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Mechanistic studies on tyrosinase-catalysed oxidative decarboxylation of 3,4-dihydroxymandelic acid

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Mushroom tyrosinase, which is known to convert a variety of *o*-diphenols into *o*-benzoquinones, has been shown to catalyse an unusual oxidative decarboxylation of 3,4-dihydroxymandelic acid to 3,4-dihydroxybenzaldehyde [Sugumaran (1986) Biochemistry **25**, 4489–4492]. The mechanism of this reaction was re-investigated. Although visible-region spectral studies of the reaction mixture containing 3,4-dihydroxymandelic acid and tyrosinase failed to generate the spectrum of a quinone product during the steady state of the reaction, both trapping experiments and non-steady-state kinetic experiments provided evidence for the transient formation of unstable 3,4-mandeloquinone in the reaction mixture. The visible-region spectrum of mandeloquinone resembled related quinones and exhibited an absorbance maximum at 394 nm. Since attempts to trap the second intermediate, namely α,2-dihydroxy-*p*-quinone methide, were in vain, mechanistic studies were undertaken to provide evidence for its participation. The decarboxylative quinone methide formation from 3,4-mandeloquinone dictates the retention of a proton on the α-carbon atom. Hence, if we replace this proton with deuterium, the resultant 3,4-dihydroxybenzaldehyde should retain the deuterium present in the original substrate. To test this hypothesis, we chemoenzymically synthesized α-deuterated 3,4-dihydroxymandelic acid and examined its enzymic oxidation. Our studies reveal that the resultant 3,4-dihydroxybenzaldehyde retained nearly 90% of the deuterium, strongly indicating the transient formation of quinone methide. On the basis of these findings it is concluded that the enzymic oxidation of 3,4-dihydroxymandelic acid generates the conventional quinone product, which, owing to its unstability, is rapidly decarboxylated to generate transient α,2-dihydroxy-*p*-quinone methide. The coupled dienone–phenol re-arrangement and keto–enol tautomerism of this quinone methide produce the observed 3,4-dihydroxybenzaldehyde.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a mono-oxygenase of widespread occurrence in Nature and is responsible for the biosynthesis of melanin pigments in a vast array of organisms. It usually catalyses the conversion of monophenols into *o*-diphenols and the oxidation of diphenols to quinones (Lerch, 1983; Robb, 1984). A few years ago we reported an unusual oxidative decarboxylation of 3,4-dihydroxymandelic acid (DHMA) to 3,4-dihydroxybenzaldehyde (DHBAld) catalysed by mushroom tyrosinase (Sugumaran, 1986). Since we could not detect the formation of a stable conventional quinone product during the steady state of the reaction, we proposed a direct enzyme-catalysed oxidative decarboxylation of DHMA to the transient, α,2-dihydroxy-*p*-quinone methide. The quinone methide formed, being unstable, rapidly undergoes both dienone–phenol re-arrangement and keto–enol tautomerism to generate DHBAld as the stable final product (Scheme 1). Following our studies, Ortiz *et al.* (1988) examined the chemical and enzymic oxidation of DHMA and proposed that tyrosinase catalysed the oxidation of substrate to 3,4-mandeloquinone, which rapidly reacted with a molecule of DHMA, oxidizing it to the quinone methide, while itself reducing back to DHMA. The quinone methide was converted rapidly into DHBAld, as proposed by us (Sugumaran, 1986). Although electrochemical and kinetic data were presented as evidence for the transient production of mandeloquinone, no direct spectral evidence was provided. In a related study Cabanes *et al.* (1988) performed stopped-flow experiments with mushroom tyrosinase and DHMA at relatively acidic non-physiological conditions (pH 3.5) and obtained evidence for a rapid burst at 400 nm, which they attributed to the production of mandeloquinone. From their chemical and enzymic studies they proposed that the enzymic oxidation of DHMA produced 3,4-mandeloquinone, which was rapidly decarboxylated to yield DHBAld via the quinone methide. On the other hand, Bouheroum *et al.* (1989) performed pulse-radiolysis studies on the chemical oxidation of DHMA and presented for the first time the spectral properties of the unstable mandeloquinone. Moreover, these authors also demonstrated that the transient mandeloquinone was decarboxylated with first-order kinetics to generate DHMAld as the end product. These studies are in agreement with the proposal made by Cabanes *et al.* (1988), and discount the mandeloquinone-mediated decarboxylation of DHMA proposed by Ortiz *et al.* (1988).

