Tyrosinase kinetics: failure of the auto-activation mechanism of monohydric phenol oxidation by rapid formation of a quinomethane intermediate

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When 3,4-dihydroxybenzylcyanide (DBC) is oxidized by mushroom tyrosinase, the first visible product, identified as the corresponding quinomethane, exhibits an absorption maximum at 480 nm. Pulse-radiolysis experiments, in which the *o*-quinone is formed by disproportionation of semiquinone radicals generated by single-electron oxidation of DBC, showed that the quinomethane (A_{480} 6440 $M^{-1}\cdot cm^{-1}$) is formed through the intermediacy of the *o*-quinone with a rate constant at neutral pH of 7.5 s^{-1}. The oxygen stoichiometry of the formation of the quinomethane by tyrosinase-catalysed oxidation of DBC was 0.5:1. On the basis of oxygen utilization rates the calculated V_{max} was 4900 $nmol\cdot min^{-1}$ and the apparent K_m was 374 μM. The corresponding monohydric phenol, 4-hydroxybenzylcyanide (HBC), was not oxidized by tyrosinase unless the enzyme was pre-exposed to DBC, the maximum acceleration of HBC oxidation being obtained by approximately equimolar addition of DBC. These results are consistent with tyrosinase auto-activation on the basis of the indirect formation of the dihydric phenol-activating cofactor. The rapid conversion of the *o*-quinone to the quinomethane prevents the formation of the catechol by reduction of the *o*-quinone product of monohydric phenol oxidation from occurring in the case of the compounds studied. In the absence of auto-activation, the kinetic parameters for HBC oxidation by tyrosinase were estimated as V_{max} 70 $nmol\cdot min^{-1}$ and K_m 309 μM. The quinomethane was found to decay with a rate constant of $2k$ 38 $M^{-1}\cdot s^{-1}$, as determined both by pulse-radiolysis and tyrosinase experiments. The second-order kinetics indicate that a dimer is formed. In the presence of tyrosinase, but not in the pulse-radiolysis experiments, the quinomethane decay was accompanied by a steady-state oxygen uptake concurrently with the generation of a melanoid product measured by its A_{650}, which is ascribed to the formation of an oligomer incorporating the oxidized dimer.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is an enzyme widely distributed in nature that oxidizes tyrosine and related compounds to their respective *o*-quinones. Tyrosinase is the principal enzyme of the pathway of biosynthesis of the pigment melanin and possesses unusual kinetic behaviour. The oxidation of monohydric phenols by tyrosinase exhibits an induction (or lag) period during which the rate of oxidation accelerates [1]. This is due to the obligatory requirement of binding of molecular oxygen to the bicuprous active site to enable the enzyme to convert phenolic substrates to the corresponding *o*-quinone. The redox potential of the active-site copper atoms results in a significant proportion of isolated tyrosinase having a bicupric active site unable to bind molecular oxygen and thus being incapable of phenol oxidation. This so-called met-tyrosinase requires a cofactor able to reduce the active-site copper atoms to give deoxytyrosinase, which is able to bind oxygen and take part in phenol oxidation [2]. Although direct reduction may occur, e.g. by electron donation from reduced metals [3], it is generally the consequence of catechol oxidation by the enzyme that can occur without oxygen:

$$\text{catechol} + 2\ Cu^{2+} \rightarrow \textit{o}\text{-quinone} + 2\ Cu^{+} + 2\ H^{+}$$

The acceleration of phenol oxidation occurs because of the recruitment of met-tyrosinase by Cu(II) reduction by the catecholic substrate that is generated as a product of the phenol oxidation, i.e. the reaction is auto-catalytic. This behaviour and the conditions influencing the rate of acceleration are well understood [4]. There has, however, been controversy concerning the source of the catechol. According to one model of tyrosinase kinetics, the catechol is formed as an intermediate by *o*-hydroxylation of the phenolic substrate [5]. The alternative view [6] is that the catechol is indirectly formed from the *o*-quinone by a combination of a reductive addition and a subsequent redox exchange. In the case of the natural substrate, tyrosine, this process involves an endo-cyclization by addition to the ring of the side chain amino group to form cyclodopa (2,3-dihydro-5,6-dihydroxyindole), which then undergoes a redox-exchange reaction with dopaquinone to give dopa (the activating catechol) and dopachrome, as proposed by Evans and Raper [7]. We have recently adduced evidence for this mechanism by demonstrating a failure of tyrosinase activation kinetics when there is interference with the redox-exchange mechanism postulated by the indirect model [8].

