Oxidation of monohydric phenol substrates by tyrosinase: effect of dithiothreitol on kinetics

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The effect of thiol compounds on the monophenolase activity of tyrosinase was investigated using 4-hydroxyanisole as the substrate and dithiothreitol (DTT) as the model thiol compound. We have demonstrated three actions of DTT on tyrosinase-catalysed reactions: (1) direct reduction of the copper at the active site of the enzyme; (2) generation of secondary, oxidizable species by adduct formation with the *o*-quinone reaction product, 4-MOB, which leads to an increase in the total oxygen utilization by the reaction system; and (3) reversible inhibition of the enzyme. We confirm our previous observation that, at approx. 10 mol of DTT/mol of enzyme, the lag phase associated with monohydric phenol oxidation by tyrosinase is abolished. We suggest that this is due to reduction of the copper at the active site of the enzyme by DTT, since (a) reduction of active-site copper *in situ* by DTT was demonstrated by [Cu(I)]₂-carbon

monoxide complex formation and (b) abolition of the lag at low DTT concentration occurs without effect on the maximum rate of reaction or on the total amount of oxygen utilized. At concentrations of DTT above that required to abolish the lag, we found that the initial velocity of the reaction increased with increasing DTT, with a concomitant increase in the total oxygen utilization. This is due to the formation of DTT–4-methoxy-*o*-benzoquinone (4-MOB) adducts which provide additional dihydric phenol substrate either directly or by reducing nascent 4-MOB. We present n.m.r. evidence for the formation of mono- and di-aromatic DTT adducts with 4-MOB, consistent with a suggested reoxidation scheme in the presence of tyrosinase. Inhibition of the enzyme at concentrations of DTT above 300 pmol/unit of enzyme was released on exhaustion of DTT by adduct formation with 4-MOB as it was generated.

INTRODUCTION

Tyrosinase [monophenol,L-dopa (dihydroxyphenylalanine): oxygen oxidoreductase, EC 1.14.18.1] is a copper-containing enzyme which catalyses the oxidation of tyrosine, the products of which reaction, by a series of subsequent enzymic and non-enzymic steps, polymerize to form melanin [1,2]. Tyrosinase-catalysed oxidation of tyrosine and of other monohydric phenols involves oxidation of the phenol to the corresponding *o*-quinone in a single step [3–6].

The active site contains a binuclear copper cluster in mushroom (*Agaricus bisporus*) and in human malignant-melanoma tyrosinase [6,7]. Most of the enzyme in a fresh tyrosinase preparation is in the 'resting' or met form in which the active site is bicupric. Only a relatively small proportion of the enzyme is present as the active monophenolase, oxytyrosinase, which is produced when the reduced, bicuprous active centre binds oxygen to form a bicupric-peroxide complex [5,8]. The initial rate of monohydric phenol oxidation is therefore slow unless a suitable two-electron reducing agent is exogenously supplied (e.g. dopa [9] or a thiol compound [10]). Generation of dihydric phenols from the *o*-quinone oxidation products provides an auto-activating mechanism for recruitment of the met enzyme to the monophenolase form and results in a gradually accelerating rate of monohydric phenol oxidation. When all the met-tyrosinase is recruited into the reaction, a maximum rate of monohydric phenol oxidation is achieved. This period of acceleration is referred to as the 'lag phase' [11]. Competition by the monohydric phenol substrate for the met enzyme results in reduction of the rate of recruitment of

the met enzyme by electron-donating substrates and hence increases the length of the lag phase.

The presence of the lag phase renders kinetic analysis of the enzyme difficult, as the initial velocity of the reaction at high monohydric phenol substrate concentration tends to zero. We have previously reported the use as a recruiting agent of small amounts of the thiol compound dithiothreitol (DTT) to reduce fully the met-tyrosinase to the oxygen-binding cuprous form before presentation of the monohydric phenol substrate [10]. This results in normalized kinetics in which the initial rate of reaction is directly proportional to the concentration of substrate and allows accurate calculation of Michaelis constants for oxidation of monohydric phenols [4].

