

Indirect oxidation of 6-tetrahydrobiopterin by tyrosinase

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Received 17 November 2003

Abstract

6-Tetrahydrobiopterin is known to bind to an allosteric site of tyrosinase to directly inhibit the enzyme. However, simultaneous measurements of ultraviolet–visible absorption spectra and oxygen consumption led us to conclude that the inhibition was due to oxidation of 6-tetrahydrobiopterin by dopaquinone. Immediately after addition of 6-tetrahydrobiopterin, tyrosinase stopped producing dopachrome from either tyrosine or dopa. Duration of inhibition was proportional to the concentration of added 6-tetrahydrobiopterin and the enzyme activity was fully restored after the inhibition. Surprisingly, there was a rapid consumption of oxygen during the inhibition period. In addition, absorption spectra indicated that the only reaction that occurred during the inhibition was oxidation of 6-tetrahydrobiopterin to 7,8-dihydrobiopterin. In the absence of tyrosine or dopa, tyrosinase did not oxidize 6-tetrahydrobiopterin, suggesting that a reaction intermediate between dopa and dopachrome was a target for the inhibition. We propose a new mechanism in which dopa is oxidized to dopaquinone and the latter, instead of producing dopachrome, is reduced back to dopa by 6-tetrahydrobiopterin.

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Keywords: Tyrosinase; 6-Tetrahydrobiopterin; Dopaquinone; Inhibition

Tyrosinase plays a pivotal role in melanogenesis by oxidizing tyrosine and dopa to dopaquinone (see [1] for review). Dopaquinone disproportionates via cyclodopa to dopa and dopachrome, the absorption of the latter at 480 nm being used in the activity measurements. The enzyme contains copper ions in the catalytic site and uses molecular oxygen as the oxidant [2]. Being an enzyme involved in a rate-determining step of melanogenesis, tyrosinase is likely to have a regulatory mechanism at the enzyme level. However, allosteric effector molecules that control the enzyme activity are yet to be identified [3,4].

6-Tetrahydrobiopterin (6-BH₄) is a redox-active cofactor of several enzymes including phenylalanine hydroxylase [5] and tyrosine hydroxylase [6]. These enzymes are mixed function oxidases that insert an oxygen atom of O₂ into the substrate. In the reactions, 6-BH₄ is oxidized to q-dihydrobiopterin via BH₄-4a-carbinolamine. q-Dihydrobiopterin is reduced back to 6-BH₄ by dihydrobiopterin reductase completing the regeneration cycle (see [7] for review). Failure in the

recycling system has been claimed as a cause for depigmentation of the skin [8,9].

Wood et al. [10] proposed another role for 6-BH₄ in melanogenesis: 6-BH₄ binds to an allosteric site of tyrosinase to directly inhibit the enzyme. Only reduced biopterins such as 6-BH₄ and 7-BH₄ were effective when tyrosine, but not dopa, was the substrate. In addition, unlike phenylalanine hydroxylase and tyrosine hydroxylase, tyrosinase did not appear to oxidize 6-BH₄. The enzyme activity could be restored by UVA irradiation which photooxidizes 6-BH₄ to 7,8-BH₂ [11]. Interestingly, tyrosinase was reactivated by α -melanocyte-stimulating hormone [12] which forms a tight complex with 6-BH₄ [13], making the latter dissociate from the enzyme. Recently Marles et al. [14] identified tyrosine hydroxylase isoenzyme I in the melanosomal membrane which also binds tyrosinase. This opens a possibility of a coupled interaction between tyrosine hydroxylase and tyrosinase. If 6-BH₄, a cofactor of tyrosine hydroxylase, interacts with tyrosinase, it may function as a regulator of melanogenesis.

In agreement with Wood et al. [10], we observed inhibition of tyrosinase by 6-BH₄. However, we found

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that 6-BH₄ was efficiently oxidized by tyrosinase during turnover instead of functioning as an allosteric inhibitor. The measurements of oxygen consumption and absorption spectra led us to propose an alternative mechanism for the inhibition of tyrosinase by 6-BH₄ in which dopaquinone is reduced back to dopa by 6-BH₄.

