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Redox reaction between amino-(3,4-dihydroxyphenyl)methyl phosphonic acid and dopaquinone is responsible for the apparent inhibitory effect on tyrosinase

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Amino-(3,4-dihydroxyphenyl)methyl phosphonic acid, the phosphonic analog of 3,4-dihydroxyphenylglycine, had been previously reported as a potent inhibitor of tyrosinase. The mechanism of the apparent enzyme inhibition by this compound has now been established. Amino-(3,4-dihydroxyphenyl)methyl phosphonic acid turned out to be a substrate and was oxidized to *o*-quinone, which evolved to a final product identified as 3,4-dihydroxybenzaldehyde, the same as for 3,4-dihydroxyphenylglycine. Monohydroxylated compounds (amino-(3-hydroxyphenyl)methyl phosphonic acid and amino-(4-hydroxyphenyl)methyl phosphonic acid) were not oxidized, neither was 4-hydroxy-L-phenylglycine. However, the relatively high K_m for amino-(3,4-dihydroxyphenyl)methyl phosphonic acid (0.52 mM) indicated that competitive inhibition could not entirely explain the previously reported strong inhibitory effect (K_i = 50 and 97 μM for tyrosine and 3-(3,4-dihydroxyphenyl)alanine (Dopa) as substrates, respectively). Neither was the enzyme covalently inactivated to a significant degree. Spectroscopic and electrochemical analysis of the oxidation of a mixture of Dopa and the inhibitor demonstrated that the phosphonic compound reduced dopaquinone back to Dopa, thus diminishing and delaying the formation of dopachrome. This produces an apparent strong inhibitory effect when the reaction is monitored spectrophotometrically at 475 nm. In this peculiar case Dopa acts as a redox shuttle mediating the oxidation of the shorter phosphonic homolog. Decomposition of the phosphonic *o*-quinone to 3,4-dihydroxybenzaldehyde drives the reaction against the slightly unfavorable difference in redox potentials.

Keywords: tyrosinase; redox exchange; quinone; phosphonic amino acids; 3,4-dihydroxybenzaldehyde.

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme widely distributed in nature. It catalyses the hydroxylation of monophenols to *o*-diphenols and the oxidation of the latter to *o*-quinones using molecular oxygen. Its action on the physiological substrate, L-tyrosine, produces L-3-(3,4-dihydroxyphenyl)alanine (L-Dopa) and then dopaquinone, which undergoes a series of nonenzymatic reactions leading to melanins [1]. The enzyme is responsible for melanization in animals and browning in plants. As browning in food products is an undesirable process, there has been a constant need in food industry for compounds preventing this reaction. Inhibition of mammalian tyrosinase has also been indicated as a possible approach to control human melanoma [2]. Although a large number of tyrosinase inhibitors have been described in the literature [3], the search for new natural products and synthetic compounds with such activity still continues [4]. Some of the most potent, competitive inhibitors include mimosine [5,6], tropolone [7,8], and kojic acid [9–12]. Some of us had previously shown that amino-(3,4-dihydroxyphenyl)methyl phosphonic acid, the phosphonic analog of 3,4-dihydroxyphenylglycine, was also a potent inhibitor of tyrosinase [13]. With a K_i of 50 and 97 μM for tyrosine and Dopa as substrates, respectively, it was comparable with mimosine and tropolone. The mechanism of action of this compound was not investigated at the time. However, recent demonstration that 3,4-dihydroxyphenylglycine serves as a substrate for tyrosinase [14] prompted us to test whether the phosphonic analog is also metabolized by this enzyme. Tyrosinase converts 3,4-dihydroxyphenylglycine to 3,4-dihydroxybenzaldehyde via spontaneous decarboxylation of the enzymatically generated *o*-quinone [14]. We predicted that, if the phosphonic analog served as a substrate, the P–C bond would be resistant to cleavage and the *o*-quinone would either undergo a standard nucleophilic attack on the ring or decompose by some unusual pathway leading to covalent inactivation of the enzyme. Neither of these assumptions turned out to be true. We now show that the inhibitory effect observed spectrophometrically does not result from the interaction of this compound with the enzyme but arises primarily from the reduction of dopaquinone by the shorter phosphonic diphenol.

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Abbreviations: Dopa, 3-(3,4-dihydroxyphenyl)alanine; DPV, differential pulse voltammetry; HMDE, hanging mercury drop electrode; SCE, saturated calomel electrode.

Enzyme: tyrosinase (EC 1.14.18.1).

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MATERIALS AND METHODS

Chemicals

Mushroom tyrosinase (specific activity 5584 U·mg^{-1}), 3-(3,4-dihydroxyphenyl)-L-alanine (L-Dopa), and 4-hydroxy-L-phenylglycine were purchased from Fluka. Catechol was from Sigma, 3,4-dihydroxybenzaldehyde and substrates for synthesis were purchased from Aldrich. D_2O was from Dr Glaser AG (Basel, Switzerland). All other reagents were from local suppliers and were of analytical grade.