In the work presented in this paper we show that the rapid conversion to the corresponding quinomethane of the *o*-quinone product of tyrosinase oxidation of the cyanomethyl-substituted phenol substrate prevents the production of the auto-catalytic catechol by a redox-exchange reaction and that the activation kinetics of tyrosinase are inhibited by the diversion of the *o*-quinone, as predicted by the indirect catechol-generation model.

Abbreviations used: HBC, 4-hydroxybenzylcyanide; DBC, 3,4-dihydroxybenzylcyanide.

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MATERIALS AND METHODS

Enzyme and substrates

Mushroom tyrosinase (ex *Agoricus bisporus*) [specific activity 2000 units/mg protein (manufacturer's estimate, 1 unit is ΔA_{280}/min of 0.001 at pH 6.5)] was obtained from Sigma Chemical Co., Poole, Dorset, U.K. This material has previously been shown to migrate as a single protein band in non-denaturing PAGE and to exhibit co-localized monophenolase and diphenolase activities [6]. 4-Hydroxybenzylcyanide (HBC) was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. 3,4-Dimethoxybenzylcyanide obtained from Aldrich Chemical Co. was converted to 3,4-dihydroxybenzylcyanide (DBC) using the procedure described by Carlsson et al. [9].

Pulse radiolysis

The pulse-radiolysis experiments were performed with a 9–12 MeV Vickers linear accelerator using 50–200 ns pulses with doses of up to 30 Gy and quartz capillary cells of optical path 2.5 cm [10,11]. Absorbed doses were determined from the transient $(SCN)_2^{\bullet -}$ formation in air-saturated KSCN solutions (10 mM) as described by Adams et al. [12], but using the recently updated $G\epsilon$ value of 2.59×10^{-4} m$^2 \cdot$J^{-1} obtained by Buxton and Stuart [13], G being the radiation chemical yield of $(CNS)_2^{\bullet -}$ and ϵ its molar absorption coefficient at 475 nm. Saturation of such solutions with nitrous oxide results in a doubling of the $(CNS)_2^{\bullet -}$ yield [13].

Generation of the one-electron oxidizing species $Br_2^{\bullet -}$ was achieved by irradiating nitrous-oxide-saturated aqueous solutions of 100 mM KBr. Under such conditions, $Br_2^{\bullet -}$ is formed within approx. 0.1 μs after the radiation pulse via the following reactions:

$$H_2O \rightsquigarrow e^-_{aq} + HO^\bullet$$

$$e^-_{aq} + N_2O + H_2O \rightarrow HO^\bullet + N_2 + HO^-$$

$$HO^\bullet + Br^- \rightarrow HO^- + Br^\bullet$$

$$Br^\bullet + Br^- \rightarrow Br_2^{\bullet -}$$

Combined oximetry and spectrophotometry

Reactions were carried out at 28 °C in a stirred quartz cuvette of 3.65 ml vol. adapted to hold the tip of a Clark-type oxygen electrode (Yellow Springs Instruments, Model 5300) and positioned in the light path of a diode-array spectrophotometer (Hewlett-Packard, Model 8452A).

Tyrosinase was used, except where otherwise stated, at 300 units/ml in sodium phosphate buffer (0.1 M, pH 7.4). The enzyme solution was added to the reaction vessel and the oxygen meter adjusted to 100 %. The absorbance of the enzyme solution was used as the blank. Additions were made with a fine-tipped pipette through the stopper aperture and recordings made of the time-dependent changes in oxygen levels and spectral absorbance. The oxygen electrode was calibrated as previously described [8].

RESULTS

Studies by pulse radiolysis

Since tyrosinase-catalysed oxidation HBC and DBC is likely to involve the corresponding *o*-quinone and pulse radiolysis is an established method [14] of studying such unstable species, this technique was applied to the oxidation of DBC.

