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THE STOICHIOMETRY OF TYROSINASE-CATALYZED OXIDATION OF 4-HYDROXYANISOLE

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Oxygen utilisation during tyrosinase-catalysed oxidation of 4-hydroxyanisole was investigated using an electron spin resonance technique which employs quantitative changes in the characteristics of the electron spin resonance spectrum of the spin label 3-carbamoyl-2,5-dihydro-2,2,5-5-tetramethyl-1-H-pyridoyl-1-yloxy (CTPO) to follow changes in the oxygen concentration. Reaction mixtures containing mushroom tyrosinase (15 $\mu g\, ml^{-1}$) and differing initial concentrations of 4-hydroxyanisole in aerated phosphate buffer at pH 6.8 were incubated at room temperature. The ratio of utilisation of oxygen was found to be in approximately 1:1 molar ratio with the initial 4-hydroxyanisole concentration in the reaction mixture between 50 and 200 μmol/l 4-hydroxyanisole. The results are consistent with the stoichiometry of oxygen utilisation being accounted for by the oxidation of 4-hydroxyanisole to anisyl quinone.

KEY WORDS: Semiquinone, oxygen, tyrosinase, stoichiometry, 4-hydroxyanisole, ESR; spin label.

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INTRODUCTION

The biological actions of 4-hydroxyanisole (4-HA) may be divided into two main categories, direct and indirect. Direct actions, which appear to be independent of the metabolism of the phenol, include effects on cells which are ascribed to the anti-oxidant action of hydroxyanisole, influences on the composition of the cell surface,[1] and inhibition of DNA synthesis.[2] Indirect actions include those phenomena which stem from the effects of the metabolites of 4-HA. In general, the active metabolites of 4-HA are its oxidation products. In the case of metabolism occurring in the endoplasmic reticulum or in mitochondria, the nature of the oxidation products has not been established.[3] In the case of tyrosinase-catalysed oxidation of 4-hydroxyan-isole it has been shown that the first oxidation product is the anisyl orthoquinone.[4] A number of possible reactions of the orthoquinone leading to other products, including oligomers and polymers of the quinone, have been proposed. In particular, there is an area of controversy regarding the possible formation of a paraquinone derivative through the para hydroxylation of the orthoquinone to give a trihydric

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phenol[5,6] and it has previously been pointed out[7] that if all the substrate is converted to paraquinone there should be a ratio of approximately 3:2 in the utilisation of oxygen compared with 4-hydroxyanisole because a re-oxidation of the trihydroxyanisole would be involved (Figure 1). An alternative indirect mechanism involving a redox interaction between the anisyl quinone and the trihydric phenol yielding a paraquinone species and 3,4-dihydroxyanisole which is re-oxidised by tyrosinase to anisyl quinone[6,7] results in the same stoichiometry. For simplicity the reaction sequence illustrated assumes direct oxidation of the postulated trihydric phenol by tyrosinase to generate the corresponding orthoquinone. The experiments reported in this communication were carried out to investigate the stoichiometry of oxygen utilisation during the tyrosinase-catalysed oxidation of 4-hydroxyanisole.

MATERIALS AND METHODS

The oxygen utilisation during the tyrosinase-catalysed oxidation of 4-HA was investigated using an electron spin resonance (ESR) technique described by Backer *et al.*[8] and modified by Sarna *et al.*[9] Mushroom tyrosinase (Grade III, obtained from the Sigma Chemical Co.) and 4-hydroxyanisole (obtained from Koch-Light Ltd. and recrystallised from water) were dissolved in 0.2 mol/l phosphate buffer, pH 6.8. Tyrosinase was used at a concentration of 15 μg/ml, equivalent to 33.45 units/ml (one unit is defined as a difference in optical absorbance at 280 nm of 0.001 per minute in a 3 ml reaction mixture containing L-tyrosine at pH 6.5 at 25°C). The spin label 3-carbamoyl-2,5-dihydro-2,2,5,5-tetramethyl-1-H-pyridoyl-1-yloxy (CTPO, Aldrich Chemical Co.) was dissolved in the solution of 4-hydroxyanisole and used at a final concentration of 1×10^{-4} mol/l. Electron spin resonance measurements were carried out on a Varian E3 X band spectrometer. Small volumes of 4-hydroxyanisole solution