Synthesis of 1-aminophosphonic acids

The synthesis was performed according to the methodology described by Soroka [15]. Acetyl chloride (0.05 mol) was added dropwise to a vigorously stirred mixture of 0.1 mol of acetamide dissolved in 20 mL of acetic acid at 0 °C. After 15 min, 0.05 mol of an appropriate aldehyde (3-methoxy-, 4-methoxy- or 3,4-dimethoxybenzaldehyde) was added. The reaction mixture was stirred for 0.5 h at 0 °C, for 4 h at room temperature, then cooled again to 0 °C and 0.05 mol of PCl_3 was added. The mixture was heated and refluxed for 2 h to complete the reaction. Acetic acid was removed by rotary evaporation and the oily residue was refluxed for 10 h in 8 M HCl. The mixture was evaporated under reduced pressure and the residue was dissolved in ethanol. Precipitated ammonium chloride was filtered off. The filtrate was treated slightly with propylene oxide until the pH reached 6. The precipitated 1-aminophosphonic acid was filtered off, washed with ethanol and re-crystalized from water. Deprotection of the methoxy groups was performed by refluxing in HI for 6 h. Precipitation with propylene oxide and re-crystalization from water gave the final product (compounds 1–3, Fig. 1).

Spectrophotometric analysis

Enzymatic reactions were routinely carried out at room temperature in 2.6 mL of 100 mM sodium phosphate buffer, pH 6.8, containing 20 μg of tyrosinase and 0.1 mM of substrates. Spectrophotometric measurements were performed either in Specord M40 (Carl Zeiss, Jena, Germany) or Beckman DU 640B UV/Vis spectrophotometers. When phosphonic analogs of phenylglycine were tested as substrates, the reactions were monitored for 90 min and spectra from 250 to 600 nm were recorded at 3 min intervals at 1200 nm·min^{-1}. When the reaction of a mixture of compound 1 and Dopa was analyzed, spectra were recorded every 40 s. Each of the substrates was also tested separately under the same conditions.

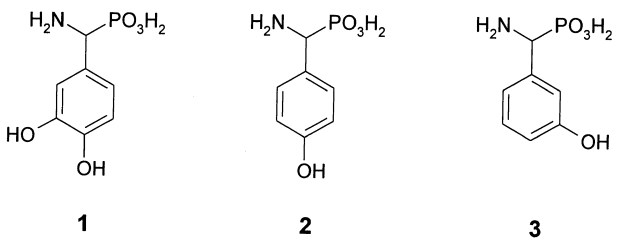

Fig. 1. Phosphonic amino acids used in this study.

K_m and V_{max} for compound 1 were determined graphically from Lineweaver–Burk plots. Reactions were monitored at 320 nm with substrate concentration from 0.1 to 1.2 mM. The extinction coefficient for 3,4-dihydroxybenzaldehyde was determined as 9685 M^{-1}·cm^{-1}.

To test the extent of suicide inactivation of the enzyme, 50 μg of tyrosinase was incubated in 1 mL of 100 mM phosphate buffer with 1.5 mM Dopa, catechol or compound 1 for 1 h and then the high- and low-molecular mass components were separated by gel filtration on a 15 mL Sephadex G-25 column. Protein containing fractions were pooled and used in enzymatic assays with 0.5 mM Dopa. Dopachrome formation was monitored at 475 nm.

Chemical oxidation of amino-(3,4-dihydroxyphenyl) methyl phosphonic acid was also performed in 100 mM sodium phosphate buffer either at pH 6.8 or 8.0 with a stoichiometric amount of sodium periodate (0.1 mM). Spectra were recorded every 40 s.

Identification of the oxidation product of amino-(3,4-dihydroxyphenyl)methyl phosphonic acid

Chemical oxidation of amino-(3,4-dihydroxyphenyl)methyl phosphonic acid with stoichiometric amount of sodium periodate was performed in 1 mL of D_2O at 25 mM concentration of the substrates. ^1H and ^{31}P NMR spectra were recorded directly from the reaction mixture 5 and 30 min after addition of $NaIO_4$ on a Bruker Avance™ DRX 300 MHz NMR spectrometer. For identification of the product of enzymatic oxidation 10 mg of the substrate (1.1 mM) was incubated with 700 μg of tyrosinase in 40 mL of 100 mM sodium phosphate buffer, pH 6.8, for 4 h with vigorous stirring. The reaction was stopped by addition of trichloroacetic acid to a final concentration of 5% and centrifuged at 12 000 ***g*** for 5 min. The supernatant was extracted twice with ethyl acetate and the solvent was evaporated under vacuum. The residual trichloroacetic acid and acetic acid released from the solvent were neutralized with NaOD, the volume of the sample was brought up to $\approx$1 mL with D_2O and the proton NMR spectrum was recorded. The spectrum of the commercial 3,4-dihydroxybenzaldehyde was taken under the same conditions.