One-electron oxidation of DBC was carried out by irradiation of an N_2O-saturated 1.0 mM solution in the presence of 100 mM KBr buffered to pH 7.1 with 10 mM phosphate. The initial product, completely formed 100 μs after the pulse, was the semiquinone of DBC with a wavelength maximum at 310 nm and a shoulder at 360 nm, which decayed by second-order kinetics to a species, or mixture of species that, at 18 ms, absorbed more weakly at longer wavelengths than the semiquinone, decreasing monotonically with increasing wavelength in the visible spectrum. Over longer times (up to 1 s after the pulse) a further stronger absorption, with peaks at 320 nm and 480 nm, grew unimolecularly with a rate constant of 7.5 s^{-1}. The same rate constant was obtained at pH 7.4. Regarding the spectrum recorded 18 ms after the pulse, although not inconsistent with the presence of a mixture of residual semiquinone, *o*-quinone and the subsequent product absorbing strongly in the visible spectrum (see below), no discrete peak at approx. 400 nm

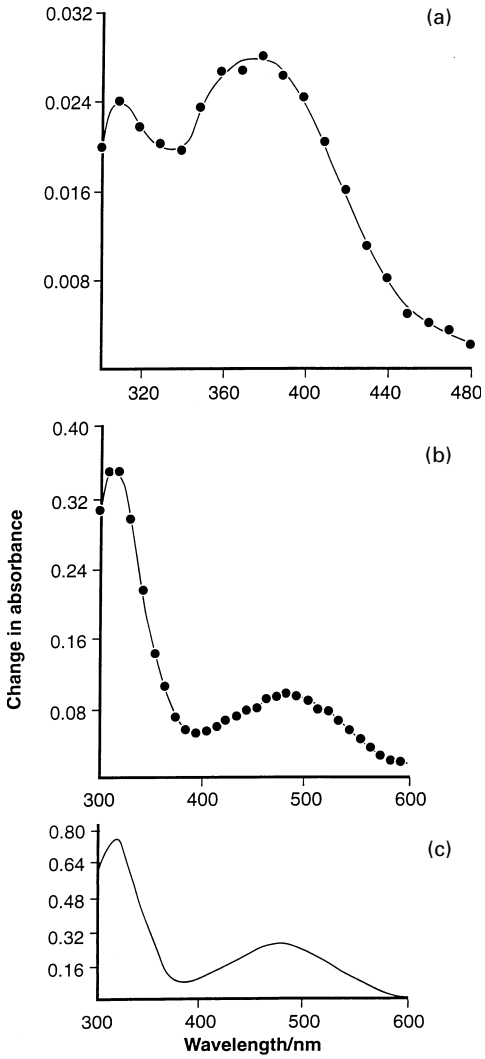
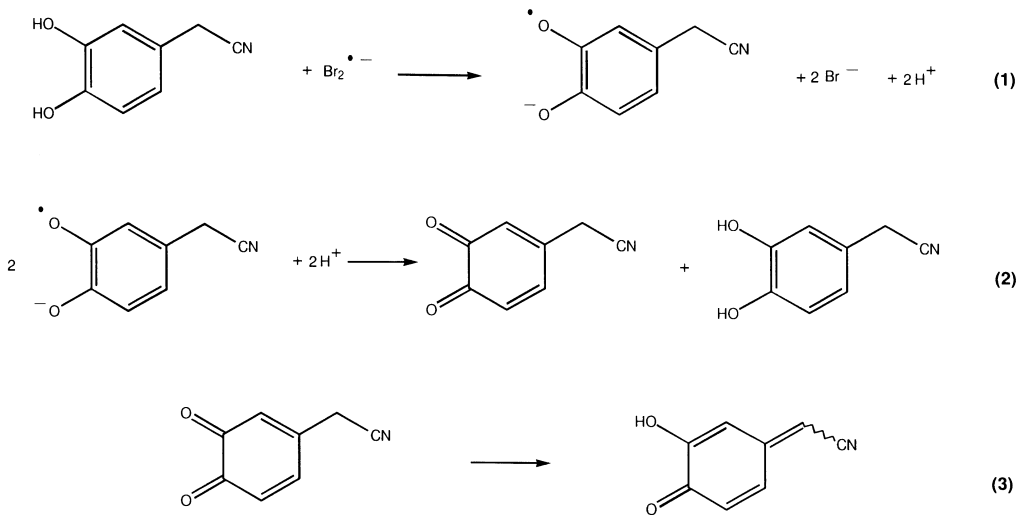


Figure 1 Absorption spectra of DBC oxidation products

(a) Absorbance as a function of wavelength over the range 300–480 nm of the initial *o*-quinone product generated by pulse radiolysis of DBC at pH 5.9. The difference spectrum at 9.0 ms is shown. (b) Spectral absorbance (300–590 nm) of the secondary quinomethane product generated from DBC by pulse radiolysis at pH 7.1. The difference spectrum at 1 s is shown. (c) Absorbance spectrum of tyrosinase oxidation product of DBC. The absorbance (300–600 nm) at 5 s incubation is shown.