In order to obtain direct evidence for mandeloquinone formation in the mushroom tyrosinase-catalysed reaction under physiological conditions and to obtain evidence for the quinone methide intermediacy in the enzyme-catalysed oxidative decarboxylation, we re-examined the tyrosinase-catalysed oxidation of DHMA. The results of our mechanistic studies are presented in this paper.

MATERIALS AND METHODS

Synthesis of α-deuterated DHMA

Sodium 4-hydroxybenzoylformate (5.6 g, 30 mmol) was reduced with NaB^2H_4 (1.5 g, 36 mmol) in ^2H_2O (30 ml) for 1 h at room temperature. The mixture was acidified with acetic acid and freeze-dried. The residue was extracted with ethyl acetate, dried over anhydrous $MgSO_4$ and concentrated on a rotary evaporator. The resulting mixture was chromatographed on a Bio-Gel P-2 column with 0.2 M-acetic acid as the eluent. Fractions

Abbreviations used: DHMA, 3,4-dihydroxymandelic acid; DHBAld, 3,4-dihydroxybenzaldehyde.

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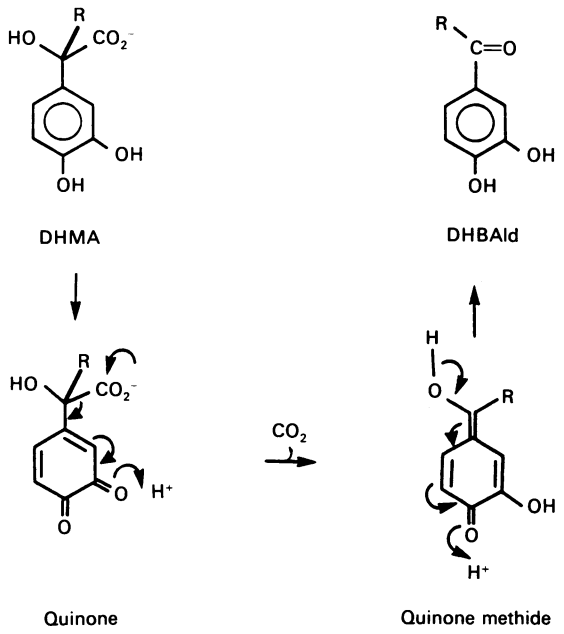

Scheme 1. Mechanism for the oxidative decarboxylation of DHMA

Oxidative decarboxylation of DHMA (R = H) generates DHBAld (R = H) as the stable end product. Initially we proposed a direct oxidative decarboxylation of DHMA to the quinone methide (Sugumaran, 1986). The quinone methide thus formed, being unstable, undergoes both dienone–phenol re-arrangement and keto–enol tautomerism to produce DHBAld. However, later workers have shown that the reaction goes through the quinone intermediate (Cabanes *et al.*, 1988; Ortiz *et al.*, 1988; Bouheroum *et al.*, 1989).

containing the required product were pooled and freeze-dried. α-Deuterated 4-hydroxymandelic acid was obtained as a white solid (1.8 g; yield 33%), m.p. 90–92 °C: i.r. (Nujol mull), ν = 3450, 3350, 2650 (broad, CO_2H+OH), 1720 and 1700 cm^{-1} (C=O); ^1H n.m.r., δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 6.50–6.93 (2H, d, J = 8 Hz, Ar*H*), 7.00–7.40 (2H, d, J = 8 Hz, Ar*H*) and 7.60–9.10 (3H, broad, O*H*+CO_2H), exchanged with ^2H_2O.