The use of thiols for this purpose, however, has drawbacks, since there are other potential effects on the reaction system, e.g. direct inhibition of the enzyme by copper binding [11] or adduct formation by nucleophilic addition of thiol to the quinone reaction products, resulting in a change in absorbance maxima [12]. Anomalies in estimation of enzyme activity due to the latter effect are avoided by use of oximetric in addition to spectrophotometric techniques.

In this paper we report investigations of the effects of DTT on tyrosinase-catalysed oxidation of 4-hydroxyanisole as determined by oximetry with simultaneous spectrophotometry.

EXPERIMENTAL

General

A. bisporus tyrosinase, specific activity 2200 units/mg of protein

Abbreviations used: 4HA, 4-hydroxyanisole; 4-MOB, 4-methoxy-*o*-benzoquinone; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent); n.O.e., nuclear Overhauser effect; dopa, 3,4-dihydroxyphenylalanine; PBSA, phosphate-buffered saline (Dulbecco's A).

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(manufacturer's estimate), DTT and bathocuproine disulphonate were obtained from Sigma. 4-Hydroxyanisole (4HA) was obtained from Koch-Light, Haverhill, Suffolk, U.K., recrystallized from water (99% pure by g.l.c.) and stored at 4 °C. Except where stated, solutions were routinely made in PBS (Dulbecco's A) (PBSA; Oxoid, Basingstoke, Hants., U.K.), pH 7.4, which was treated with Chelex-100 chelating resin (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.). Sodium dithionite was obtained from BDH.

Measurement of $[Cu(I)]_2$ -CO fluorescence

Tyrosinase at 330 units/ml in PBSA with or without a 10 μ l aliquot of 2 mM DTT (20 pmol/unit of enzyme) in a final volume of 3 ml was de-aerated by exhaustive bubbling with nitrogen and sealed to exclude air. The solutions were then sparged for 5 min with CO (Air Products GB, Darlaston, West Midlands, U.K.), sealed to prevent re-aeration and the solution monitored to observe the development of the fluorescent $[Cu(I)]_2$ -CO complex in a Perkin-Elmer LS-3 fluorescence spectrometer over 15 min ($\lambda_{\text{excitation}}$ 285 nm; $\lambda_{\text{max., emission}}$ 522 nm; cf. *Neurospora* tyrosinase: $\lambda_{\text{excitation}}$ 285 nm; $\lambda_{\text{max., emission}}$ 550 nm [13]).

Oximetry

The standard reaction mixture most commonly employed comprised 2 ml of 100 μ M 4HA (200 nmol/reaction) and 165 units/tyrosinase added in a 200 μ l aliquot (330 units total). 4HA was used as a substrate rather than tyrosine, since the stoichiometry of the reaction is 1 mol of oxygen/mol of 4HA, whereas the stoichiometry of tyrosine oxidation is 1.5 mol of oxygen/mol of substrate [4]. This is due to the intramolecular cyclization of the initial product of tyrosine oxidation, dopaquinone, to form cyclodopa, from which, by redox reaction with dopaquinone, dopachrome and dopa are formed [14]. Dopa is a substrate for tyrosinase and is oxidized with the utilization of a further 0.5 mol of oxygen. 4HA is not subject to intramolecular cyclization, which, in addition to simplifying the stoichiometry and kinetics of the reaction, also leaves the electrophilic ring position of the *o*-quinone product available for other interactions, e.g. nucleophilic addition by DTT [10], which might be masked in the case of dopaquinone by competition with the cyclizing amino group on the side chain. The use of this model monohydric phenol substrate thus facilitates detection and allows accurate quantitation of thiol-quinone adducts by the measurement of the increased oxygen consumption by the enzyme due to their subsequent oxidation.

