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STUDIES ON THE KINETICS OF OXIDATION OF 4-HYDROXYANISOLE BY TYROSINASE

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Abstract—The potential use of 4-hydroxyanisole as a chemotherapeutic agent in the treatment of malignant melanoma led us to investigate the kinetics of oxidation of this tyrosine analogue by tyrosinase. We found that addition of amino acids accelerated the reaction, resulting in a reduction in length of the characteristic lag period of monohydric phenol oxidation. The lag period was abolished completely by an aliquot of exhausted 4-hydroxyanisole/tyrosinase reaction mixture and by very low concentrations of thiol-containing compounds. We conclude that the reaction-accelerating property of non-thiol amino acids is due to the reductive addition of the ortho-quinone reaction product to nucleophilic groups of the amino acids. The dihydric phenol product which results is capable of met-tyrosinase recruitment by electron donation to the cupric active site generating the cuprous form of the enzyme which binds oxygen and is able to oxidise monohydric phenols. Abolition of the lag period by an aliquot of exhausted reaction mixture is probably due to recruitment of the met-enzyme by catecholic oligomers of the quinone product. Thiol containing compounds are able to abolish the lag period due to the ability of these compounds to reduce met-tyrosinase directly.

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing enzyme widely distributed in nature from bacteria to mammals and is mainly involved in melanin biosynthesis and the synthesis of other polyphenol compounds [1]. Tyrosinase is a monooxygenase or mixed function oxidase, so called because it incorporates one atom of oxygen per mole of substrate, the other atom of oxygen being reduced to form water in the course of catalysing both the ortho-hydroxylation of monohydric phenols and the oxidation of the resulting ortho-dihydric phenol to the corresponding ortho-quinone [2].

The copper-containing active site is binuclear in mushroom (*Agaricus bisporus*) tyrosinase [3–5] and in tyrosinase from human malignant melanoma [6]. Most of the tyrosinase in a fresh preparation is in the oxidised or met-form in which copper is present as the cupric ion [7]. Only a small proportion, from 2 to 30%, of the cuprous enzyme which binds oxygen to form oxy-tyrosinase which is then able to oxidise monohydric phenols, is present; hence the initially slow rate of quinone production from monohydric phenols. Dihydric phenols (e.g. dopa; [8]) act as accelerators of monohydric phenol oxidation by reducing met-tyrosinase to the cuprous enzyme which binds oxygen to form oxy-tyrosinase. The dihydric phenol is concomitantly oxidised to the corresponding quinone.

Oxidation of monohydric phenols by tyrosinase is independent of exogenous dihydric phenols once the met-enzyme is activated by an appropriate electron donor. However, increasing monohydric phenol concentration lengthens the lag period because of competition for met-tyrosinase, so reducing the rate of activation by the dihydric phenol. There is also some competition by dihydric phenols for oxy-tyrosinase, resulting in quinone formation and the regeneration of the met-enzyme, although the net effect of dihydric phenols is, in all known cases, stimulatory with respect to the rate of monohydric phenol oxidation [9].

We have investigated the kinetics of mushroom tyrosinase using 4-hydroxyanisole (4HA), a tyrosine analogue of potential chemotherapeutic benefit in malignant melanoma [10–12]. This compound, like tyrosine, is a monohydric phenol and a substrate for tyrosinase. Oxidation of 4HA by tyrosinase *in vitro* produces 4-methoxy ortho benzoquinone [13], an extremely cytotoxic species in a model system [14] and which is strongly implicated in the anti-melanoma activity of 4HA. An understanding of the control of tyrosinase activity would allow potentiation of the conditions for 4HA administration and permit maximum therapeutic benefit to be gained.

MATERIALS AND METHODS

Mushroom (*Agaricus bisporus*) tyrosinase, specific activity 2300 units per mg protein (manufacturer's estimate) was obtained from Sigma Ltd. (Poole, Dorset, U.K.). 4-Hydroxyanisole was obtained from Koch-Light Ltd. (Poole, Dorset, U.K.) and recrystallised from water to remove a brown oily residue which forms on storage, probably from autoxidation. The recrystallised form was shown to be >99% pure by gas chromatography. The purified form was stored at 4°. Lysine, arginine, cysteine and dithiothreitol (DTT) were obtained from Sigma Ltd. Solutions of reagents were made in phosphate buffered saline (Dulbecco's A) (PBSA) (Oxoid Ltd., U.K.) at pH 7.4.