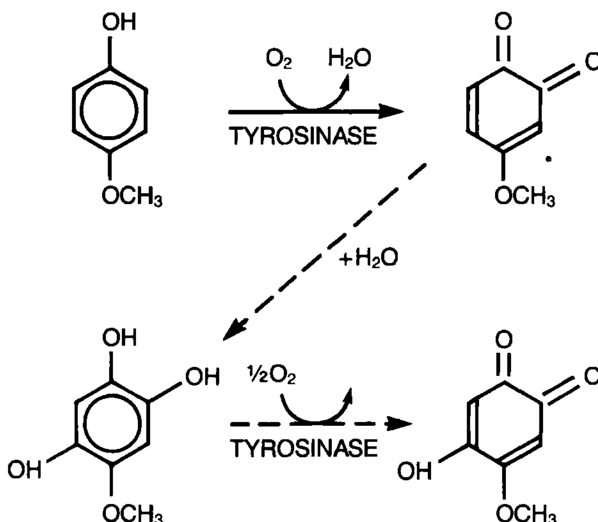

FIGURE 1 *Schematic outline of the possible reaction sequence leading to the formation of the postulated trihydric phenol.* The first step shows the tyrosinase-catalysed oxidation of 4-hydroxyanisole to the corresponding orthoquinone. The second step shows a water addition to the molecule with the formation of the trihydric phenol and the third step involves the reoxidation of the trihydric phenol.

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containing the CTPO and tyrosinase solution were mixed on a microscope slide and 100μl immediately transferred using a micro syringe into a quartz sample container. The sample chamber has internal dimensions of 0.5 × 8 × 24 mm with 0.5 mm diameter injection ports at each end and was mounted on a perspex strip enabling the chamber to be aligned in the resonance cavity. The sample injection ports were sealed with wax and the chamber placed in the resonance cavity of the spectrometer. The initial concentration of oxygen dissolved in the reaction mixture was calculated taking into account the atmospheric pressure and the concentration of the dissolved reagents. Changes in the concentration of oxygen in the reaction mixture were established on the basis of quantitative characteristics of the CTPO electron spin resonance spectrum, using a calibration curve as previously described by Sarna *et al.*[9]

RESULTS AND DISCUSSION

The total amount of oxygen utilised was examined at different substrate concentrations, taking the final point of the oxidation as the plateau value of oxygen concentration. The results are illustrated in Figure 2, which shows the stoichiometry of