The reaction was allowed to proceed in an oxygen-electrode chamber at 25 °C. Oxygen utilization was monitored with a Biological Oxygen Monitor (YSI Inc., Yellow Springs, OH, U.S.A.; model 5300) using a standard oxygen probe (YSI; model 5331). The oxygen concentration in air-saturated PBSA was calculated from standard data to be 245 μ M (245 nmol/ml; 539 nmol/reaction). Electrical zero oxygen concentration was periodically verified using 2% sodium dithionite. No oxygen uptake was observed from a reaction mixture containing DTT and tyrosinase only.

(a) Preincubation of tyrosinase with DTT

The effect of preincubation of tyrosinase with a range of concentrations of DTT on the kinetics of tyrosinase-catalysed

oxidation of 4HA was investigated; 330 units tyrosinase in 200 μ l of PBSA was preincubated with a 10 μ l aliquot of one of a range of concentrations of DTT from 0.2 mM to 20 mM (6–600 pmol of DTT/unit of enzyme). The resulting mixture, after 5 min incubation at 25 °C, was added to 2 ml of 4HA in the oxygen-electrode chamber and oxygen consumption monitored.

(b) Determination of the DTT-4-methoxy-*o*-benzoquinone (4-MOB) adduct absorbance spectrum and measurement of DTT concentration during the reaction

A standard reaction mixture comprising 100 μ M 4HA and 165 units of tyrosinase/ml (preincubated with 600 pmol of DTT/unit of enzyme) in a total volume of 3 ml was incubated in a stirred quartz cuvette with a side arm into which was introduced an oxygen electrode. The progress of the reaction was monitored by oximetry and spectrophotometric scanning from 200 to 700 nm every 30 s (after an initial 30 s mixing time) by a Hewlett-Packard 8452A diode-array spectrophotometer and data processed by Hewlett-Packard HP 89532 u.v.-visible software in order to correlate appearance of the absorbance spectrum of 4-MOB with progress of the reaction. The absorbance spectrum of the DTT-4-MOB adduct was determined by subtracting the initial-product absorbance-spectrum scan (30 s) from the final scan before acceleration of the reaction rate (8 min).

For a series of identical reaction mixtures, repetitive spectrophotometric scans from 200 to 600 nm were made at 1 s intervals commencing 10 s before addition to the cuvette of 0.5 ml of a 5 mM solution of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent [15]) in PBSA. Scans continued until a stable absorbance at 412 nm was observed (about 20 s). This procedure allowed measurement and subtraction of any product absorbance at 412 nm (4-MOB; $\lambda_{\text{max.}}$ 420 nm) present immediately before addition of DTNB. The concentration of DTT was determined from a standard curve of A_{412} versus [DTT], which was linear between 1 and 100 μ M DTT (results not shown).

(c) Post-reaction addition of DTT

The formation of an oxidizable adduct by reaction of the primary product of tyrosinase-catalysed oxidation of 4HA with DTT was investigated. A standard reaction mixture was allowed to go to completion, i.e. when no further oxygen uptake is observed. This post-reaction mixture is effectively a solution of the product of tyrosinase-catalysed oxidation of 4HA, 4-MOB and tyrosinase. To this mixture was added immediately a 10 μ l aliquot of one of a range of concentrations of DTT and the subsequent oxygen consumption monitored.

N.m.r. spectroscopy

A 30 μ mol portion of DTT was dissolved in 0.6 ml of [2H_4]methanol and the n.m.r. spectrum determined at 35 °C, using the residual proton peak in the solvent at δ 3.30 p.p.m. for referencing. The n.m.r. spectrum was obtained in the presence of 10, 30 and 60 μ mol of 4-MOB. Addition of 4-MOB in excess was indicated by a permanent yellow colour due to the 420 nm absorbance of 4-MOB. Nuclear-Overhauser-effect (n.O.e.) and decoupling experiments were used to elucidate the nature of the products observed.

4-Methoxycatechol was prepared as described in [16]. Briefly, 10 ml of a 10% solution of 4-MOB in chloroform was shaken with an aqueous solution of Na_2HPO_4 (2.2 g) and sodium dithionite (6 g) in water (30 ml). The chloroform layer was separated and the aqueous phase extracted with 50 ml chloro-

form. The combined extracts were dried (over anhydrous MgSO_4), filtered and rotary-evaporated at 30 °C to give a black oil. The catechol was purified by flash chromatography on silica gel, detected with iodine vapour (red-brown colour reaction) and evaporated to give a near-white solid.