Scheme 1

Reactions 1–3 are shown.

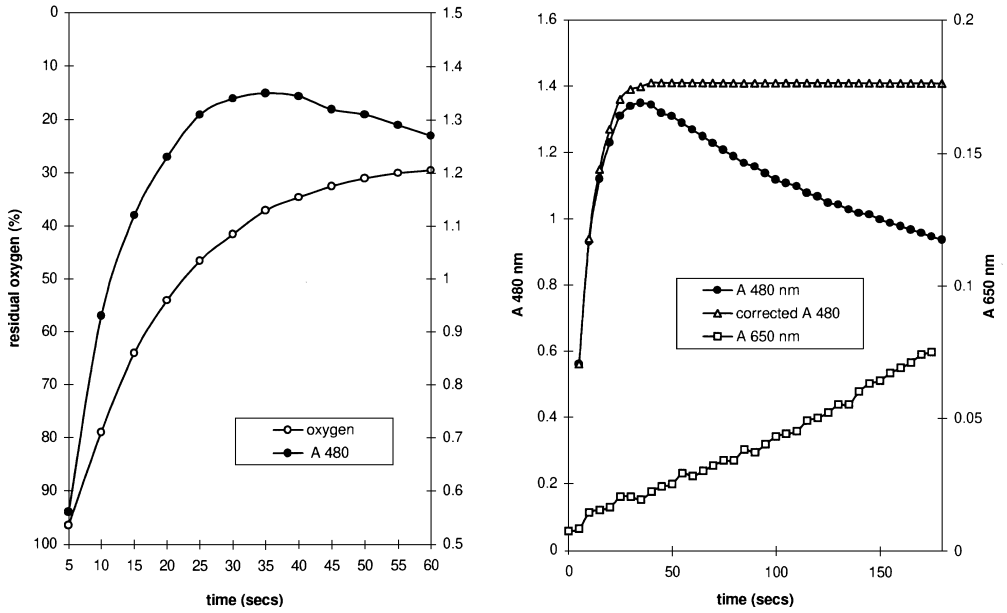


Figure 2 Tyrosinase-catalysed oxidation of DBC

The left-hand panel shows the oxygen uptake (○) by 600 nmol of DBC over a period of 1 min together with the A_{480} of the product measured (●). The measurements were taken at 5 s intervals starting at 5 s, to allow mixing of the substrate introduced into the enzyme solution. The right-hand panel shows spectral changes over a period of 180 s showing the fall in A_{480} (●) due to the decay of the quinomethane and the rise at 650 nm (□) due to the formation of the melanoid product. The A_{480} corrected for zero decay of the quinomethane (△) was calculated using the equation $A(corr)_t = A_t + 2kA^2_{t-1}$, where $2k = 38\ M^{-1} \cdot s^{-1}$.

was obtained where the corresponding *o*-quinone might be expected to have a maximum [14].

Since *o*-quinones tend to become longer-lived in more acidic conditions [15], the above experiment was repeated at pH 5.9 in order to try to obtain clear evidence of the *o*-quinone. In this case, after 9.0 ms, when all the semiquinone had disappeared, a species with a peak at 380 nm was observed (Figure 1a), which is assigned to the *o*-quinone formed via the reactions shown in reactions (1) and (2). At this pH, the *o*-quinone absorption was eventually replaced (k 1.0 sec^{-1}) with a more strongly absorbing species similar to that found at neutral pH.

The full spectrum of the species that formed over approx. 1 s at pH 7.1 is given in Figure 1(b). On the basis of the similarity of this spectrum to that of the quinomethane of 1,2-dehydro-*N*-acetyldopamine [16] and of morpholine-trapping experiments after treatment of DBC with the mild chemical oxidant di-anisyltellurium oxide [17], the spectrum shown in Figure 1(b) is assigned to the corresponding quinomethane formed via re-