Polarographic analysis

Cathodic voltammetry was performed with a pulse polarograph PP-04 (Unitra Telpod, Kraków, Poland) with digital data acquisition. The measurements were performed using a two-electrode measuring system: saturated calomel electrode (SCE) as the reference and hanging mercury drop electrode (HMDE) as the recording electrode. Voltammetric curves were recorded in the potential range from 0.0 to −2.0 V. Measurements were performed with the DPV technique using the following parameters: amplitude of pulses (ΔE) = 20 mV, pulse duration time (t_p) = 40 ms, potential speed (v) = 0.025 V·s^{-1}, mercury drop area (S) = 0.0326 cm^2. Cyclic voltammetry was performed with a multifunctional electrochemical device EMU (constructed at the Institute of Physical and Theoretical Chemistry, Wrocław University of Technology). Cyclic voltammograms were recorded in the potential range from −0.4 to +0.9 V using a three-electrode system: silver-saturated silver chloride electrode as the reference, platinum wire

electrode as the recording electrode and graphite electrode as the auxiliary electrode. Redox potentials for Dopa and amino-(3,4-dihydroxyphenyl)methyl phosphonic acid were estimated after semilogarithmic conversion of data from intersection points of oxidation and reduction curves. To improve the accuracy, the cathodic current was corrected by multiplying it by factor $\alpha = i_{pA}/i_{pC}$ (maximum anodic current/maximum cathodic current).

Oxygen consumption measurements

Measurements were performed with a multifunctional electrochemical device CX-551 (ELMETRON, Zabrze, Poland) equipped with an oxygen (Clark-type) sensor CTN-9202 (ELSENT, Wrocław, Poland) connected to a microcomputer. The sensor was calibrated according to manufacturer's instruction using a two-point method with saturated sodium sulfite solution (0% point) and air-saturated distilled water (100% point). All measurements were corrected for buffer concentration, temperature, and actual barometric pressure. Reactions were carried out in 9.0 mL of 100 mM sodium phosphate buffer, pH 6.8, with 0.1 mM of substrates and 69 μg of the enzyme to maintain conditions identical to spectrophotometric assays.

RESULTS

Our screening of phosphonic analogs of aromatic amino acids as tyrosinase inhibitors demonstrated that amino-(3,4-dihydroxyphenyl)methyl phosphonic acid (compound 1, Fig. 1) was at least an order of magnitude more potent than other compounds [13,16]. The striking difference in activity between this compound and the monohydroxylated derivatives [amino-(4-hydroxyphenyl)methyl phosphonic acid (compound 2) and amino-(3-hydroxyphenyl)methyl phosphonic acid (compound 3)] was particularly intriguing. We have therefore tested these compounds as substrates for tyrosinase. We have found that compound 1 was indeed metabolized by the enzyme, whereas the monohydroxylated derivatives were not (Fig. 2). We have also tested 4-hydroxy-L-phenylglycine, which was not metabolized, either (data not shown). Oxidation of compound 1, either by tyrosinase or sodium periodate, generated a product with an absorption maximum at 320 nm, closely resembling the UV spectrum of 3,4-dihydroxybenzaldehyde [14]. No such spectroscopic changes were observed for compounds 2 and 3 even after 24 h of incubation with tyrosinase. When the reactions were monitored by oxygen consumption, a substantial drop in oxygen concentration was also registered only for the diphenolic derivative (compound 1).

To identify the oxidation product(s) we originally performed a reaction of compound 1 with sodium periodate in D_2O and subjected it immediately to NMR analysis. The ^1H spectrum clearly demonstrated the appearance of an aldehydic proton at $\delta = 9.5$ p.p.m and the ^{31}P spectrum showed the release of a free phosphate at $\delta = 1.5$ p.p.m. Retention of the benzylic proton in D_2O demonstrates that the decomposition of the *o*-quinone (at least the one generated chemically) does not proceed via the quinone methide tautomer of the phosphonic acid. This pathway has also been discounted for the decomposition of the *o*-quinone generated from 3,4-dihydroxyphenylglycine [14]. The NMR spectrum of the oxidation product produced