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Ultraviolet/visible spectra were recorded using a Pye-Unicam SP8-400 dual beam spectrophotometer. The reaction was monitored by the appearance of the product of the reaction, 4-methoxy ortho benzoquinone, which absorbs at 260 nm, against a blank cuvette containing PBSA alone.

Enzyme kinetic studies

A standard reaction mixture contained 100 μM 4HA and 100 μg/ml tyrosinase in a final reaction volume of 3 ml. The volume of the reaction mixture and concentrations of the reactants were adjusted to allow for any further additions made.

Analysis of enzyme kinetics

Figure 1 shows a typical trace of absorbance at 260 nm (4-methoxy ortho benzoquinone accumulation) versus time for a reaction mixture containing 100 μM 4HA and 100 μg/ml tyrosinase. The length of the lag period was measured as the time from initiation of the reaction to the maximum rate of reaction, R_{max}. R_{max} was determined as the point at which the curve of 260 nm absorbance met the tangent to the steepest part of the curve.

(i) *Effect of reaction products.* A reaction mixture containing 100 μM 4HA and 30 μg/ml tyrosinase was allowed to proceed to completion, so that no further increase in absorbance at 260 nm was observed. A 200 μl aliquot was immediately removed from this mixture and added to a 100 μM solution of 4HA and 100 μg/ml tyrosinase added. The rate of reaction of the mixture was monitored at 260 nm.

(ii) *Effect of non-thiol amino acids.* The reaction was allowed to proceed in the presence of alanine, lysine or arginine at concentrations of 100, 200, 500 or 1000 μM and alanine at 2000 μM and the rate of product formation monitored at 260 nm. Results were expressed as a percentage of the length of the lag phase in a control standard reaction mixture.

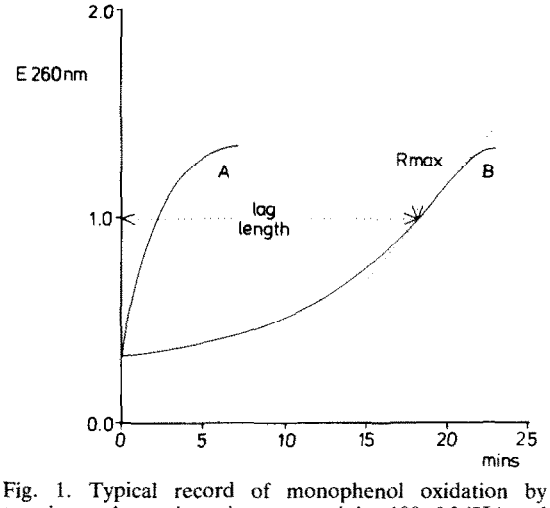

Fig. 1. Typical record of monophenol oxidation by tyrosinase. A reaction mixture containing 100 μM 4HA and 100 μg per ml tyrosinase (total volume 3 ml) was allowed to proceed at room temperature in a spectrophotometer cuvette. Trace A: plus 200 μl exhausted reaction mixture (see Materials and Methods (i)); trace B: plus 200 μl PBSA. The absorbance of the quinone product was monitored at 260 nm.

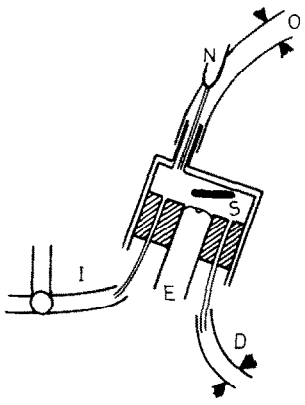

Fig. 2. Schematic outline of the arrangement of the oxygen electrode chamber. E, electrode tip; S, stirrer bar; I, inlet tube; D, drainage tube; O, air outlet tube; N, syringe needle.