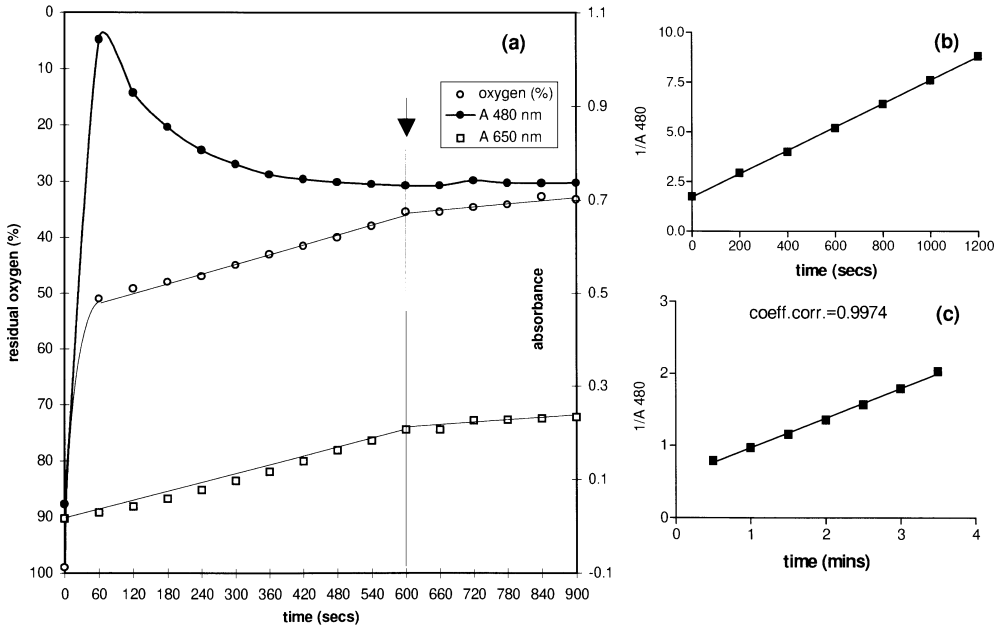
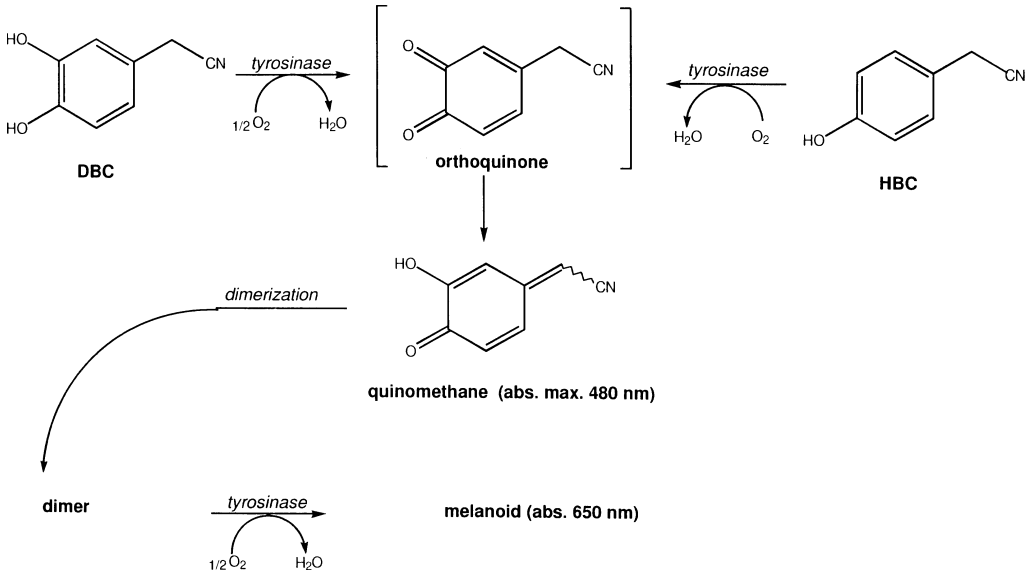


Figure 3 Decay of quinomethane and subsequent oxidation

(**a**) Data from an experiment in which 196 nmol DBC was oxidized by tyrosinase under the standard conditions of the assay. The Figure shows the A_{480}, the residual oxygen in the cuvette and the A_{650}, all at 1 min intervals. The oxidation of DBC is complete by the end of the first minute with the utilization of about 50% of the dissolved oxygen in the system (398 nmol). Thereafter, oxygen utilization (○) is nearly linear until 600 s, when the addition of acetic acid to the system halts the reaction. Decay of the quinomethane (●) is second order ($2k$ 38 $M^{-1} \cdot s^{-1}$). The increase in the A_{650} (□) parallels the oxygen uptake. The right-hand panels show the second-order kinetics of the initial quinomethane decay determined (**b**) for the product of radiation chemical oxidation of DBC and (**c**) for the tyrosinase-oxidation product. coeff. corr., correlation coefficient.