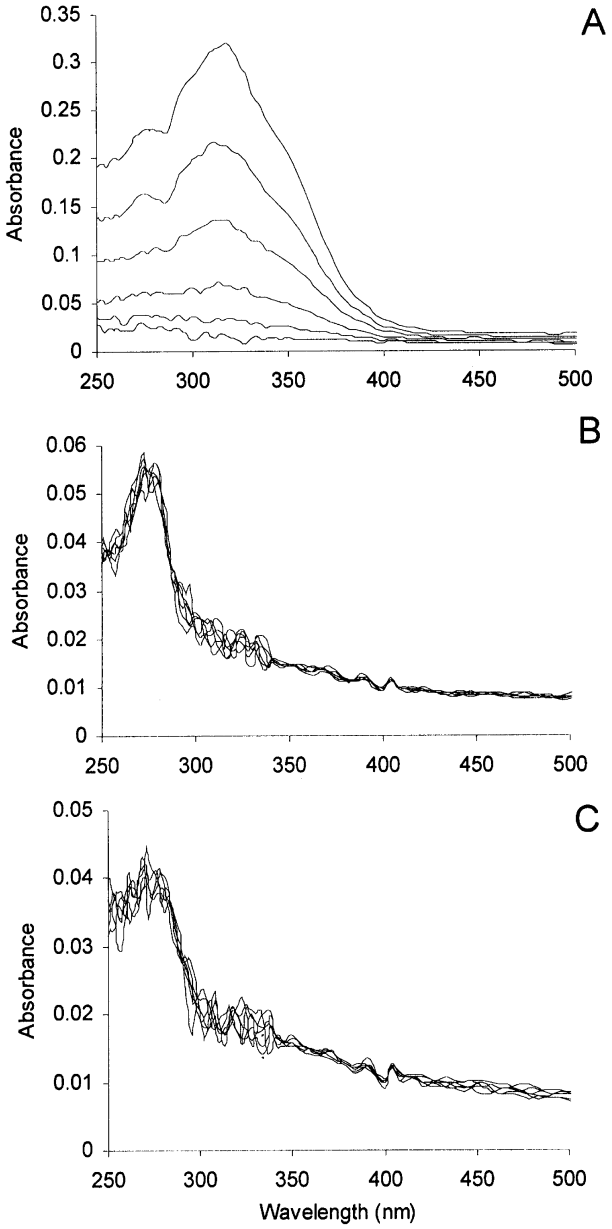

Fig. 2. Spectral changes associated with oxidation of phosphonic analogs of phenylglycine by tyrosinase. Each compound at 0.1 mM concentration was incubated with 20 μg of the enzyme. The spectra displayed were recorded at 15 min intervals from 0 to 75 min. The reference cuvette contained the substrate without the enzyme. (A) Amino-(3,4-dihydroxyphenyl)methyl phosphonic acid; (B) amino-(4-hydroxyphenyl)methyl phosphonic acid; (C) amino-(3-hydroxyphenyl)methyl phosphonic acid.

enzymatically matched the spectrum of 3,4-dihydroxybenzaldehyde (9.48 p.p.m., 1 H, singlet; 7.40 p.p.m., 1H, doublet, $J = 8.2$ Hz; 7.32 p.p.m., 1H, singlet; 6.78 p.p.m., 1H, doublet, $J = 8.2$ Hz). Reduction potentials for both aldehydes were also identical (−1.46 V, SCE as reference).

We have monitored the appearance of 3,4-dihydroxybenzaldehyde polarographically for all three compounds at 0.1 and 0.5 mM concentration in reactions with tyrosinase. After 2 h, the aldehyde was detectable only in the reaction