RESULTS

Measurement of $[\text{Cu(I)}]_2\text{-CO}$ fluorescence

After sparging de-aerated solutions of tyrosinase with CO , an increase in fluorescence at 522 nm, attributable to the $[\text{Cu(I)}]_2\text{-CO}$ complex, was observed. In the presence of DTT, the rate of fluorophore formation and peak fluorescence emission intensity was increased with respect to control levels in the absence of DTT (Figure 1). The increase in fluorescence in the non-reduced control enzyme preparation may be ascribed to exchange of oxygen in pre-existing oxytyrosinase for CO .

Oximetry

(a) Pre-exposure of tyrosinase to DTT

Pre-incubation of tyrosinase with DTT produced marked effects on the course of the subsequent enzyme-catalysed oxidation of 4HA (Figure 2a). DTT at a level of 30 pmol/unit of tyrosinase completely abolished the lag phase of the reaction without effect on the total oxygen uptake of the system. Addition of DTT above this level was accompanied by an increase in the total oxygen consumption of the reaction which was associated with an increase in the initial velocity up to the DTT level where inhibition of the enzyme became evident. A plot of excess oxygen consumed versus pmol of DTT/unit of enzyme added is linear, with a slope corresponding to 212 pmol of O_2 /pmol of DTT per

Figure 1 Demonstration of *in situ* reduction of active-site copper ions in tyrosinase by DTT by formation of $[\text{Cu(I)}]_2\text{-CO}$ complex under anoxic conditions

Plot of the rate of formation of the bicuprous-CO complex in tyrosinase measured by fluorescence (excitation 285 nm, emission 550 nm) in the presence of 2 mM DTT (—) and in the absence of reductant (.....).

Figure 2 Preincubation of tyrosinase with DTT

(a) The effect on tyrosinase-catalysed reaction kinetics of pre-incubation of tyrosinase with a range of concentrations of DTT. Tyrosinase (330 units) was preincubated with an aliquot of each of a range of concentrations of DTT, and oxygen consumption was monitored on addition of 200 nmol of 4HA. $\circ$, Control, no DTT in preincubation; $\triangle$, 30 pmol of DTT/unit of enzyme; $\square$, 150 pmol of DTT/unit of enzyme; $\bullet$, 300 pmol DTT/unit of enzyme; $\blacktriangle$, 600 pmol DTT/unit of enzyme. (b) Plot of excess oxygen consumed by a tyrosinase-catalysed reaction mixture containing 200 nmol of 4HA after preincubation of tyrosinase with a range of concentrations of DTT. $r^2 = 0.992$; slope = 212 pmol of O_2 /pmol of DTT/unit of enzyme; molar ratio $\text{DTT}/\text{O}_2 = 1:0.61$.

unit of enzyme ($r^2 = 0.992$), corresponding to a molar ratio (DTT/O_2) of 1:0.61. The plot does not pass through the origin (Figure 2b), which reflects the fact that no increase in oxygen consumption occurs until sufficient DTT has been added to abolish the lag phase. At 300 pmol of DTT/unit of enzyme some reversible inhibition of the enzyme became evident as the initial rapid rate of reaction gave way to a slower, intermediate phase, after which the rate of reaction accelerated to completion; at 600 pmol of DTT/unit of enzyme the intermediate phase was much more pronounced (Figure 2a).