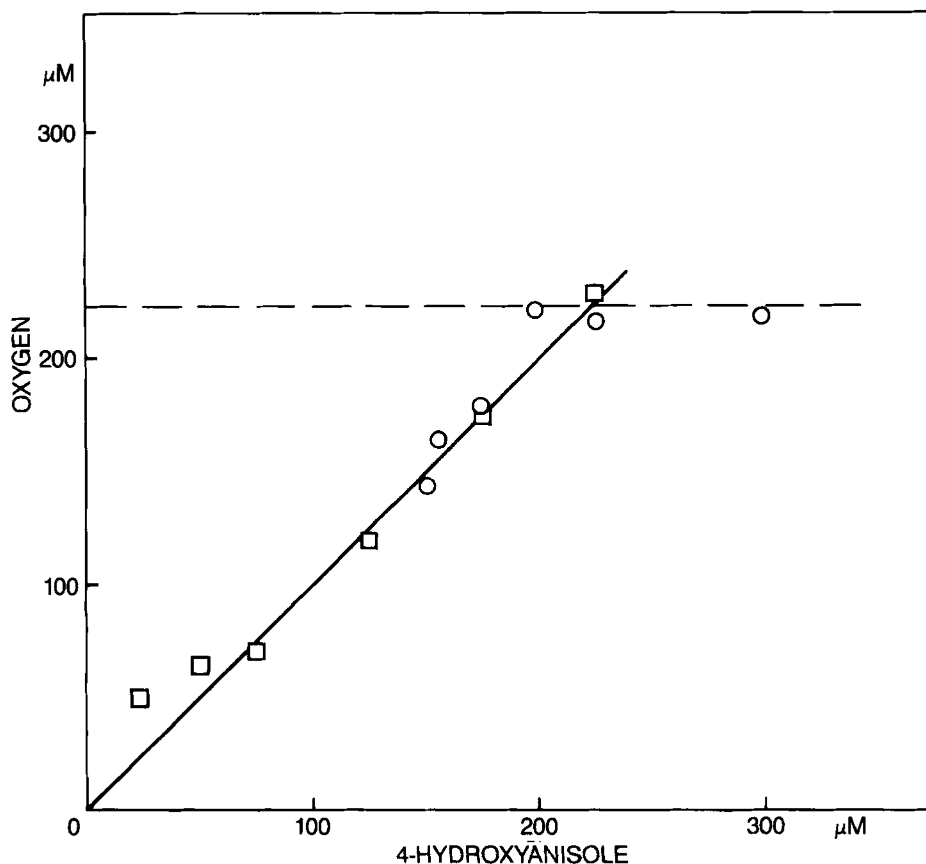

FIGURE 2 *Oxygen utilisation as a function of the initial concentration of 4-hydroxyanisole.* The data from two sets of experiments are shown (○ and □). The broken line indicates the oxygen saturation of the incubation mixture (233 μmol/l).

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oxygen utilisation as a function of the initial hydroxyanisole concentration in the tyrosinase-catalysed oxidation mixture. In two separate sets of experiments a range of 4-hydroxyanisole concentrations between 20 and 300 μmol/l in 0.2 mol/l buffer were incubated at 24°C in the presence of 15 μg/ml of mushroom tyrosinase and the total oxygen utilised measured at the end of the reaction. Air-saturated conditions were used giving an initial oxygen concentration in the incubation mixture of approximately 223 μmol/l which prevented further oxidation of 4-HA beyond this concentration. With the possible exception of the lowest concentration of 4-hydroxyanisole (20 μmol/l) the ratio of utilisation of oxygen was found to be in an approximately 1:1 molar ratio with the initial 4-hydroxyanisole concentration in the reaction mixture. We ascribe the deviation from linearity at low concentrations of 4-hydroxyanisole to difficulties in the accurate measurement of small reductions in oxygen concentration but we cannot exclude the possibility of an altered mechanism of oxygen utilization at 4-hydroxyanisole concentrations below 50 μmol/l. The ratio of substrate concentration to tyrosinase in our experiments was between 1.3 μmoles/mg of enzyme at the lowest substrate concentration and 13 μmoles/mg at the highest substrate concentration. The concentration ratio in the experiments of Nilges and Swartz[10] was 20 μmoles per mg of tyrosinase. Thus, their conditions resemble those pertaining to the upper range of substrate concentrations in our experiments, in which the results demonstrate 1:1 stoichiometry. In control experiments, in which no enzyme was present, no autoxidation of 4-hydroxyanisole was detected. We conclude that the oxygen used during the tyrosinase-catalysed oxidation of 4-hydroxyanisole is required only for the production of the orthoquinone and is not implicated in any subsequent reactions. Thus, the proposal that oxygen is required for the reoxidation of the putative trihydric phenol does not receive support from these results. Nilges and Swartz[10] have demonstrated that the semiquinone radical signal produced during the oxidation of 4-HA by tyrosinase[6] is inversely related to the oxygen concentration and have interpreted this finding in terms of a cycle of reduction of anisyl quinone to the corresponding hydroquinone with simultaneous oxidation of the postulated trihydric phenol intermediate. Nilges and Swartz[10] have proposed that the anisyl semiquinone is formed by reverse dismutation of the anisyl 3,4-quinone and the corresponding quinol and that, as a consequence, the steady-state concentration of semiquinone would remain low while oxygen is available because the hydroquinone concentration would be negligible due to its rapid reoxidation by tyrosinase to the quinone. However, such a process would consume additional oxygen and the results of the present study of the stoichiometry of oxygen utilisation indicate that the oxygen used is accounted for by the oxidation of 4-HA to anisyl quinone. Recent pulse radiolysis studies[11] of anisyl-3,4-semiquinone have demonstrated that the semiquinone is unreactive towards oxygen with a rate constant $\leqslant 10^5 M^{-1} s^{-1}$. Thus, the role of oxygen as a regulator of the semiquinone concentration is obscure.

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