(iii) *Effect of thiol-containing compounds.* The reaction was allowed to proceed in the presence of cysteine at 1, 10, 50, 100, 200 and 500 μM and DTT at 5, 10, 50, 100, 150 and 200 μM and the appearance of product monitored. The results were expressed as % control lag phase length.

(iv) *Oxygen electrode study of the effect of DTT on reaction kinetics.* To enable studies to be undertaken that required a high concentration of enzyme (due to inactivation of the enzyme by products of the reaction) an oxygen electrode chamber of small volume was constructed. The probe tip of an EIL oxygen electrode, comprising an oxygen sensor capsule (8012 17C) connected to a PTI-401 dissolved oxygen meter, was inserted into a nylon ring and sealed with Teflon tape inside the end of a polycarbonate 20 ml disposable syringe. The chamber volume was adjusted to 2 ml and mixing was ensured by placing a 6 mm Teflon coated metal stirrer bar inside the chamber and agitating it with a magnetic stirrer placed vertically beside the chamber. The substrate solutions, after saturating with air, were introduced into the chamber through thin tubing inserted through a hole in the nylon ring. Another tube inserted through a similar hole at 180° to the inlet tube acted as a drain. The chamber was mounted at an angle of about 15° to the vertical so that the drainage tube was on the lower side and the inlet tube situated underneath the syringe outlet which was fitted with a short length of clamped silicon tubing. The enzyme solution was injected from a graduated 1 ml syringe through a fine needle threaded through the wall of the syringe outlet tubing so that the point of the needle was level with the end of the syringe barrel (Fig. 2). The procedure for filling the electrode chamber was to clamp the lower outlet tubing and release the clamp on the upper outlet tubing to allow displacement of air. The substrate solution was carefully injected via a three-way tap and bubbles eliminated through the top tube which was then clamped and the lower drainage tube unclamped to ensure that the chamber remained at atmospheric pressure during subsequent additions. When the output signal from the oxygen electrode

had stabilised, the enzyme solution was introduced through the needle into the top of the chamber. With the stirrer setting employed, the mixing time was about 1 sec. To minimise dilution effects the enzyme was added in 100 μl aliquots in most of the experiments reported. The displaced fluid from the bottom of the chamber passed into the drainage tube. The time of the addition of the enzyme was marked on the chart recorder linked to the output of the oxygen electrode. On termination of the experiment the chamber was washed through several times with distilled water and allowed to drain. The instrument was calibrated to give a reading of 100 mV when the buffer solution was air saturated (approx. 210 μM O_2).

A mixture containing 200 μM 4HA and 50 μg/ml tyrosinase was allowed to react in the oxygen electrode chamber. A molecular weight of 30,000 Da was assumed for each monomer of the enzyme, each monomer bearing one copper atom [9]. This gives an enzyme concentration in the reaction mixture of 0.0017 μM or 1.7 nmoles/ml, a total in the reaction volume of 3.4 nmoles. Assuming one binuclear copper active site per 2 enzyme monomers, 1.7 nmoles of active sites are predicted. The effect of a range of concentrations of DTT on the kinetics of the reaction was investigated.

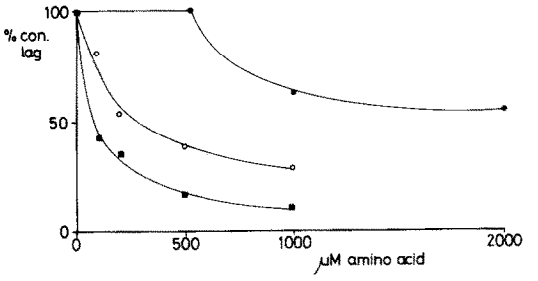

Fig. 3. The effect on lag phase length of the addition to a standard reaction mixture of a range of concentrations of the amino acids alanine, lysine and arginine: ●, alanine; ○, lysine; ■, arginine.

RESULTS

4HA/tyrosinase kinetics studies

(i) *Effect of reaction products.* Addition of a 200 μl aliquot of a fully oxidised reaction mixture completely abolished the lag period of the standard reaction mixture. R_{max} occurred at the beginning of the reaction and was greater than in the control reaction (see Fig. 1).