Materials and methods

Materials. Mushroom tyrosinase was obtained from Sigma (St. Louis, MO, USA). 6-BH₄ and other pterin derivatives were purchased from Schirks Laboratories (Jona, Switzerland). All other chemicals were from Sigma. Buffer solutions (0.1 M potassium phosphate, pH 7.4) were treated with Chelex-100 to remove metal ions. Diethylenetriaminepentaacetate (0.5 mM) was also included in the buffer solutions to chelate residual metal ions. Stock solutions of 6-BH₄ and pterin derivatives were prepared in 0.1 M HCl.

UV-Visible absorption spectra. Buffer was filled up to the top of a magnetically stirred cuvette fitted with a screw cap. No air was allowed in the cuvette so that the reaction system uses only the water-dissolved oxygen to oxidize a substrate. A small volume of the substrate (tyrosine or dopa) was injected into the cuvette to a final concentration of 100 μM. The reaction was initiated by adding tyrosinase (50 U/ml final concentration) using a syringe. 6-BH₄ (100 μM) was also added when necessary. The reaction was followed in real time using a UV-Vis absorption spectrophotometer equipped with a photodiode array detector (Hewlett-Packard Model 8453).

Measurements of oxygen consumption. Samples were prepared in duplicate to measure both absorption spectra and oxygen consumption. Again the sample chamber of an oxygen electrode (Rank Brothers, Digital Oxygen System Model 10) was filled up to the top to keep air from contacting the reaction solution. Electrical signals from the oxygen electrode were processed with an AD converter which transferred digital data to a personal computer. All the absorption and oxygen consumption measurements were carried out at 25 °C.

Results

Wood et al. [10] reported that 6-BH₄ inhibited both human and mushroom tyrosinase by binding to an allosteric site. We repeated their experiments with mushroom tyrosinase using a photodiode array spectrophotometer, instead of a conventional scanning instrument, to follow the reactions in real time at all wavelengths. In addition, oxygen consumption during the reaction was simultaneously measured using an oxygen electrode, which provided crucial information for elucidation of the inhibition mechanism.

Inhibition of tyrosinase by 6-BH₄ when dopa is the substrate

Enzymatic conversion of dopa, a substrate of tyrosinase, to dopachrome was measured by the unique absorption of dopachrome at 480 nm as shown in Fig. 1. In the absence of 6-BH₄ (solid line), tyrosinase (50 U/ml) converted 100 μM dopa to dopachrome in ~500 s. When 100 μM 6-BH₄ was included in the reaction mixture at