α-Deuterated 4-hydroxymandelic acid was enzymically hydroxylated to α-deuterated DHMA by using the general aromatic hydroxylation procedure developed by Klibanov *et al.* (1981). A mixture of α-deuterated 4-hydroxymandelic acid (0.336 g, 2 mmol), horseradish peroxidase (0.5 mg) and dihydroxyfumaric acid (0.6 g) in 100 ml of 50 mM-sodium acetate buffer, pH 5.2, was continuously stirred with a constant supply of O_2 at 0 °C for 1 h. Two batches (0.6 g each) of dihydroxyfumaric acid were added at 1 h intervals and the reaction was continued for a total period of 3 h. At the end of this period, the contents were acidified, filtered and freeze-dried. The residue was extracted with methanol/diethyl ether (1:2, v/v) mixture and concentrated in a rotary evaporator. Chromatography of the mixture on a Bio-Gel P-2 column with 0.2 M-acetic acid as the eluent gave the required α-deuterated DHMA (70 mg), m.p. 128–130 °C: i.r. (Nujol mull), ν 3450, 3350, 3200, 2700, 2600 (broad, CO_2H+OH), 1710 and 1700 cm^{-1} (C=O); ^1H n.m.r., δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 6.40–7.00 (3H, m, Ar*H*) and 8.00–9.50 (4H, broad, O*H*+CO_2H), exchanged with ^2H_2O. The electron-impact mass spectrum confirmed approx. 90% retention of deuterium in the product.

Large-scale reaction

A reaction mixture containing 24 mg of α-deuterated DHMA and 2.4 mg of mushroom tyrosinase in 150 ml of 100 mM-sodium phosphate buffer, pH 6.0, was incubated at 25 °C for 100 min. At the end of this period, the reaction was arrested by the addition of 2 ml of acetic acid and the contents of the reaction mixture were freeze-dried. The residue was taken up in a minimum amount of water and chromatographed on a Bio-Gel P-2 column (2.5 cm × 95 cm) with 0.2 M-acetic acid as the eluent at a flow rate of 1 ml/min. Under these conditions unchanged DHMA was eluted between 650 and 775 ml and DHBAld was eluted between 810 and 900 ml. The fractions containing the product were pooled, freeze-dried and used for structural elucidation studies.

Other procedures

H.p.l.c. analysis of the reaction mixtures was carried out as outlined previously (Sugumaran, 1986). U.v. and visible-region spectra were recorded with a Milton–Roy spectronic 3000 array spectrophotometer. I.r. spectra were taken in a Perkin–Elmer model 137 spectrophotometer, and ^1H-n.m.r. spectra were obtained with a 60 MHz Perkin–Elmer model R-24 spectrometer. Electron-impact mass spectra were recorded with Finnigan MAT model 4510B mass spectrometer.

Materials

Mushroom tyrosinase (specific activity 4000 units/mg of protein), horseradish peroxidase (specific activity 220 units/mg of protein), DHMA, 3,4-dihydroxyphenylacetic acid and DHBAld were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) supplied sodium 4-hydroxybenzoylformate and NaB^2H_4. Other chemicals and solvents were procured from commercial sources.

RESULTS

In accordance with previous results (Sugumaran, 1986), a reaction mixture containing DHMA and mushroom tyrosinase readily generated DHBAld as the major product. The formation of quinone could not be readily observed in such a reaction mixture (Fig. 1). Under the same conditions, however, quinone production from the substrate analogue 3,4-dihydroxyphenylacetic acid could readily be detected, although it has been shown that carboxymethyl-*o*-benzoquinone is also unstable (Sugumaran *et al.*, 1989*b*). Cabanes *et al.* (1988) used stopped-flow studies with excess tyrosinase at pH 3.5 and monitored the formation of a reactive species at 400 nm that they have ascribed to the transiently formed mandeloquinone. However, to obtain direct evidence for quinone formation under physiological conditions, we resorted to using pre-steady-state conditions. Fig. 2 shows the

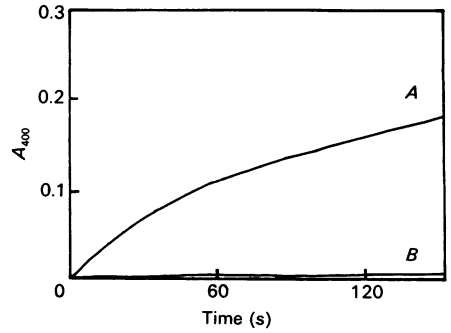

Fig. 1. Time course of quinone formation under steady-state conditions

A reaction mixture containing 200 μg of substrate and 10 μg of mushroom tyrosinase in 1 ml of 100 mM-sodium phosphate buffer, pH 6.0, was incubated at room temperature and the appearance of quinone in the reaction mixture was monitored continuously at 400 nm. Curve *A*, 3,4-dihydroxyphenylacetic acid; curve *B*, DHMA.

