

Tyrosinase enzyme is able to catalyze two different reactions (*vide supra*): the hydroxylation of monophenols to *o*-diphenols (monophenolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). Both types of reactions are catalyzed by the same active site. Therefore, if the photosensitized process modified significantly the active site of tyrosinase, both activities should be affected. To explore this point, after treatment with radiation in the presence of pterin, the two activities of the enzyme were measured separately.

To determine the diphenolase activity, the experimental procedure was exactly the same as that employed for the determination of the overall activity (L-tyrosine as the substrate), but, in this case, DOPA was used as the substrate. As expected taking into account the kinetic behavior of tyrosinase (see Section 2), the lag period was not observed under these conditions and, for a given sample, the diphenolase activity was higher than that corresponding to the overall activity.

Therefore, air-equilibrated solutions containing the enzyme (*ca* 14 μg/mL) and pterin (95 μM) were irradiated for different periods of time. For each treated sample, the overall activity and the diphenolase activity were determined. Results showed that the diphenolase activity decreased when the enzyme is exposed to UV-A radiation in the presence of pterin. Moreover, the corresponding curve of percentage of the remaining activity plotted against the irradiation time was similar to that obtained for the overall activity (Fig. 2).

The spectrophotometric assay (Material and methods Section) to determine the overall and the diphenolase activities cannot be used to measure selectively the monophenolase activity. Therefore, in this case, the concentrations of L-tyrosine, DOPA and L-dopachrome, determined by high-performance liquid chromatography (HPLC), were followed as a function of the elapsed time after adding L-tyrosine to the sample.

A solution of tyrosinase was irradiated for 10 min in the presence of pterin (100 μM) (as a control, the photoinactivation in this experiment was checked by determining the overall activity by the spectrophotometric assay). L-tyrosine was added to both the irradiated and the control solutions and the concentrations L-tyrosine, DOPA and L-dopachrome were determined at different times by HPLC (Fig. 3). For the irradiated solution the rates of substrate consumption and L-dopachrome formation were much lower than for

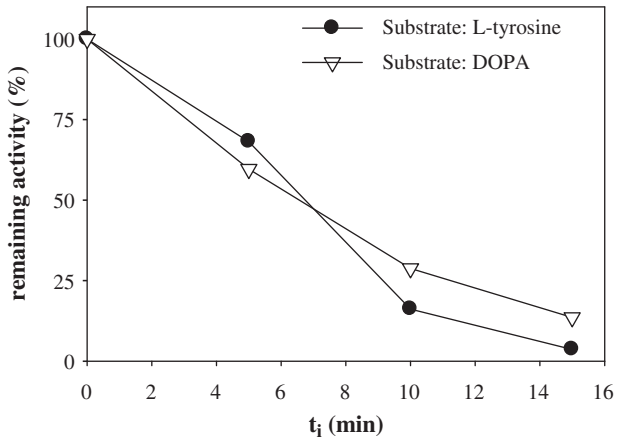


Fig. 2. Determination of the diphenolase activity (substrate: DOPA) and the overall activity (substrate: L-tyrosine) of tyrosinase as a function of time (t_i) in solutions (1 mM KH_2PO_4, pH 6.5) irradiated in the presence of pterin (95 μM) at room temperature. For comparative purposes, the data are expressed as the percentage of the initial enzyme activity. For each sample the formation of L-dopachrome was followed measuring the absorbance at 475 nm as a function of the time elapsed after adding the substrate (37 °C).

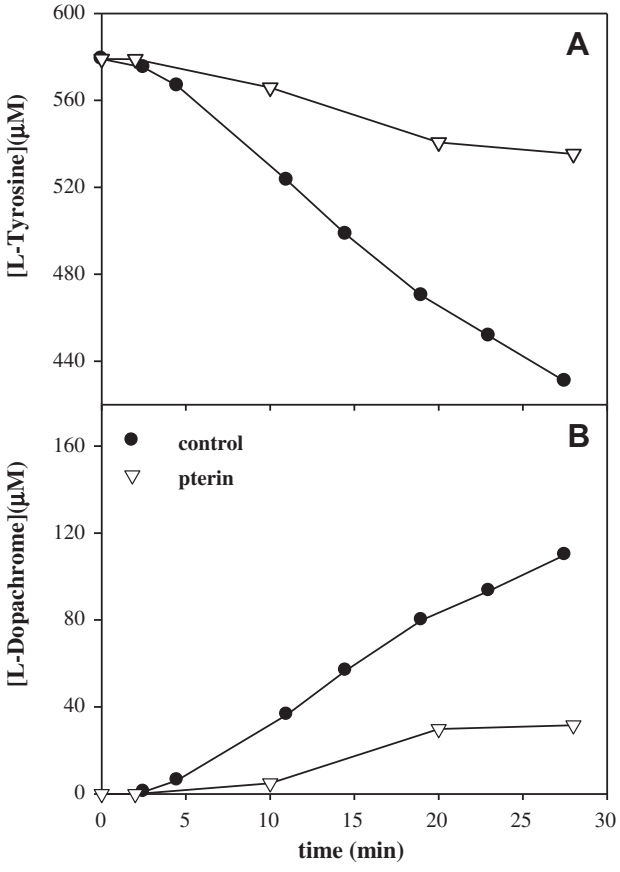


Fig. 3. Evaluation of the monophenolase activity in solutions of tyrosinase irradiated in the absence (control) and in the presence of pterin (100 μM). Evolution of (A) L-tyrosine and (B) L-dopachrome concentrations as a function of the elapsed time after addition of L-tyrosine to the sample. Irradiation time 10 min, pH 6.5, concentrations were determined by HPLC analysis.

the control (tyrosinase solution irradiated in the absence of pterin). For both samples no DOPA was detected. These results reveal that, as expected, the monophenolase activity is also strongly affected by the treatment with UV-A radiation in the presence of pterin.

The results presented in this work unequivocally demonstrate for the first time that tyrosinase is inactivated by pterin through a photosensitized process and suggest that such a process is initiated by an electron transfer reaction from the enzyme to the triplet excited state of pterin. The photochemical process affects both the monophenolase and the diphenolase activities of tyrosinase. Taking into account that pterins are present in the human skin and that, in particular, they accumulate in regions were the protection against the UV radiation fails, the inactivation of the enzyme that catalyzes the first and rate-limiting step of melanin biosynthesis photoinduced by pterins is relevant from a biomedical point of view and can have important ramifications. Finally, most of the studies on photosensitizing properties of photoactive endogenous biological compounds are performed using nucleic acids or their components as targets. However, the results of the present work show that other macromolecules such as enzymes should also be considered as relevant targets of photosensitized processes.

Acknowledgments

The present work was funded by CONICET (Grant PIP 112-200901-00425), ANPCyT (Grants PICT 33919 and PICT 06-01482), and UNLP (Grant X586). A.D.G. thanks CICPBA for a fellowship for undergraduate students. M.L.D. and A.H.T. are research members of CONICET.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.155.

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