Scheme 2

Diagrammatic outline of tyrosinase-catalysed oxidation of HBC and DBC showing the rearrangement of the *o*-quinone product of the enzymic reaction to form the quinomethane product (absorbance maximum at 480 nm) with subsequent decay, possibly to a dimer, which is oxidized by tyrosinase to a melanoid product absorbing significantly at 650 nm.

actions (1) and (2), followed by the rearrangement shown in reaction (3).

In these pulse-radiolysis experiments, the quinomethane appeared to be stable for > 10 s. On the basis of thiocyanate dosimetry and taking into account the fact that the yield of quinomethane is half that of the initial yield of $Br_2^{\bullet -}$ radical [see reactions (1)–(3)], molar absorption coefficients of 23800 $M^{-1} \cdot cm^{-1}$and 6440 $M^{-1} \cdot cm^{-1}$ were obtained for the quinomethane wavelength maxima at 320 and 480 nm respectively.

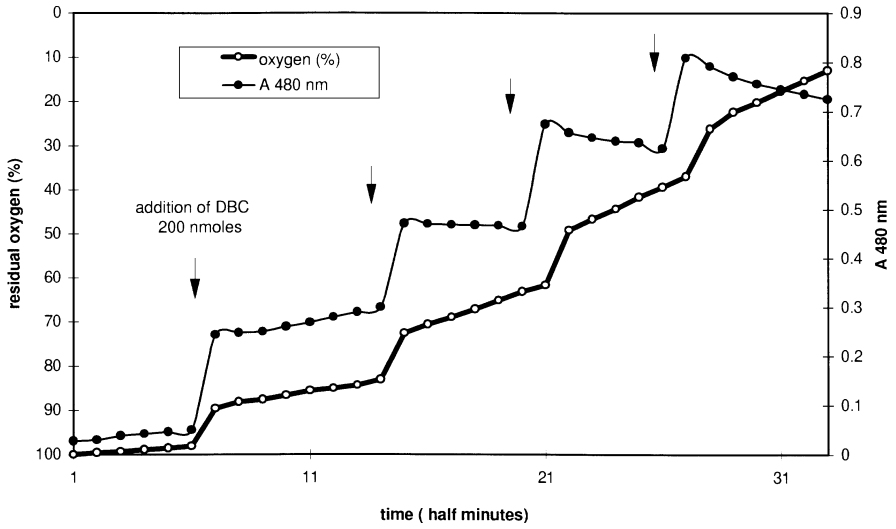


Figure 4 Tyrosinase-catalysed oxidation of HBC (600 nmol)

The graph shows the gradually increasing oxygen utilization rate (○) after successive additions of DBC (points of addition shown by arrows). The decay of the product measured at 480 nm (●) shows concentration dependence.

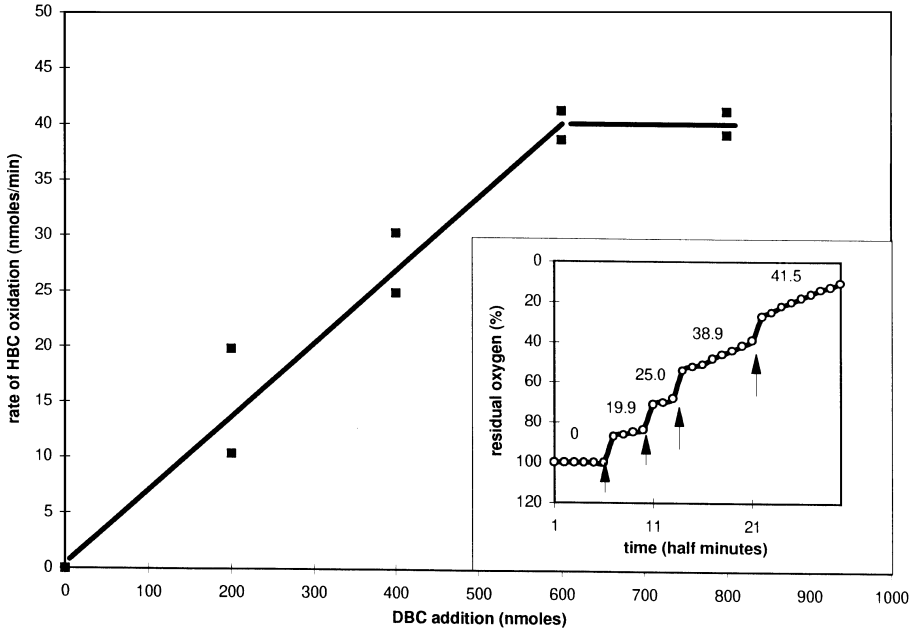


Figure 5 Effect of DBC addition on oxidation rate of HBC (600 nmol)

The calculated rates (■) of HBC oxidation after total DBC additions up to 800 nmol are shown. The data were derived from two separate experiments of the kind shown in the inset.