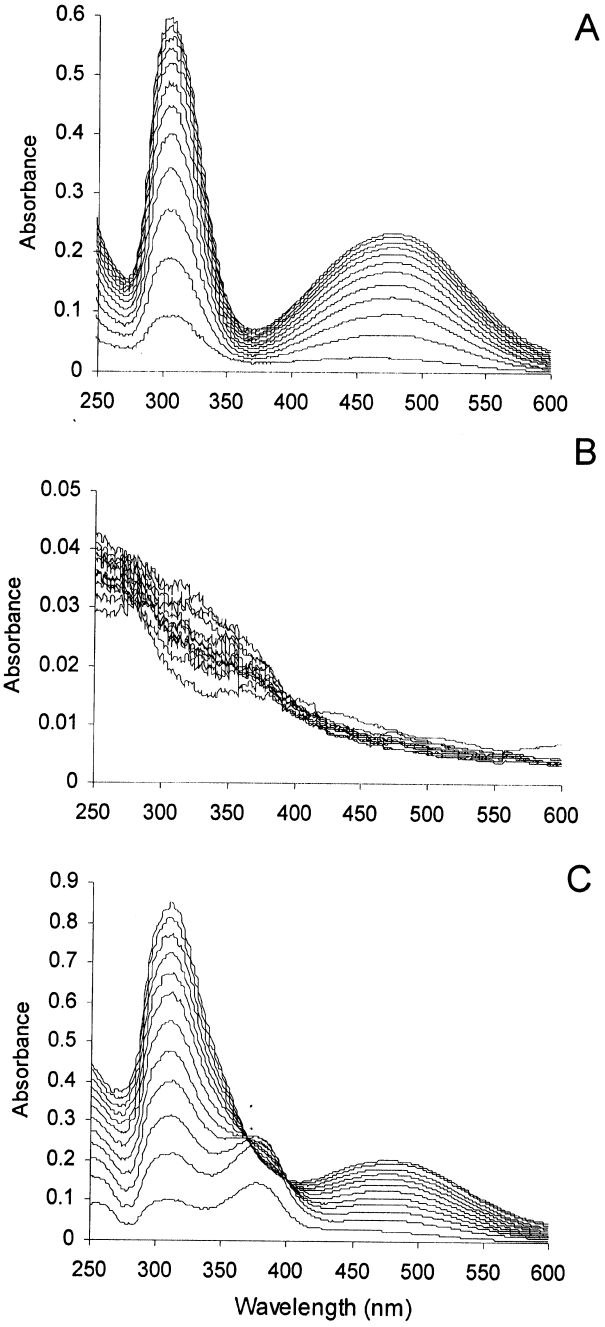

Fig. 3. Spectral changes associated with oxidation of amino-(3,4-dihydroxyphenyl)methyl phosphonic acid (A), Dopa (B) and their equimolar mixture (C) by tyrosinase. Substrates at 0.1 mM concentration each were incubated with 20 μg of the enzyme and the spectra were recorded at 40 s intervals.

with compound 1. This result confirmed the spectrophotometric data and the results of the oxygen consumption experiments, which demonstrated that the monohydroxylated compounds (2 and 3) are not oxidized by tyrosinase. The batch of the enzyme, which was used for these experiments, was routinely tested with standard monophenolic substrates, such as tyrosine, and showed full monophenolase activity. Therefore, we concluded that compounds 2 and 3 are not substrates or that the reaction is so slow that it was not detectable by our methods.

We have determined the Michaelis constant for compound 1 to see whether its inhibitory effect on tyrosinase was competitive in nature. However, K_m equal 0.52 ± 0.10 mM indicated that this was rather unlikely (K_m for Dopa under our conditions was 0.37 ± 0.08 mM). We have not observed a decrease in the enzymatic activity during the course of the reaction (90 min) of tyrosinase with compound 1, which indicated that covalent inactivation of the enzyme was not significant. However, to provide further evidence, we have also preincubated the enzyme with either compound 1, Dopa or catechol, a well-known suicide substrate of tyrosinase [17], and then separated the high- and low-molecular mass components on Sephadex G-25 columns. The enzymatic activity of the protein fractions was then assayed with Dopa. Inactivation of the enzyme by compound 1 (V_o = 183 ± 10 nmol·min^{-1}) was about three times weaker then by Dopa (V_o = 55 ± 8.7 nmol·min^{-1}) and 24 times weaker then by catechol (V_o = 7.5 ± 1.9 nmol·min^{-1}).

We have therefore speculated that compound 1 may interfere with chemical transformation of dopaquinone following the enzymatic oxidation of Dopa, as has already been demonstrated for other compounds [11,18–20]. The previously reported inhibition constants [13] were based on the appearance of dopachrome measured at 475 nm. If compound 1 reduced the enzymatically generated dopaquinone, it would prevent the formation of dopachrome. Changes in the UV/Vis spectra of a mixture of compound 1 and Dopa oxidized by tyrosinase indicated that this was indeed the case (Fig. 3C). In the initial phase of the reaction

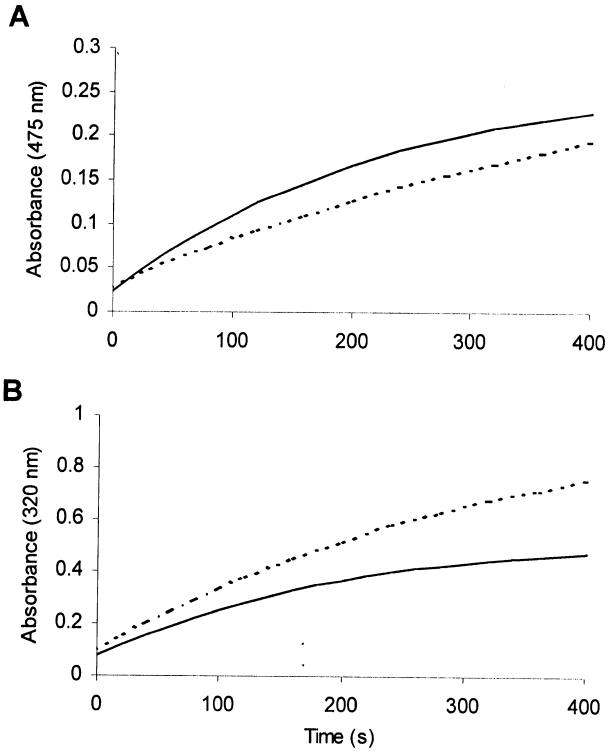

Fig. 4. Changes of absorbance at 475 nm (A) and 320 nm (B) recorded during oxidation of Dopa (solid line) and its equimolar mixture with amino-(3,4-dihydroxyphenyl)methyl phosphonic acid (dashed line, 0.1 mM each) by tyrosinase.