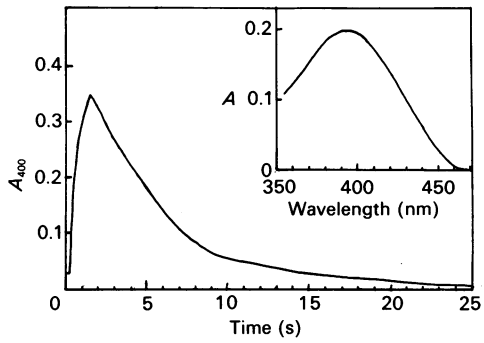

Fig. 2. Time course of quinone formation under pre-steady-state conditions

A reaction mixture containing 10 mmol of DHMA and 900 μg of mushroom tyrosinase in 1 ml of 100 mM-sodium phosphate buffer, pH 6.0, was incubated at room temperature and the appearance of quinone in the reaction mixture was monitored continuously at 400 nm. Inset: visible-region spectrum of quinone formed in the reaction mixture at about 1 s.

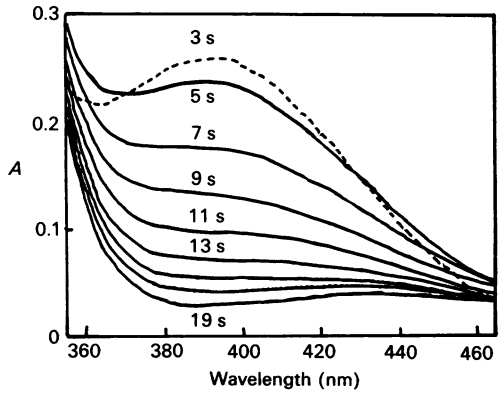

Fig. 3. Spectral changes associated with the transformation of mandeloquinone

The spectral changes associated with the reaction mixture described in Fig. 2 were monitored at 2 s intervals.

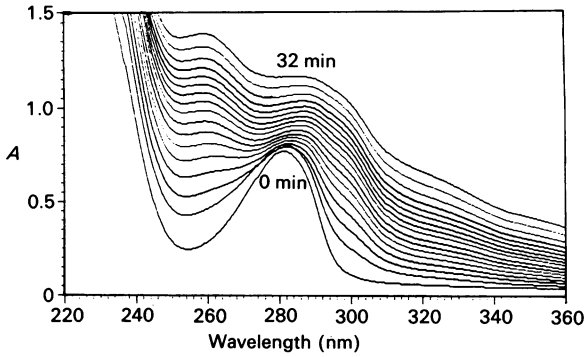

Fig. 4. U.v.-spectral changes associated with the enzymic oxidation of DHMA in the presence of *N*-acetylcysteine

A reaction mixture containing 0.2 μmol of DHMA, 0.4 μmol of *N*-acetylcysteine and 25 μg of tyrosinase in 1 ml of 100 mM-sodium phosphate buffer, pH 6.0, was incubated at room temperature and the u.v.-spectral changes associated with the enzymic oxidation were recorded at 2 min intervals.

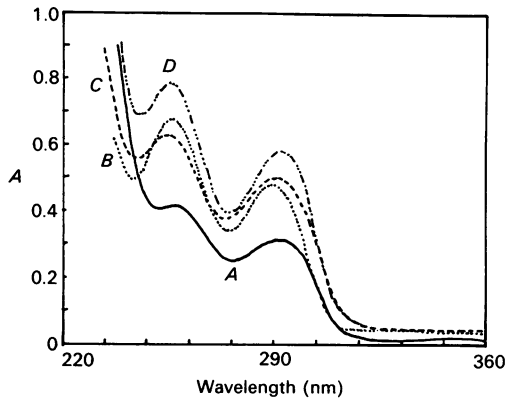

Fig. 5. U.v. spectra of *N*-acetylcysteinyl-quinone adducts

Curve *A* is the u.v. spectrum of the *N*-acetylcysteinyl-mandeloquinone adduct. For comparison, the spectra of (*B*) *N*-acetylcysteinyl-*o*-benzoquinone adduct, (*C*) *N*-acetylcysteinyl-4-methyl-*o*-benzoquinone adduct and (*D*) *N*-acetylcysteinyl-*N*-acetyldopamine quinone adduct are also shown in the Figure.