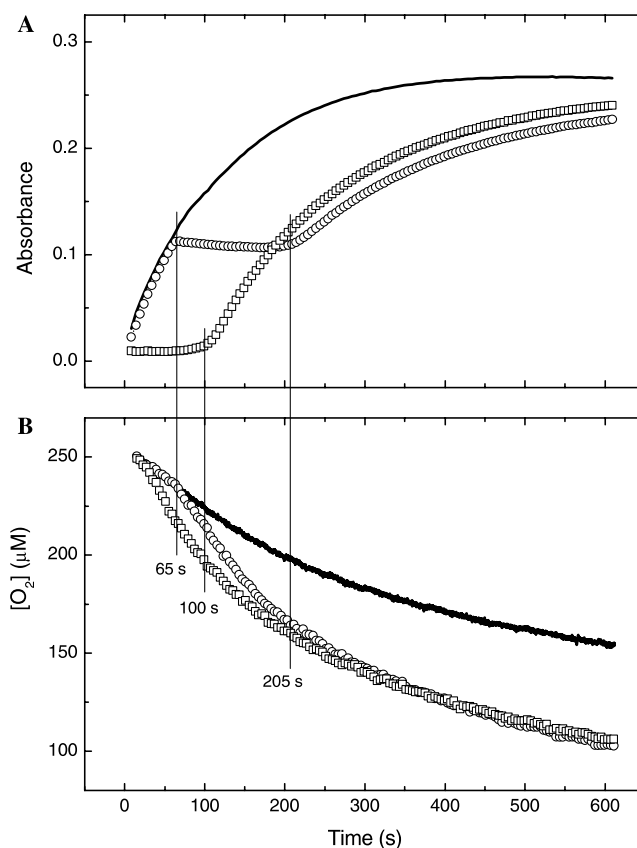


Fig. 1. Dopachrome formation by tyrosinase using dopa as the substrate is inhibited by 6-BH₄. Dopa (100 μM) was oxidized to dopachrome by tyrosinase (50 U/ml) in the absence of 6-BH₄ (solid lines). Absorption at 480 nm (A) and decrease in O₂ concentration (B) were measured for identical samples. Same measurements were repeated with 6-BH₄ (100 μM) added at the beginning ($t = 0$ s, squares) and in the middle ($t = 65$ s, circles) of the reaction. The time points 100 and 205 s refer to the end of inhibition period when 6-BH₄ was added at $t = 0$ and 65 s, respectively.

the beginning (squares), dopachrome was not produced for the first ~100 s and thereafter the enzyme activity was almost fully restored. If 6-BH₄ was added in the middle of the reaction (at $t = 65$ s, circles), the inhibition period lasted slightly longer (~140 s). In both cases, the duration of inhibition increased with the concentration of 6-BH₄ (not shown).

We also monitored the corresponding oxygen consumption rates as shown in Fig. 1B. Tyrosinase consumed ~100 μM of O₂ for complete oxidation of 100 μM dopa (solid line). This stoichiometry of 1:1 for dopa:O₂ is consistent with a previously proposed mechanism [15]. When 100 μM 6-BH₄ was present from the start (squares), the oxygen consumption rate was even faster than that in the absence of 6-BH₄. A similar result was obtained when 6-BH₄ was added in the middle of the reaction (circles). If 6-BH₄ inhibited tyrosinase by binding to an allosteric site of the enzyme as Wood et al. [10] suggested, no oxygen should be consumed during the inhibition period. In other words, the mechanism

proposed by Wood et al. is not consistent with the result we obtained from the oxygen consumption measurements.

Inhibition of tyrosinase by 6-BH₄ when tyrosine is the substrate

Fig. 2 shows the same measurements as those in Fig. 1 except that tyrosine was used as a substrate instead of dopa. Formation of dopachrome from tyrosine was much slower than that from dopa (compare the solid lines in Figs. 1 and 2) with a lag phase. Complete conversion of 100 μM tyrosine to dopachrome consumed $\sim 150 \mu\text{M}$ O₂, in agreement with a previous report [15].

When 100 μM 6-BH₄ was added at the beginning to the reaction mixture containing tyrosinase and tyrosine (Fig. 2A, squares), dopachrome formation was completely inhibited for ~ 310 s, which is significantly longer than the corresponding value (~ 100 s) for an experiment

with dopa as the substrate (see Fig. 1A). This can be accounted for by the fact that tyrosine must be converted to dopa before dopa can be oxidized to dopaquinone. Small amount of dopa thus formed is then oxidized to dopaquinone, which in turn is reduced back to dopa by 6-BH₄. Therefore, when tyrosine was the substrate, the slower formation of dopaquinone was responsible for the longer inhibition period. Similar results were obtained when 6-BH₄ was added in the middle of the reaction (Fig. 2A, circles). The inhibition lasted for 270 s, which is comparable to the value for the initial addition of 6-BH₄ (310 s) if the lag phase (~ 40 s) is taken into consideration.

When 6-BH₄ was added at the beginning (Fig. 2B, squares), $\sim 60 \mu\text{M}$ O₂ was consumed before 6-BH₄ was exhausted. About 10 μM of O₂ was used in the lag phase (i.e., the first 40 s in Fig. 2B), in which inactive met form is converted to active oxytyrosinase [16]. This stoichiometry of 1:0.5 for 6-BH₄:O₂ implies that the slow production of dopa from tyrosine and subsequent oxidation of dopa to dopaquinone were the only pathways that oxidized 6-BH₄ during the inhibition period. We obtained similar results when 6-BH₄ was added in the middle of the reaction (Fig. 2B, circles).

Interaction of 6-BH₄ with a turnover intermediate

Oxygen consumption during the inhibition of dopachrome formation (Figs. 1 and 2) prompted us to consider the following two alternatives for the inhibitory mechanism. First, 6-BH₄ is a better substrate than dopa so that tyrosinase selectively oxidizes 6-BH₄ using O₂ during the inhibition period. Once 6-BH₄ is exhausted, the enzyme starts to oxidize dopa. Second, 6-BH₄ reacts with an intermediate (dopaquinone, for example) that leads to the formation of dopachrome. We tested these two possibilities.