(ii) *Effect of non-thiol amino acids.* Addition of an amino acid to a standard reaction mixture shortened the lag period length (Fig. 3). The shortening of the lag was directly but not linearly proportional to the concentration of a given amino acid. Arginine was more effective than lysine, which was more effective than alanine, although in no case was the lag phase completely abolished. The relative effectiveness of the amino acids probably depends on the relative nucleophilicity of the molecules. This property of the amino acids is difficult to predict from the structures and the p*K*as of the individual amino or guanido groups, since all are protonated at pH 7.4 and there is no strict relationship between basicity and nucleophilicity. Nucleophilic addition to one group in a molecule will have effects on the propensity of any remaining groups to undergo addition due to both intramolecular electron delocalization and steric effects, the latter being of particular significance in the case of arginine. On addition of an amino acid to the reaction mixture, the absorbance maxima at 260 nm and 415 nm of the primary oxidation product of 4HA, 4-methoxy ortho-benzoquinone disappear and in the case of arginine new maxima at 312 nm and 480 nm are formed. This spectrum corresponds to the addition product of the arginine and ortho benzoquinone.

(iii) *Effect of DTT and cysteine.* Figure 4 illustrates the effect of DTT and cysteine on the kinetics of a standard reaction mixture. At very low concentrations (10 and 20 μM DTT and 1 and 10 μM cysteine), both thiol containing compounds completely abolished the lag period. R_{max} occurred initially and was greater than in the control. At higher concentrations, however, the lag period appeared to return. In the case of DTT, the lag length increased in proportion to the concentration of DTT; in the case of cysteine the increase in lag length did not continue at concentrations above 200 μM. Formation of an addition product of the thiol-containing compounds and the reaction product

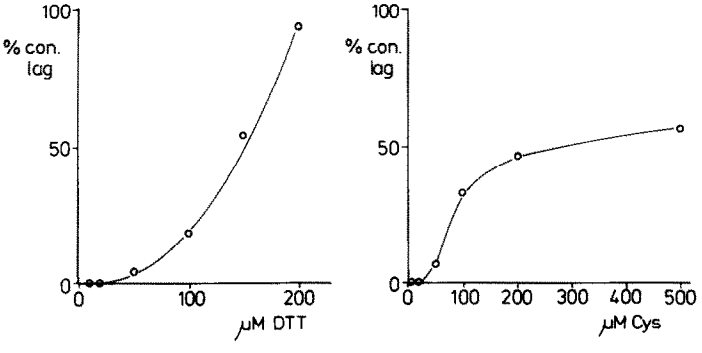

Fig. 4. The effect of the addition to a standard reaction mixture of the thiol-containing compounds DTT and cysteine.

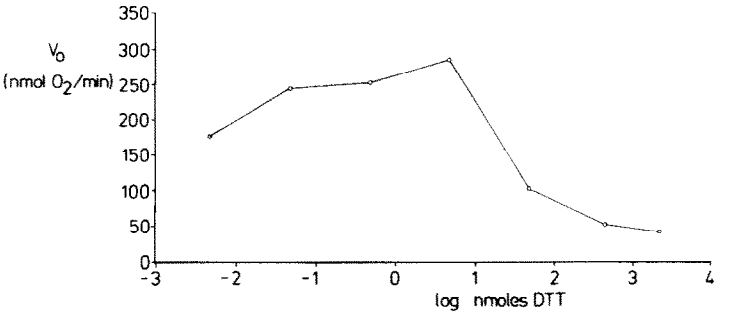

Fig. 5. The effect of dithiothreitol on 4HA/tyrosinase kinetics determined by oxygen uptake.

was shown by the loss of the typical maxima of the ortho quinone at 260 nm and 415 nm and the formation of new maxima at 310 nm and 375 nm in the case of cysteine addition and at 215 nm and 310 nm in the case of DTT addition.