(b) Determination of the DTT-4-MOB adduct absorbance spectrum and measurement of DTT concentration during the reaction

The 4-MOB-DTT adduct was shown by subtraction of the initial spectrum (30 s) from that immediately before acceleration of the reaction (8 min) to have absorbance maxima at 216, 238 and 320 nm (Figure 3a); the rate of formation of the adduct, monitored by absorbance at 320 nm is approximately linear until the reaction rate accelerates (Figure 3b). There is no accumulation of free 4-MOB until the reaction rate accelerates. The late

Figure 3 Determination of the absorbance spectrum of the DTT-4-MOB adduct and measurement of the concentration of DTT during the reaction

(a) Absorbance spectrum of the DTT-4-MOB adduct. Difference spectrum produced by subtracting the initial spectrum (30 s) from the last spectrum before acceleration of the reaction (8 min) when the adduct chromophore (A_{320}) reaches a maximum. (b) Measurement of the concentration of DTT and relationship to other reactant and product parameters through the course of the reaction: ▲, DTT (nmol; scaling factor $\times 10^{-3}$); ○, oxygen utilization (nmol; scaling factor $\times 2 \times 10^{-3}$); □, 4-MOB chromophore accumulation (A_{420} ; scaling factor $\times 1$); ■, DTT-4-MOB chromophore accumulation (A_{320} ; scaling factor $\times 1$). (c) A_{320}/A_{420} ratio in control and DTT-preincubated enzyme reaction systems. ●, Control reaction: 165 units tyrosinase/ml of reaction mixture; 100 μ M 4HA; no DTT in preincubation; ○, reaction (concentration of reactants as in control) after preincubation of tyrosinase with 600 pmol of DTT/unit of enzyme.

increase in absorbance at 320 nm is due to the bathochromicity of 4-MOB-derived polymeric species.

The concentration of DTT in the reaction mixture was observed to fall during the initial (rapid) and the intermediate phases of the reaction. Reduction of the concentration of DTT to zero coincided with acceleration in reaction rate, as indicated by both increased rate of oxygen utilization, cessation of accumulation of the DTT-4-MOB adduct chromophore at 320 nm and by the appearance of the 420 nm chromophore of free 4-MOB (Figure 3b).

A plot of the A_{320}/A_{420} ratio throughout a control reaction (tyrosinase preincubated without DTT) and a reaction catalysed by DTT-preincubated tyrosinase (600 pmol/unit of enzyme) clearly illustrates the marked contribution of the DTT-4-MOB

Figure 4 Addition of DTT to tyrosinase-catalysed reaction mixtures post reaction

(a) Oxygen-consumption curves showing the effect of post-reaction addition of a range of concentrations of DTT. ●, Oxygen consumption of a reaction mixture containing 200 nmol of 4HA and 330 units of tyrosinase. Oxygen consumption by the system on subsequent addition of: ○, 10 nmol of DTT; ■, 50 nmol of DTT; □, 100 nmol of DTT; △, 200 nmol of DTT. (b) Plot of excess oxygen consumption of a standard reaction mixture on post-reaction addition of a range of concentrations of DTT. Results are plotted from data shown in (a). $r^2 = 0.994$; slope = 0.876 nmol of O_2 /nmol of DTT.

adduct at 320 nm in the reaction catalysed by DTT-preincubated enzyme (Figure 3c). After the exhaustion of DTT at 8 min, the A_{320}/A_{420} ratio falls to that of the control. It should be noted that the DTT-inhibited reaction is of longer duration than the control.

(c) Post-reaction addition of DTT

Addition of an aliquot of DTT to a reaction mixture after complete oxidation of 4HA resulted in further oxygen consumption (Figure 4a). A direct, linear relationship (slope = 0.876, $r^2 = 0.994$) was found between DTT added and the subsequent oxygen consumed (Figure 4b). The plot passes through the origin, indicating that there is no minimum level of DTT addition before excess oxygen consumption occurs under these conditions.