time course of absorbance changes (monitored at 400 nm) occurring in the reaction mixture containing high concentrations of enzyme and substrate. It is clear from the Figure that a reactive species absorbing at 400 nm is rapidly formed and then disappears from the reaction mixture. The visible-region spectrum of this transient intermediate exhibited an absorbance maximum of 394 nm (Fig. 2 inset) and resembled the spectrum of related quinones (Sugumaran, 1986). From these studies it was concluded that this intermediate appearing transiently in the reaction mixture is mandeloquinone. Fig. 3 shows the spectral changes associated with the transformation of this quinone. As is evident, mandeloquinone is unstable and decomposed rapidly to a colourless product, which was identified as DHBAld. The above studies confirm the initial production and transient nature of mandeloquinone in the reaction mixture.

We then focused our attention on the second intermediate, namely α,2-dihydroxy-*p*-quinone methide (see Scheme 1). Since quinone methides are highly reactive intermediates, it is difficult to observe them directly, but they can be easily trapped by nucleophiles (Sugumaran, 1988). Therefore we attempted to trap the quinone methide with nucleophiles. Attempts to trap this intermediate with a powerful nucleophile such as a thiol were in vain, as it trapped the mandeloquinone instead. For instance, Fig. 4 shows the spectral changes accompanying the oxidation of DHMA by tyrosinase in the presence of *N*-acetylcysteine. The appearance of a peak at about 255 nm is consistent with the trapping of quinone as its cysteinyl adduct. These spectral changes were remarkably similar to the spectral changes reported for the tyrosinase-catalysed oxidation of catechol, 4-methylcatechol and *N*-acetyldopamine in the presence of *N*-acetylcysteine (Sugumaran *et al.*, 1989*a*). When the reaction mixture was chromatographed and analysed, a product exhibiting absorbance at about 255 nm could be separated from the reaction mixture. This product was tentatively identified as 5-(*N*-acetylcysteinyl)-DHMA on the basis of comparison of its u.v. spectrum with those of related adducts (Fig. 5) and by consideration of the well-known reactions of quinone with *N*-acetylcysteine (Sugumaran, 1988; Sugumaran *et al.*, 1989*a*). In an earlier study, after failing to trap a quinone methide with cysteine, we successfully demonstrated its formation by trapping it as a methanol adduct (Saul & Sugumaran, 1990). This technique could not be used in the present study because of the following consideration. The Michael 1,6-addition product of methanol to

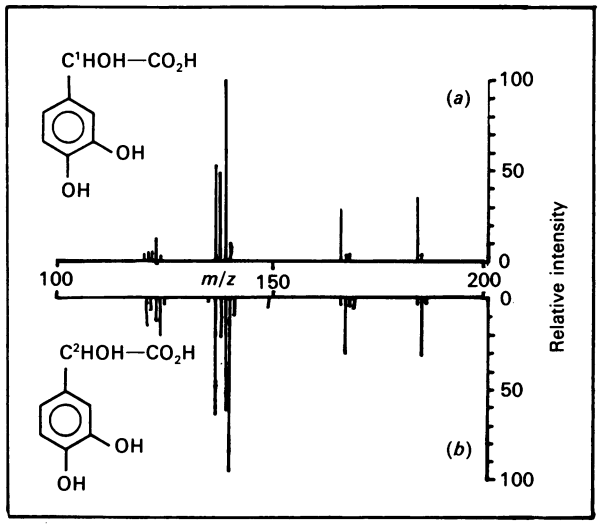

Fig. 6. Mass spectra of (*a*) DHMA and (*b*) α-deuterated DHMA

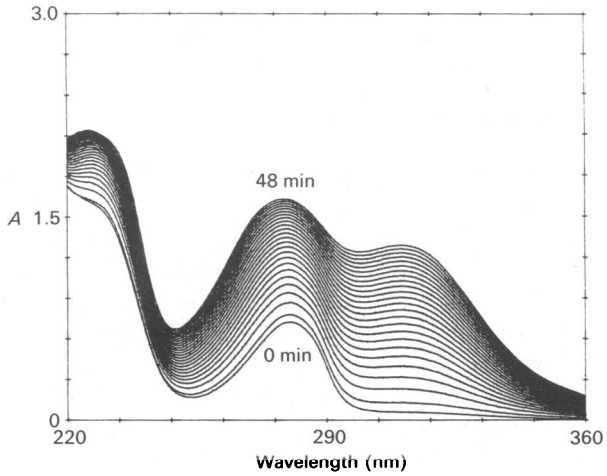

Fig. 7. U.v.-spectral changes associated with the enzymic oxidation of α-deuterated DHMA