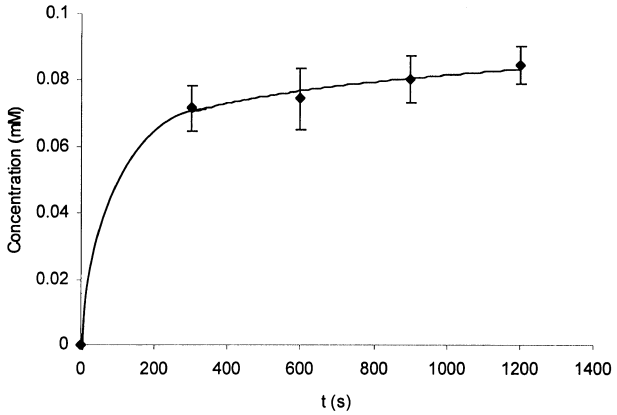

Fig. 5. Changes in the concentration of 3,4-dihydroxybenzaldehyde during oxidation of the equimolar mixture of amino-(3,4-dihydroxyphenyl)methyl phosphonic acid and Dopa (0.1 mM each) by tyrosinase. The aldehyde appearance was monitored polarographically using the SCE/HMDE system. When compound 1 was used alone, the aldehyde was not detectable within the monitored time period. Error bars represent standard deviation.

rapid accumulation of a quinone was observed (λ_{max} = 380 nm). This peak was barely detectable when compound 1 was oxidized separately (Fig. 3B), because of small reaction rate in this case. However, an identical peak appeared when compound 1 was oxidized by sodium periodate, and therefore its appearance during enzymatic oxidation of compound 1 in a mixture with Dopa has to be attributed to the phosphonic *o*-quinone produced by chemical oxidation of compound 1 by the enzymatically generated dopaquinone. This peak at 380 nm disappeared after $\approx$ 5 min. In the initial phase of the reaction it was accompanied by a steady increase of absorbance at 320 nm, where the absorption maxima of dopachrome and 3,4-dihydroxybenzaldehyde overlap. At the same time the increase of absorbance at 475 nm, the visible absorbance maximum for dopachrome, was much slower, compared to oxidation of Dopa alone (Figs 3 and 4). We have also monitored the appearance of 3,4-dihydroxybenzaldehyde polarographically during enzymatic oxidation of compound 1 either separately or in a mixture with Dopa. Results of this experiment confirmed that in a mixture the aldehyde was produced rapidly in the initial phase of the reaction (Fig. 5). Conversion of compound 1 to 3,4-dihydroxybenzaldehyde was almost complete within 5 min. Thus Dopa in fact catalyzes the oxidation of the shorter phosphonic diphenol acting as a redox shuttle. Reactions occurring in a mixture of Dopa, compound 1, and tyrosinase are summarized in Fig. 6.

We have attempted to determine the redox potentials for Dopa and compound 1 to see whether the difference would favor the redox reaction between dopaquinone and the phosphonic diphenol. Because of the irreversibility of the systems (cyclization of dopaquinone and decomposition of the phosphonic *o*-quinone) the values obtained are not very precise (150 mV for Dopa and 195 mV for compound 1), but indicate that the formation of the phosphonic *o*-quinone is not favored. Thus, two other factors drive the reaction: much slower enzymatic oxidation of compound 1 then Dopa and rapid removal of the phosphonic *o*-quinone from the system by its decomposition.

As mentioned before, the relatively high K_m for compound 1 indicated that in fact the true inhibition of the enzyme should be small. We have therefore compared oxygen consumption during enzymatic oxidation of Dopa and its mixture with compound 1 (Fig. 7). These curves were identical in the initial phase ($\approx$ 3 min), demonstrating that in fact no inhibition takes place under the conditions tested (0.1 mM of each compound). Total oxygen consumption in each reaction mixture corresponded approximately to the amount of the diphenolic substrates oxidized by the enzyme (0.8 equivalents for Dopa and 1.1 equivalents for the mixture, whereas the theoretical values are 1 and 1.5 equivalents, respectively).