In Fig. 3A, we monitored the absorption of 6-BH₄ (297 nm), dopa (283 nm), and dopachrome (480 nm) as 6-BH₄ ($t = 0$ s), tyrosinase ($t = 80$ s), and dopa ($t = 160$ s) were added sequentially. The corresponding rates of oxygen consumption are shown in Fig. 3B. 6-BH₄ alone decayed slowly due to autoxidation ($t = 0$ –80 s). Addition of tyrosinase, however, did not significantly alter either the rate of 6-BH₄ oxidation ($t = 80$ –160 s) or the oxygen consumption rate, indicating that 6-BH₄ is not a substrate for tyrosinase. This excludes the first possibility mentioned above.

Subsequent addition of dopa at 160 s greatly enhanced both the rate of 6-BH₄ oxidation (Fig. 3A, solid line) and oxygen consumption (Fig. 3B). 6-BH₄ did not react with dopa in the absence of tyrosinase (not shown), suggesting that an intermediate in the reaction of dopa and tyrosinase is involved. In addition, either oxidation of dopa (dash-dotted line) or formation of dopachrome (dashed line) was observed during this period.

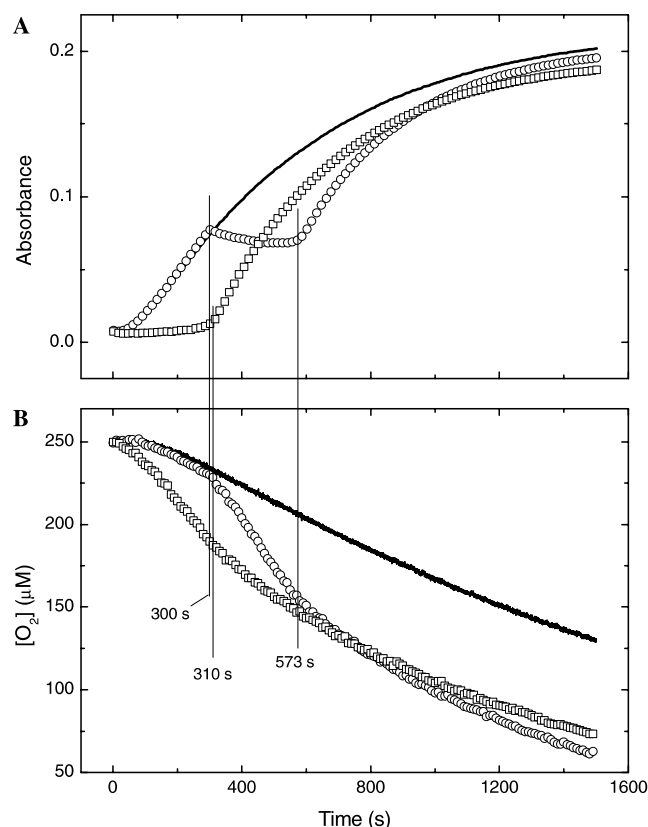


Fig. 2. Dopachrome formation by tyrosinase using tyrosine as the substrate is inhibited by 6-BH₄. Tyrosine (100 μM) was oxidized to dopachrome by tyrosinase (50 U/ml) in the absence of 6-BH₄ (solid lines). Absorbance at 480 nm (A) and decrease in O₂ concentration (B) were measured for identical samples. Same measurements were repeated with 6-BH₄ (100 μM) added at the beginning ($t = 0$ s, squares) and in the middle ($t = 300$ s, circles) of the reaction. The time points 310 and 573 s represent the end of inhibition period when 6-BH₄ was added at $t = 0$ and 300 s, respectively.