A reaction mixture containing 100 μg of α-deuterated DHMA and 10 μg of mushroom tyrosinase in 1 ml of 50 mM-sodium phosphate buffer, pH 6.0, was incubated at room temperature, and the change in absorbance in the u.v. region was recorded at 2 min intervals.

this quinone methide is an unstable semiacetal of DHBAld, whose formation can also be accounted for by the non-enzymic addition of methanol to DHBAld.

After failing to trap the quinone methide, we resorted to the following mechanistic studies to provide evidence for its formation. DHBAld could be formed either by unimolecular decarboxylation of mandeloquinone to the quinone methide and its subsequent aromatization (Cabanes *et al.*, 1988; Brouheroum *et al.*, 1989) or by mandeloquinone-mediated oxidative decarboxylation of DHMA (Ortiz *et al.*, 1988). Whereas the former possibility dictates the quantitative retention of the α-proton of DHMA in the product, the latter possibility calls for either 50% retention (if the reaction goes through the 3,4-dihydroxybenzyl alcohol intermediate) or total loss (if the reaction goes through the 3,4-dihydroxybenzylformic acid intermediate) of this proton. The earlier studies discounted both 3,4-dihydroxybenzyl alcohol and 3,4-dihydroxybenzoylformic acid as freely generated intermediates, but did not rule them out as enzyme-bound intermediates (Sugumaran, 1986). In order to rule out these routes and to gather evidence for quinone methide route, we chemoenzymically synthesized α-deuterated DHMA and studied the enzyme-catalysed oxidation with this compound.

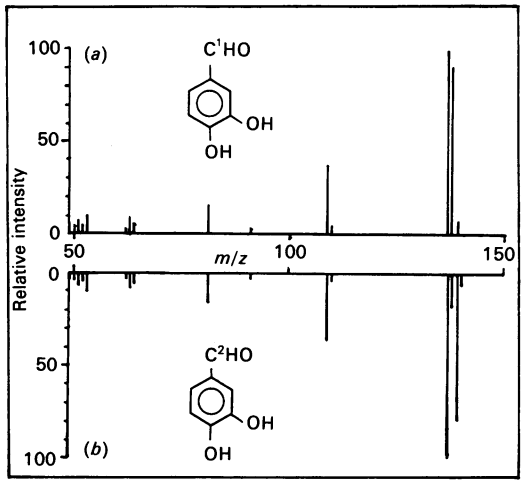

Fig. 8. Mass spectra of enzymic products formed from (*a*) DHMA and (*b*) α-deuterated DHMA

Fig. 6 shows the mass spectrum of both the protiated and deuterated forms of DHMA. From the Figure it is evident that the chemoenzymic synthesis generated α-deuterated DHMA. The deuterated DHMA also served as a substrate for mushroom tyrosinase. Fig. 7, for example, shows the spectral changes associated with the oxidation of this compound with tyrosinase. These spectral changes are the same as those occurring during the oxidation of DHMA (Sugumaran, 1986). From a large-scale reaction mixture, the DHBAld formed was isolated and subjected to mass-spectral analysis. If quinone methide is indeed formed as the transient intermediate, then its transformation into DHBAld should involve retention of α-deuterium, as both dienone–phenol re-arrangement and the keto–enol tautomerism of quinone methide do not require the removal of this atom from the molecule (see Scheme 1). From the mass spectrum (Fig. 8) it can be seen that the DHBAld formed retained nearly 90% of deuterium in its side chain.

DISCUSSION

The unusual oxidative decarboxylation of DHMA to DHBAld was first reported from this laboratory (Sugumaran, 1986). On the basis of the available evidence at that time, we proposed a direct tyrosinase-catalysed oxidative decarboxylation of DHMA to a quinone methide and subsequent transformation of the quinone methide to account for the observed reaction. However, in subsequent years three groups of workers who examined both the chemical and enzymic oxidation of DHMA established that the reaction indeed goes through the conventional quinone intermediate (Cabanes *et al.*, 1988; Ortiz *et al.*, 1988; Bouheroum *et al.*, 1989).