N.m.r. spectroscopy

The initial spectrum of 30 μ mol of DTT in [2H_4]methanol showed peaks at δ 4.77 (s, exchangeable protons), δ 3.67 (t, J 5.2 Hz, $CHOH$), δ 3.3 (m, residual C^2H_2H in [2H_4]methanol) and δ 2.63 (d, J 5.2 Hz). The addition of 10 μ mol of 4-MOB, gave two new compounds (I) and (II) in the ratio of 56:44 estimated from the relative areas of the peaks. Compound (I) had 1-proton doublets at δ 6.48 and δ 6.37 (J 2.9 Hz), a methoxyl peak at δ 3.69 and

Figure 5 N.m.r. spectra in the regions (i) 2.85–3.10 and (ii) 6.19–6.53 p.p.m. of the products of the reaction between 4-MOB and DTT with various concentrations of 4-MOB

Spectrum a, 30 μ mol of DTT + 10 μ mol of 4-MOB; spectrum b, 30 μ mol of DTT + 30 μ mol of 4-MOB; spectrum c, 30 μ mol of DTT + 60 μ mol of 4-MOB.

CH_2S protons at δ 3.04 (dd, J 5.4, 13.4 Hz) and δ 2.91 (dd, J 7.6, 13.5 Hz). Compound (II) had 1-proton doublets at δ 6.44 and δ 6.36 (J 2.9 Hz), a methoxyl peak at δ 3.67 and CH_2S protons at δ 3.03 (dd, J 5.4, 13.4 Hz) and δ 2.90 (dd, J 7.6, 13.5 Hz) [Figure 5, (i) and (ii); trace a]. A second addition of 4-MOB, bringing the total amount added to 30 μ mol, caused the relative amounts of the two new compounds to change to 42:58 [Figure 5, (i) and (ii), trace b]. The putative structures of compounds (I) and (II) were confirmed by decoupling experiments in which selective and separate irradiation of the two lowest-field doublets reduced the corresponding higher-field doublets to singlets and an n.o.e. experiment in which each of the methoxyl peaks was selectively and separately irradiated showed an enhancement of both the aromatic protons of compounds (I) and (II). In addition to the peaks from compounds (I) and (II), others were also visible, of which one set could be positively identified as 4-methoxycatechol, δ 6.39 (d, 2.8), 6.23 (dd, 2.8, 8.6) and 6.65 (d, 8.6) by comparison with the reference sample. After the third addition of 4-MOB,

bringing the total amount to 60 μ mol (excess 4-MOB; permanent yellow colour due to the 420 nm chromophore), only peaks due to compound (II) were visible [Figure 5, (i) and (ii), trace c]. The proposed reaction mechanism for the n.m.r. experimental system and the structures of compounds (I) and (II) (structures [A] and [E] respectively) are shown in Scheme 1.

DISCUSSION

We have identified three discrete interactions of the thiol compound DTT in tyrosinase-catalysed oxidations. Small amounts of DTT abolish the characteristic lag phase of monohydric phenol oxidation without effect on the maximum rate of reaction or on the total oxygen consumption of the reaction mixture. We suggest that this effect is due to direct reduction of the copper at the active site of met-tyrosinase by the thiol, effecting the $\text{Cu(II)} \rightarrow \text{Cu(I)}$ transition required for oxygen binding to the active site in the recruitment of the enzyme to the active

Scheme 1 Proposed reaction mechanism for the production of species I and II in the n.m.r. reaction system (i.e. in the absence of tyrosinase)

Addition of DTT to the 6-position of 4-MOB (route *a*) results in the formation of the catecholic adduct [A], which is identical with species I identified by n.m.r. spectroscopy. As the relative concentration of 4-MOB is increased, the probability of addition of a second 4-MOB to the free thiol group of [A] increases (route *b*) to give structure [E], which is identical with species II identified by n.m.r. spectroscopy. Redox exchange (route *d*) between further 4-MOB and [E] results in the production of 4-methoxycatechol and structure [C]. 4-Methoxycatechol may also result from reduction of 4-MOB by DTT with the concomitant formation of oxidized DTT [DTT(ox)] (route *e*). Key to routes indicated in Schemes 1 and 2: (*a*) addition of DTT to 4-MOB; (*b*) addition of 4-MOB to the catecholic DTT monoadduct [A]; (*c*) addition of 4-MOB to the quinonoid DTT monoadduct [B]; (*d*) redox exchange at the expense of reduction of 4-MOB to 4-methoxycatechol; (*e*) reduction by DTT without adduct formation (to form oxidized DTT).