The decay of the quinomethane was examined over a longer period and was found to be second order (see Figure 3b) with $2k$ 38 $M^{-1} \cdot s^{-1}$.

Tyrosinase-catalysed oxidation

When DBC was used as substrate, tyrosinase-catalysed oxidation was rapid. The spectral characteristics of the product were ascribed to the quinomethane (Figure 1c). On the basis of complete conversion of the substrate to the quinomethane, the molar absorption coefficient at the absorption maximum of 480 nm was estimated as 5900 $M^{-1} \cdot cm^{-1}$ and comparison of the rate of oxygen utilization and generation of the product (Figure 2) gave a reaction stoichiometry of 0.504 ± 0.002 mol O_2 per mol of substrate, consistent with catechol oxidation.

The observed quinomethane product with absorbance peaks at 320 and 480 nm decayed with second-order kinetics, consistent with a dimerization reaction taking place (Figure 3c). This putative dimeric product acts as a secondary substrate for tyrosinase although the oxygen utilization by this secondary

reaction was slow compared with the initial DBC oxidation. The highest secondary oxidation rate measured was 16.6 nmol oxygen per min. The secondary oxygen utilization was linear and occurred concomitantly with the generation of a product absorbing at 650 nm (r 0.984). This product exhibited a broad melanin-like absorption spectrum and did not form in the comparable pulse-radiolysis experiments. Addition of acetic acid to denature the enzyme halted oxygen uptake, indicating that the oxidation of the putative dimer is principally the result of enzymic activity (Figure 3a). An experiment in which the oxygen consumption was followed to completion gave an overall stoichiometry of 0.72, consistent with the reactions:

$2\ DBC + O_2 \rightarrow 2\ QM + 2\ H_2O$ (tyrosinase-catalysed oxidation)

$2\ QM \rightarrow D$ (spontaneous dimerization)

$D + 0.5\ O_2 \rightarrow M + H_2O$ (tyrosinase-catalysed oxidation)

$2\ DBC + 1.5\ O_2 \rightarrow M + 3\ H_2O$ (overall)

where QM is quinomethane, D is dimer and M is melanoid product.

The nature of the soluble melanoid product absorbing at 650 nm was not determined, but we propose that it is formed by oxidation of the dimeric product of the quinomethane as suggested in Scheme 2. The structure of the dimer is currently under investigation.

When aliquots of the DBC were added to the enzyme solution each addition produced a transient rapid utilization of oxygen (Figure 4) in each case with a stoichiometry of approx. 0.5. The rate of product formation could not be accurately ascertained because of the decay of the quinomethane, the rate of which increased with increasing concentration, but from the rate of oxygen utilization the kinetic parameters for DBC oxidation were calculated as V_{max} 4900 ± 313 nmol·min^{-1} and K_m 374 ± 4 μM. The rate of secondary oxygen consumption between catechol additions was measured and the steady-state rate calculated from the linear slope.

When the corresponding monohydric phenol substrate (HBC) was added to the enzyme solution, in concentrations up to 164 μM there was negligible oxygen utilization. However, with the phenol present in the cuvette, successive additions of DBC resulted in an increased rate of HBC oxidation calculated as the difference between the actual oxygen utilization rate and the secondary oxidation of the DBC-derived dimer. From these data, a plot of the monohydric phenol-oxidation rate against the catechol addition (Figure 5) showed that the maximum rate of HBC oxidation was achieved after addition of equimolar DBC. This was the same as found for the initial oxygen consumption rate when the enzyme was pre-exposed to the catechol. These experiments gave estimated parameter values for HBC oxidation by activated enzyme of V_{max} 73 ± 2 nmol/min and K_m 309 ± 4 μM.