DISCUSSION

The 3-dimensional structure of tyrosinase still remains unknown. Although the general architecture of the active site has been deduced mainly by analogy with catechol

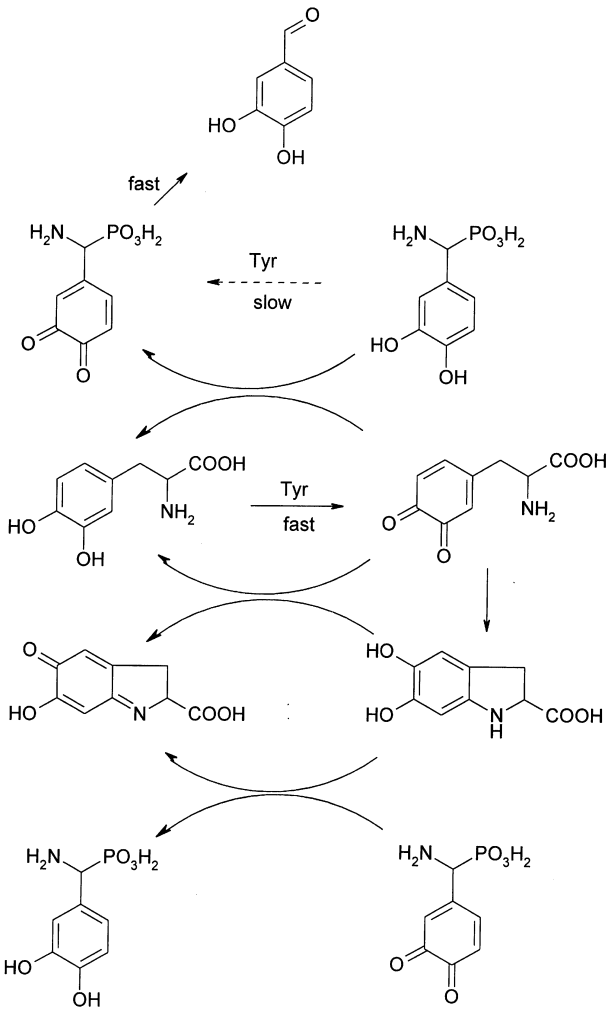

Fig. 6. Reactions occurring during oxidation of a mixture of Dopa and amino-(3,4-dihydroxyphenyl)methyl phosphonic acid by tyrosinase. Dopaquinone produced rapidly by the enzyme oxidizes the phosphonic diphenol to its *o*-quinone, which decomposes to 3,4-dihydroxybenzaldehyde. Most likely both quinones participate in oxidation of leukodopachrome to dopachrome.

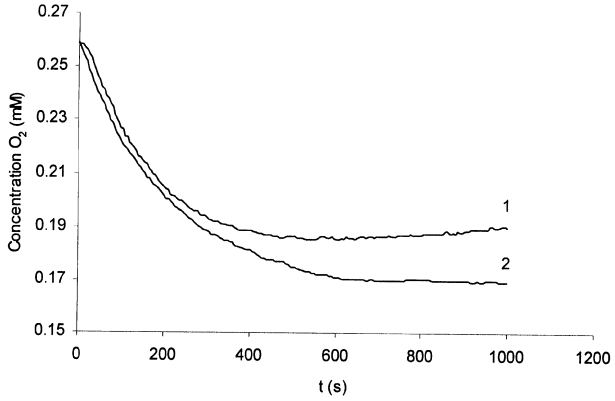

Fig. 7. Oxygen consumption during oxidation of Dopa (curve 1) and its equimolar mixture with amino-(3,4-dihydroxyphenyl)methyl phosphonic acid (curve 2) by tyrosinase. The reaction volume was 9 mL and contained 0.1 mM of each substrate and 69 μg of tyrosinase.