monohydric phenolase, oxytyrosinase. Evidence for reduction *in situ* is provided by the effect of DTT on the formation of the fluorescent $[\text{Cu(I)}]_2\text{-CO}$ complex formed by displacement of oxygen in the oxytyrosinase complex by CO under anoxic conditions [13]. Deoxygenation of oxytyrosinase $\{[\text{Cu(II)}]_2\text{-O}_2^{2-}\}$ leaves the binuclear active centre in the bicuprous $\{[\text{Cu(I)}]_2\}$ state, allowing fluorophore development on binding of CO. The rate and extent of fluorophore formation reflects the amount of oxytyrosinase in the enzyme sample. The formation of the fluorophore in the control may be ascribed to the displacement of oxygen from the control level of oxytyrosinase by CO; in the DTT-exposed enzyme this is augmented by the presence of additional oxytyrosinase which is formed as a result of reduction of met-tyrosinase under the initial aerobic conditions.

A second effect of DTT on the tyrosinase-catalysed oxidation of 4HA was evident from the kinetics of oxygen consumption in the presence of higher concentrations of DTT. After the abolition of the lag phase, higher concentrations of DTT led to an increase in the total oxygen consumption of the system and to an increase in the initial velocity of the reaction until inhibition of the

enzyme became evident (Figure 2a). Increased oxygen consumption denotes an increase in available, oxidizable substrate; increased initial velocity indicates the presence of a substrate of higher affinity for tyrosinase than 4HA. Since *o*-dihydric phenols (catechols) are substrates for met- as well as for oxy-tyrosinase, and hence oxidized without an intervening lag phase [17], both these criteria are met by the proposal that a thiol-quinone adduct is formed which either (i) is itself a dihydric phenol substrate for tyrosinase, or (ii) undergoes a redox reaction with 4-MOB to generate 4-methoxycatechol, which is then reoxidized rapidly to 4-MOB (see Scheme 2). N.m.r. spectroscopy confirmed the formation of thiol-quinone adducts, with initial addition of the thiol at position 6 of the quinone ring (see Scheme 1). Steric hindrance due to the bulky group at position 6 might interfere with the ease of access of the catechol-thiol adduct to the active site of the enzyme, favouring the proposition that additional dihydric phenol substrate is generated via a redox mechanism (Schemes 1 and 2, route *d*). The excess oxygen utilization (above that required to oxidize all the 4HA present to 4-MOB) was directly proportional to the amount of DTT added in a DTT/ O_2

Scheme 2 Proposed reaction mechanism for DTT–4-MOB adduct formation and subsequent oxygen utilization by tyrosinase

Addition of DTT to the 6-position of 4-MOB results in the formation of the catecholic adduct [A], as in Scheme 1. [A] may act directly as a substrate for tyrosinase, requiring $\frac{1}{2}\text{O}_2$ for reoxidation to the *o*-quinone, [B], or, which is the more likely due to the potential steric effects of the 6-position substitution, may undergo a redox reaction with free 4-MOB to give 4-methoxycatechol and [B]. 4-Methoxycatechol is then reoxidized to 4-MOB. When 4-MOB is in relative excess, the probability increases of addition of a second 4-MOB to the free thiol group of [B] (route *c*), potentially resulting in the formation of a DTT-bridged structure [C]. [C] may act as a direct catecholic substrate for tyrosinase, requiring $\frac{1}{2}\text{O}_2$ for reoxidation, but steric effects again favour redox exchange with 4-MOB (route *d*) to form 4-methoxycatechol and compound [D]. This results, theoretically, in the utilization of a total of 1 mol of O_2 /mol of DTT. Due to the oxidative activity of tyrosinase the oxidation state of the adducts in the presence of the enzyme tends toward the quinonoid rather than the catecholic form.

molar ratio of 1:0.61, indicating that, due to the relative excess of DTT, a single reductive addition to 4-MOB per DTT molecule predominates (route *a*, Scheme 2), producing directly, or by redox reaction, the corresponding catechol (route *d*), which requires 0.5 molecule of O_2 for reoxidation (Scheme 2).