DISCUSSION

The data presented confirm the proposal that the lag period exhibited by tyrosinase in oxidizing monohydric phenol substrates is due to acceleration of the reaction by recruitment of inactive enzyme by oxidation of a catecholic intermediate substrate. The inactive monophenolase is considered to possess a bicupric active site (met-tyrosinase in the terminology of Lerch [2]), which is reduced by reaction with a catecholic substrate that is simultaneously oxidized to the corresponding quinone. Since the oxidation of the dihydric phenol substrate is mainly accounted for by oxygen utilization, only a small proportion can be oxidized by conversion of bicupric enzyme into the active oxygen-binding bicuprous (deoxytyrosinase) form. This may account for the results obtained when successive additions of DBC resulted in gradual enhancements of the rate of HBC oxidation, but measurements of the oxygen consumption during DBC oxidation steps were insufficiently consistent to enable the extent of the non-oxygen-requiring oxidation to be estimated. Given that the Michaelis constants of HBC and DBC are similar, the comparatively diminished activation effectiveness of smaller additions of the catechol might be interpreted as indirect evidence that there is competition by the monohydric phenol present in the reaction system for the active site of the met-enzyme. However, we have no evidence bearing directly on this point.

The monohydric phenol oxidation rate after each catechol addition was linear and showed no acceleration, i.e. exhibited no lag period. The explanation is that no significant amount of intermediate catechol is generated during the oxidation of HBC because of the rapid rearrangement of the o-quinone to form the corresponding quinomethane (Scheme 2), leaving no o-quinone that could be converted to a catecholic substrate by reduction.

The quinomethane has an optical absorption maximum at 480 nm with a molar extinction coefficient of 6440 M^{-1}·cm^{-1}. Pulse-radiolysis experiments demonstrate that the quinomethane forms rapidly by isomerization of the o-quinone. The spectral characteristics of the product formed enzymically were identical to those of the quinomethane generated by pulse radiolysis. The quinomethane is relatively stable, but the 480 nm peak decayed with a second-order rate constant $2k$ of 38 M^{-1}·s^{-1}. In the presence of tyrosinase it gave rise to a brown solution absorbing significantly at 650 nm. The nature of this product remains to be determined, but it is likely to consist of, or incorporate, an oxidized dimer of the quinone methide.

We have recently shown that if the cyclization product of the o-quinone is unable to reduce the quinone the lag kinetics of monohydric phenol oxidation by tyrosinase are abolished and acceleration of the reaction requires the addition of a catecholic substrate [8]. The experiments with cyano-substituted substrates provide further evidence that the unusual kinetic features of tyrosinase-catalysed oxidation of monohydric phenols derive from the indirect generation of a catecholic substrate, which is able to accelerate the reaction by a process of enzyme recruitment. This recruitment mechanism involves the two-electron reduction of the bicupric active site of met-tyrosinase by the catechol [18–21]. The catechol is formed indirectly by reduction of the o-quinone product of enzyme action. In the case of oxidation of the natural substrate (tyrosine), the o-quinone product, dopaquinone, undergoes a spontaneous reductive cyclization to cyclodopa, which acts as an endogenously generated reductant for dopaquinone. This rapid redox exchange gives rise to dopachrome and dopa [22]. Dopa is a substrate for tyrosinase and is able to be oxidized with the concomitant reduction of the copper atoms at the active site of enzyme in the bicupric (or met) state. In this way, enzyme molecules are converted to the 'deoxy' form, in which state they are able to bind molecular oxygen and oxidize the monohydric phenol substrate [23].

In the case of the lag phase exhibited by the oxidation of the non-cyclizing tyrosinase substrate, 4-hydroxyanisole, we have proposed that the reduction of the stable o-quinone product of tyrosinase oxidation [24,25] occurs by reaction of the o-quinone with nucleophilic species present in the reaction mixture, including the enzyme [26,27]. In the incubation mixture, the catechol can be formed by electrophilic attack on thiol and amino groups of the enzyme (or any other protein present). However, the resultant catechol is bound and thus unable to diffuse to the active site. It can nevertheless undergo a redox-exchange reaction with free o-quinone to form a diffusible

catechol able to reach the active site and activate the enzyme by reduction of the copper atoms in the manner we propose. The same argument applies to the reaction of enzyme with the quinomethane to give a bound catechol, but in this case there is no free *o*-quinone to react with it; hence no activation can take place.

The use of these tyrosinase substrates with a side chain substitution leading, on oxidation to the *o*-quinone, to rapid formation of the corresponding quinomethane and, hence, failure of auto-activation, enables the separate determination of the kinetic parameters of monohydric and dihydric phenol oxidation. The rate of HBC oxidation (V_{max} 73 nmol·min^{-1}) was found to be much slower than the corresponding rate of DBC oxidation (V_{max} 4900 nmol·min^{-1}). The apparent Michaelis constants for the two substrates were found to be similar [K_m (HBC) 309 μM; K_m (DBC) 374 μM].

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