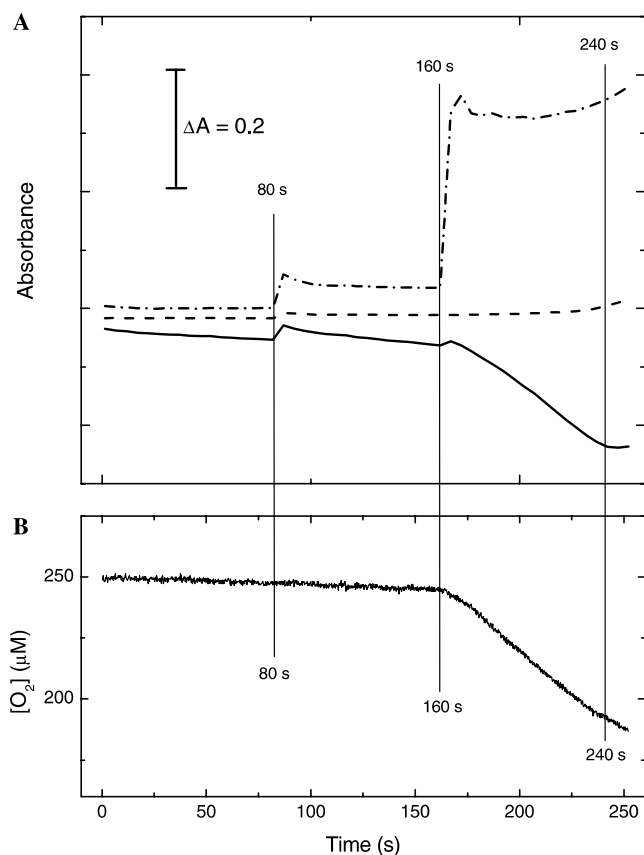


Fig. 3. BH_4 is not directly oxidized by tyrosinase but by a turnover intermediate. 6- BH_4 (100 μM) was added to a buffer solution at $t = 0$ s and allowed to stand to confirm that autoxidation is not significant. At $t = 80$ s tyrosinase (50 U/ml) was added but 6- BH_4 was not oxidized. Upon addition of dopa (100 μM) at $t = 160$ s, a rapid oxidation of 6- BH_4 was observed until 6- BH_4 was exhausted and the enzyme restarted to produce dopachrome ($t = 240$ s). (A) Absorption at 297 nm (solid line), 480 nm (dashed line), and 283 nm (dash-dotted line) for the measurement of 6- BH_4 , dopachrome, and dopa, respectively. (B) Corresponding O_2 consumption curve.

The data in Fig. 3 suggest that an intermediate between dopa and dopachrome reacted with 6- BH_4 . A good candidate for the intermediate is dopaquinone. If dopaquinone is reduced back to dopa by 6- BH_4 , the concentration of dopa would be kept constant and dopachrome, which is produced by disproportionation of two molecules of dopaquinone, would not be formed. However, 0.5 mol O_2 is needed for the oxidation of 1 mol dopa to dopaquinone [15] so that 50 μM O_2 would be consumed in the oxidation of 100 μM 6- BH_4 by dopaquinone. Indeed, as shown in Fig. 3B, 50 μM O_2 was consumed during the inhibition period ($t = 160$ –240 s). Therefore, the mechanism given in Scheme 1 is fully consistent with these observations.

Oxidation of 6- BH_4 during the inhibition period

We next measured UV–Vis absorption spectra at various times during the reactions. As shown above in