The plot of DTT versus O_2 (Figure 2b) does not pass through the origin. We interpret this as a further demonstration of direct reduction of the enzyme copper by DTT. No excess oxygen consumption occurs, indicating that no reduction of 4-MOB takes place until a threshold amount of DTT has been exceeded. In this instance, the stoichiometry is approx. 10 mol of DTT/mol of tyrosinase, although the absolute value is dependent on the redox state of the enzyme preparation used. Despite this clear demonstration of *in situ* copper reduction by DTT and the subsequent 'normalizing' effect on the kinetics of monohydric phenol oxidation by tyrosinase, there are practical limitations to

the use of DTT as a tyrosinase-recruiting strategy, owing to both the variability of preparations of the enzyme and to the complicating effects of excess thiol on the products of the reaction.

The production of an additional oxidizable substrate for tyrosinase on reaction of DTT with 4-MOB was investigated by addition of DTT to a reaction mixture after complete oxidation of 4HA to 4-MOB. No evidence for reduction of the quinone by intramolecular cyclization of DTT to the oxidized form was adduced, since no increase in A_{284} was observed; at this wavelength oxidized DTT has a pronounced maximum [18]. A burst of further oxygen consumption was produced on addition of DTT, owing to DTT–4-MOB adduct formation followed immediately by oxidation of the adduct or of another catechol generated by redox reaction. The amount of oxygen consumed on addition of DTT was directly proportional to the amount of DTT added. The molar ratio of DTT added to oxygen consumed

was, in this case, 0.86:1, which reflects the finding from n.m.r. data that the frequency of a second reduction per DTT molecule increases in the presence of excess 4-MOB (route *b*, Scheme 1).

Thirdly, we observed an apparently reversible inhibition of the enzyme by higher concentrations of DTT. In the preincubation experiments, in addition to the abolition of the lag phase, concentrations of DTT above 150 pmol/unit of enzyme produced a change in the progress of the reaction, such that the initial rate was not maintained and a slower phase ensued, followed by an acceleration to a rate similar to the initial velocity (Figure 2a). The ability of thiol compounds to inhibit tyrosinase by chelating the copper at the active site has been known for some time [11]. From these data it is clear, however, that the inhibition is reversible and is associated with increased overall oxygen consumption of the system in a manner directly proportional to the concentration of the thiol. In the preincubation at 600 pmol of DTT/unit of enzyme the oxygen utilization trace shows that the enzyme is inhibited after a short, initial lag-free phase. It may be that the oxytyrosinase active site is not accessible to DTT, possibly due to the presence of oxygen, and that only after the first round of substrate oxidation is thiol-binding possible and hence the inhibition is not immediately exerted. The slow second phase of the reaction represents the residual substrate oxidation rate of the DTT-inhibited enzyme, which persists until sufficient enzyme-generated *o*-quinone has been formed to remove DTT from the mixture in adduct form. During the slow inhibited phase of the reaction the DTT-quinone adduct (A_{320}) accumulated in a linear manner, whereas no free 4-MOB (A_{420}) was evident (Figure 3b). On exhaustion of free DTT, the A_{320} reaches a plateau and the reaction accelerates to a rapid, uninhibited rate. Further increase in A_{320} is due to the bathochromicity of 4-MOB-derived polymeric species. This phenomenon is reflected by the return to the control ratio A_{320}/A_{420} (Figure 3c).

This study extends the observations on the effects of thiol

compounds on tyrosinase-catalysed oxidation reactions previously reported [10]. We have now demonstrated directly three actions of DTT on tyrosinase-catalysed reactions: (1) direct reduction of the copper at the active site; (2) formation of oxidizable species subsequent to adduct formation with the *o*-quinone primary reaction product; and (3) reversible inhibition of the enzyme by DTT.

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