(iv) *Oxygen electrode investigation of the effect of DTT.* Since the addition products of thiols and 4-methoxy ortho benzoquinine effectively remove the chromophore by the accumulation of which the rates of reaction had been measured, we found it necessary to examine the effects of thiol-containing compounds on the reaction kinetics by monitoring the rate of oxygen utilisation of the reaction mixture, since this constitutes an index of the oxidative activity of the enzyme which is unaffected by subsequent reactions of the product.

The biphasic effect of increasing concentrations of DTT was confirmed by measurements of the rate of reaction in terms of oxygen uptake. The results are presented in Fig. 5. The point at which enhancement of the initial velocity (V_0) of the reaction gave way to inhibition was after a molar ratio of between 5 and 10 DTT:1 tyrosinase. Above this optimal concentration the enzyme was profoundly inhibited with respect to the rate of oxygen utilisation but the residual reaction showed no lag phase.

DISCUSSION

Although there is a mechanism for the production of a met-tyrosinase recruiting diphenolic species from tyrosine, there is no such intramolecular rearrangement available to the quinone product of 4HA oxidation, 4-methoxy ortho benzoquinone (4-MOB). Despite this, the reaction proceeds with similar kinetics and as shown in this study, there is a species present in the reaction products which, when added to a fresh reaction mixture, can completely abolish the lag. The presence of an unidentified diphenolic species produced by polymerisation of 4-MOB may account for this effect. A range of diphenolic species may be produced by dimerisation (or polymerisation) of 4-MOB; resulting in a dibenzofuran, as suggested by [15] or a dibenzoparadioxy species.

The lag period length of 4HA oxidation was shown by this study to be shortened by the addition of nucleophilic compounds. The non-thiol amino acids, alanine, lysine and arginine were able to reduce the lag period but not abolish it. The effect of these amino acids lies in the nucleophilic nature of the amino group which leads to the formation of Michael addition products with 4-MOB, which are diphenolic species. The nucleophilic attack would result initially in addition to the 5-position with subsequent addition to the 6-position [16]. The lag period is never completely removed by these compounds, probably because they exert their effect via reduction of reaction products, the initial production of which is subject to the inherent lag phase.

In contrast to the above, it was found spectrophotometrically that at very low concentrations of cysteine or of dithiothreitol the lag phase was completely removed, although, at higher concentrations, an apparent lag phase returned. The concentration dependence of this effect was found to differ between DTT and cysteine. Addition products were also observed to form between 4-MOB and thiol groups. These addition compounds do not absorb at 260 nm, the wavelength at which the reaction is monitored spectrophotometrically, resulting in an apparent disappearance of the product. The reaction product can then only accumulate when the thiol is exhausted, resulting in an apparent lag period. This problem was overcome by monitoring the reaction in the presence of DTT by the rate of oxygen consumption, using an oxygen electrode. Again a biphasic action of the thiol was evident but although inhibition of the enzyme was observed at higher concentrations of thiols there was no lag period. In these instances the mechanism of enhancement of the reaction must differ from that produced by Michael addition products. We propose that the redox potential of these thiol compounds is sufficient to permit direct reduction of met-tyrosinase. The redox potential of DTT is -0.33 V and that of cysteine is -0.21 V at pH 7.0 [17], whereas the redox potential of met-tyrosinase is $+0.36$ V [9]. The maximum positive effect of DTT appears to be at a molar ratio of DTT:tyrosinase of between 5:1 and 10:1. Above this level progressive inhibition of the enzyme was found. The mechanism of thiol inhibition of tyrosinase, at least in the case of beta-mercaptoethanol and *Neurospora* tyrosinase has been shown to be due to the formation of an esr-detectable half-met derivative of the enzyme, containing a complex between a half-reduced copper active site and the thiol [18].

Since, in principle, Michaelis–Menten kinetic analysis of tyrosinase would be possible if the met-

form of the enzyme were reduced prior to incubation with monohydric phenol substrates, such that the initial velocity of the reaction is R_{max}, the potential of the DTT titration procedure in the kinetic analysis of tyrosinase is of interest. Further, the possibility that the direct activation as well as inhibition of tyrosinase by variations in local thiol concentration offers new insight into mechanisms of biological control of melanogenesis.

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