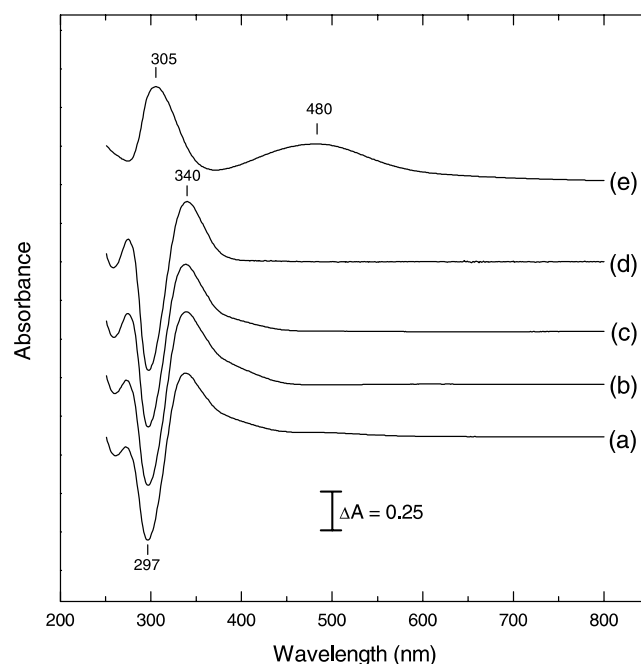
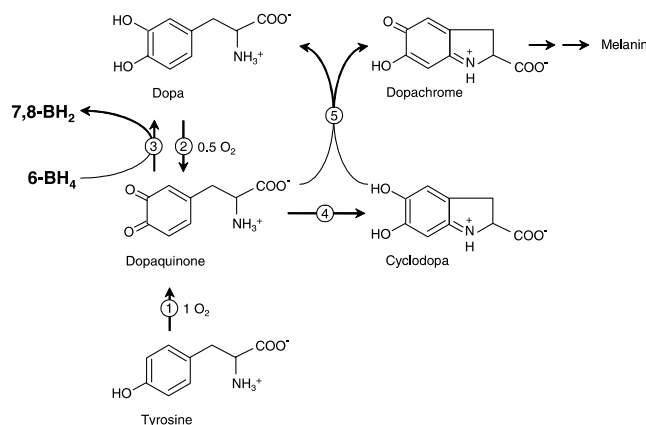


Fig. 4. Difference absorption spectra demonstrate that only 6- BH_4 was oxidized during the inhibition period. Difference spectra were calculated from the spectra corresponding to (a) 100 and 0 s of Fig. 2 (squares), (b) 205 and 65 s of Fig. 2 (circles), (c) 300 and 0 s of Fig. 4 (squares), and (e) 500 and 0 s of Fig. 1 (solid line). Spectrum (d) represents a genuine difference spectrum of 6- BH_4 minus 7,8- BH_2 , which is nearly identical to (a–c), suggesting that during inhibition only 6- BH_4 was oxidized without consumption of other reactants in the reaction mixture.

Fig. 1, the enzyme was inhibited at $t = 0$ –100 s and $t = 65$ –205 s when 6- BH_4 was added at the beginning and in the middle of the reaction, respectively. We measured the spectra at these time points and calculated difference spectra to obtain net changes during the inhibition period. Fig. 4A is a net spectral change between the end and beginning of the inhibition when dopa was added at the beginning. A similar result was obtained when dopa was added in the middle (Fig. 4B) or tyrosine was the substrate replacing dopa (Fig. 4C, see also Fig. 2). The spectrum is almost identical to an authentic difference spectrum of oxidized and reduced bipterins, i.e., 7,8- BH_2 minus 6- BH_4 (Fig. 4D). There was no other significant change in the spectra suggesting that during the inhibition only 6- BH_4 was oxidized without forming dopachrome or consuming dopa. For comparison, we also presented the spectra of dopachrome minus dopa (Fig. 4E).

A mechanism for the 6- BH_4 oxidation by tyrosinase in turnover

Based on the results discussed above, we propose a mechanism for the indirect oxidation of 6- BH_4 by tyrosinase (Scheme 1). Tyrosinase oxidizes dopa to dopaquinone (Reaction 2). Then two molecules of



Scheme 1. A proposed mechanism for the inhibition of tyrosinase by 6-BH₄ based on this study. Tyrosinase oxidizes dopa to dopaquinone with 0.5 mol O₂ (Reaction 2). Two molecules of dopaquinone then disproportionate (via cyclodopa formation; Reaction 3) to dopa and dopachrome (Reaction 5). In the presence of 6-BH₄, however, dopaquinone is reduced back to dopa by 6-BH₄ (Reaction 3). Our data indicate that Reaction 3 is much faster than Reaction 4, whose rate constant was determined recently to be 3.8 s⁻¹ [17].

dopaquinone (via cyclodopa) disproportionate to one molecule each of dopa and dopachrome (Reaction 5) in which the cyclization step (Reaction 4) is rate-limiting with a rate constant of 3.8 s⁻¹ [17]. In the absence of 6-BH₄ dopachrome is used as a precursor for melanin synthesis. When 6-BH₄ is present, dopaquinone is reduced back to dopa by 6-BH₄ (Reaction 3). Since the dopa concentration was kept constant and no dopachrome was produced, Reaction 3 must be much faster than Reactions 2 and 4.