oxidase and arthropod hemocyanins [21], little is known about the details determining substrate specificity. While the mammalian enzyme seems to be very specific, the most widely used model enzyme, the mushroom tyrosinase, can oxidize a broad range of monophenolic and diphenolic compounds. However, there are limits to this tolerance. Studies with the *Neurospora crassa* tyrosinase demonstrated that bulky substituents attached to the aromatic ring dramatically reduced the monophenolase, but not the diphenolase activity [22]. Although 4-*t*-butylphenol bound to the enzyme with affinity similar to tyrosine (K_m equal 0.18 and 0.59 mM, respectively), it was oxidized approximately 200 times more slowly. The oxidation rate of 4-hydroxyphenylacetic acid did not differ much from that for tyrosine. There was also little difference between the reaction rates for Dopa and 4-*t*-butylcatechol [22]. These results are explained by the requirement for the monophenols to rearrange from the axial to equatorial position in the binuclear copper site during the *ortho*-hydroxylation reaction. Bulky substituents on the ring present a barrier to this rearrangement [22,23]. Our results with the derivatives of phenylglycine, both phosphonic and carboxylate, are consistent with this hypothesis. In the case of monohydroxylated phenylglycines the steric barriers presented by the amino-carboxylate or the amino-phosphonic groups prevent the appropriate positioning of the aromatic ring within the enzyme active site for the hydroxylation reaction to occur. The same seems to hold for the derivatives of mandelic acid: 3,4-dihydroxymandelic acid is oxidized by tyrosinase, whereas 4-hydroxymandelic acid is not [24]. Although the studies with *N. crassa* tyrosinase cited above showed little effect of the side substituents on the diphenolase reaction for several substrates, in mushroom tyrosinase steric constrains influence also this reaction. It has been shown recently that *o*-diphenols with small or no substituents (e.g. methyl catechol, catechol) bind to the oxy form of mushroom tyrosinase 200 times faster than substrates with a large or charged side chain (e.g. L-α-methyl-Dopa, L-Dopa methyl ester) [25]. Also, the K_m values are higher and V_{max} values lower for α-methyltyrosine and α-methyl-Dopa than for tyrosine and Dopa, respectively [26]. However, 4-*t*-butylcatechol, which has a large but nonpolar side substituent, is oxidized by mushroom tyrosinase several times faster than Dopa [25,27]. So, although both the steric and polar characteristics of the diphenolic substrate modulate the rate of oxidation [23,25], it appears that the size of the side substituent is not the primary factor. It has been demonstrated recently that the carboxyl group plays an important role in substrate recognition by the mammalian tyrosinase [28]. The affinity of the wild type enzyme was ≈ 4-fold lower for dopamine and 10-fold lower for D-Dopa than for L-Dopa. However, esterification of the carboxyl group had little effect, thus excluding electrostatic interactions. A much smaller difference in the kinetic parameters for the H389L mutant indicated that histidine 389 is likely to be involved in interactions of the mammalian enzyme with the carboxylate group of the diphenolic substrates. However, the H389L mutation had little effect on the affinity of the enzyme for tyrosine. It appears therefore that the binding of monophenols and diphenols to the mammalian tyrosinase differs. As H389 is adjacent to H390, which coordinates CuB, it was concluded that monophenols dock to copper A but diphenols dock to copper B in the tyrosinase active site [28].

Our data also indicate that polar interactions play an important role in substrate recognition by mushroom tyrosinase and that the orientation of monophenolic and diphenolic substrates may differ.

Although the oxidation of the phosphonic analog of 3,4-dihydroxyphenylglycine does occur, it is much slower than for Dopa (V_{max} equals 0.386 ± 0.058 μmol·min^{-1} and 1.64 ± 0.35 μmol·min^{-1}, respectively, under our conditions). The *o*-quinones generated enzymatically from both 3,4-dihydroxyphenylglycine [14] and its phosphonic analog (this study) decompose to 3,4-dihydroxybenzaldehyde. The same phenomenon occurs for 3,4-dihydroxymandelic acid [24,29–33], whereas the *o*-quinone generated from α-(3,4-dihydroxyphenyl)-lactic acid decomposes to 3,4-dihydroxyacetophenone [34]. However, oxidation of these compounds by tyrosinase in a mixture with natural substrates has not been tested so far. We predict that their effect on dopachrome formation will be very similar to amino-(3,4-dihydroxyphenyl)methyl phosphonic acid.

Redox exchange reactions between dopaquinone and other molecules occur in melanogenesis and have also been reported for synthetic compounds. Besides the best-known oxidation of leukodopachrome (see Fig. 6), dopaquinone can also oxidize 5-*S*-cysteinyldopa, the precursor of pheomelanins [35]. 4-*t*-Butyl catechol, 4-methyl catechol, and catechol have been shown to have synergistic effect on the oxidation of Dopa by tyrosinase. It has been demonstrated that this effect is mediated by oxidation of Dopa by the *o*-quinones generated enzymatically from these compounds, which are better substrates for the enzyme [19,20]. A similar effect has also been observed for 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid [18], although in this case the results are controversial, as these compounds should be poor substrates for tyrosinase. On the other hand, 3,4-dihydroxybenzoic acid inhibited the dopachrome formation, although the exact mechanism of this reaction has not been investigated [18].

Amino-(3,4-dihydroxyphenyl)methyl phosphonic acid also appeared to be a potent tyrosinase inhibitor, when the reaction was assayed spectrophotometrically [13]. However, we have now shown that it does not result from its interaction with the enzyme but from chemical reactions in solution. What distinguishes our case from other redox

interactions in this system is the decomposition of the phosphonic *o*-quinone. This decomposition prevents the redox reaction from reaching equilibrium and provides a long-lasting sink for dopaquinone. Our current data also explains why both L and D isomers of compound 1 showed similar inhibitory activity *in vitro* [13] and why this compound showed only a modest activity when tested in mouse B16 melanoma and human KB carcinoma cell lines [16] – it is simply not a good inhibitor of tyrosinase.

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