In the present study we used pre-steady-state conditions and confirmed the transient formation of mandeloquinone. In addition, we were successful in obtaining the visible-region spectrum of enzymically generated mandeloquinone under physiological conditions for the first time. The spectrum of the quinone corresponded well to that of related quinones (Sugumaran, 1986) and resembled the pulse-radiolytically generated quinone (Bouheroum *et al.*, 1989). Moreover, trapping experiments with *N*-acetylcysteine also attested to the formation of a quinone product.

In general, most quinones are reasonably stable for observation. Hence the remarkable instability of mandeloquinone is quite intriguing. We have recently reported that α-methylmandeloquinone also exhibits a similar decarboxylation reaction (Sugumaran *et al.*, 1991*b*). However, the related carboxymethyl-*o*-benzoquinone, which is also known to be highly unstable, does not seem to exhibit this reaction (Sugumaran *et al.*, 1989*b*). The stability of quinones varies depending upon the conditions. Dopaquinone and related compounds, owing to the presence of an appropriately positioned internal nucleophile (amino group), rapidly undergo intramolecular Michael 1,4-addition reaction and generate ring-closed products (Sugumaran, 1988). Unexpectedly, both carboxymethyl-*o*-benzoquinone (Sugumaran *et al.*, 1989*b*) and carboxyethyl-*o*-benzoquinone (Sugumaran *et al.*, 1989*c*) exhibit similar intramolecular cyclization reactions even though the carboxy group is not as strong a nucleophile as that of an amine. In the case of mandeloquinone we could not detect such a reaction, as it was converted into DHBAld nearly quantitatively.

Isomerization of quinones to quinone methides is yet another frequently observed reaction (Sugumaran, 1990). Thus Witkop and his associates proposed the formation of quinone methides from the quinone of dopamine derivatives and their subsequent hydration as a route to the production of noradrenalines (norepinephrines) as early as 1959 (Senoh & Witkop, 1959). Although this reaction has been shown to be accomplished by a direct enzyme-catalysed hydroxylation route (Kaufman *et al.*, 1962) (dopamine β-hydroxylase reaction), we have more recently demonstrated the occurrence of this reaction for the production of *N*-acylnoradrenaline derivatives in insects (Saul & Sugumaran, 1988, 1989, 1990). During our studies we also demonstrated the ready tautomerization of a number of quinones to unstable quinone methides (Sugumaran, 1990). Thus quinone methide formation could account for (*a*) conversion of 3,4-dihydroxyphenylacetic acid into DHMA (Sugumaran *et al.*, 1989*b*), (*b*) oxidative deamination of 3,4-dihydroxybenzylamine (Sugumaran, 1990), (*c*) oxidative conversion of 3,4-dihydroxybenzyl alcohol into DHBAld (Sugumaran *et al.*, 1991*a*), (*d*) transformation of dihydrocaffeic acid derivatives into caffeic acid derivatives (Sugumaran *et al.*, 1989*c*,*d*) and (*e*) oxidative dimerization of 1,2-dehydro-*N*-acetyldopamine (Sugumaran *et al.*, 1987, 1990*b*). Finally, in the case of α-methyldopachrome methyl ester we could demonstrate its ready isomerization to quinone methide both by spectroscopy and by isolation and identification (Sugumaran *et al.*, 1990*a*; Sugumaran & Semensi, 1991).

The conversion of mandeloquinones into quinone methide by decarboxylative transformation is a unique reaction (Scheme 1). Direct evidence for the formation of quinone methide from mandeloquinone could not be obtained even by trapping techniques, as α-hydroxy-substituted quinone methides, instead of exhibiting external reactivity, show only internal reactivity. Both dienone–phenol re-arrangement and keto–enol tautomerism drive these molecules towards an internal aromatization reaction to generate oxo-substituted catechols (see Scheme 1). Fully in agreement with this conclusion is the near-quantitative retention of deuterium observed in the DHBAld formed from α-deuterated DHMA. We have witnessed a similar conversion of quinone methides derived from *N*-acetylnoradrenaline, *N*-β-alanylnoradrenaline and 3,4-dihydroxyphenyl glycol to oxo-substituted catechols (Saul & Sugumaran, 1989, 1990). Thus the unusual oxidative transformation of DHMA into DHBAld occurs via the intermediary formation of both quinone and quinone methide.

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