Discussion

According to Wood et al. [10], 6-BH₄ inhibits tyrosinase directly by binding to an allosteric site. This means that tyrosinase is inhibited indefinitely as long as 6-BH₄ is bound to the enzyme. In addition, their mechanism predicts that no O₂ should be consumed during the inhibition. We observed, however, that the enzyme was inhibited for a limited time and thereafter it became fully active. Moreover, the inhibition accompanied rapid consumption of O₂. This led us to propose an alternative mechanism for the inhibition of tyrosinase by 6-BH₄ as summarized in Scheme 1.

Although not specified in their papers [10,11], it is likely that they used a conventional scanning spectrophotometer for the activity assays. According to the method described in [10], they incubated tyrosinase and a substrate for a fixed period of time (3 min) and the activity was measured by increase in the absorption at 475 nm. As our results indicate, the enzyme activity is restored once all the added 6-BH₄ is oxidized. Therefore, one can readily expect that the enzyme activity mea-

sured after a fixed time of incubation would decrease with the concentration of 6-BH₄ if the incubation time is longer than the time for 6-BH₄ to be exhausted. This is probably why they observed a dose-dependent decrease in the enzyme activity. Our mechanism is, however, supported by the oxygen consumption measurements and spectroscopic identification of reaction products, both of which were not included in their work.

We also tested a few structurally related compounds for the inhibitory effect (not shown). Tetrahydrofolate and 5-methyltetrahydrofolate, both containing a fully reduced pterin ring like 6-BH₄, were ineffective. 7,8-BH₂, a two-electron oxidized form of 6-BH₄, was not an inhibitor, either. A racemic mixture of *R*- and *S*-forms of 6-BH₄ was as effective as the pure *R*-form. If 6-BH₄ binds to an allosteric site which usually possesses stereospecificity, a racemic mixture would be half as effective as the pure *R*-form. A simple chemical reaction between 6-BH₄ and dopaquinone would be much less sensitive to the stereochemistry of 6-BH₄ in support of our observation.

In general, the duration of inhibition was longer when 6-BH₄ was added in the middle than at the beginning of the reaction because significant amount of dopa was consumed prior to the 6-BH₄ addition. The lower dopa concentration makes the dopaquinone production slower and makes the 6-BH₄ oxidation last longer. Slightly more O₂ was consumed when 6-BH₄ was added in the middle of the reaction (70 vs 50 μM). This is probably due to oxidation of preformed dopachrome to downstream compounds [15].

Dopaquinone, an o-quinone, is a very reactive species. It undergoes an internal cyclization with a rate constant of 3.8 s⁻¹ [17] to form cyclodopa (Reaction 4 in Scheme 1). Nucleophilic addition of a cysteine residue produces a cysteinyl-dopa, a precursor in pheomelanin synthesis (see [18] for review). On the other hand, dopaquinone is a strong oxidant that can oxidize other redox-active species, as exemplified by 6-BH₄ oxidation in this work. Concomitant reduction of dopaquinone prevents dopachrome formation and the overall reaction appears to be inhibited. A similar mechanism of tyrosinase inhibition was reported by Gasowska et al. [19] in which amino-(3,4-dihydroxyphenyl)methyl phosphonic acid reduced dopaquinone.

Tyrosinase uses both tyrosine and dopa as the substrate. However, dopa can be synthesized by tyrosine hydroxylase which oxidizes tyrosine to dopa. Since the inactive mettyrosinase is activated by dopa [16,20], tyrosine hydroxylase may accelerate melanogenesis by activating tyrosinase. In this regard, Marles et al. [14] reported recently that both tyrosinase and tyrosine hydroxylase isoenzyme I colocalize in the melanosomal membrane. This implicates a possible interaction between the two enzymes. In the present work, 6-BH₄ was oxidized by tyrosinase in turnover. Since 6-BH₄ is

required for the enzyme activity of tyrosine hydroxylase, it is tempting to postulate that tyrosinase downregulates tyrosine hydroxylase by a feedback mechanism: if tyrosine hydroxylase produces too much dopa, then tyrosinase will oxidize 6-BH₄ and the tyrosine hydroxylase activity will decrease. This hypothesis needs to be tested in future studies.

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