Oxidation of monohydric phenol substrates by tyrosinase

An oximetric study

Sandra NAISH-BYFIELD*† and Patrick A. RILEY†

*Department of Biology and Biochemistry, Brunel University, Uxbridge, Middx. UB8 3PH, and †Department of Chemical Pathology, University College and Middlesex Medical School, Windeyer Building, Cleveland Street, London W1P 6DB, U.K.

The purity of commercially available mushroom tyrosinase was investigated by non-denaturing PAGE. Most of the protein in the preparation migrated as a single band under these conditions. This band contained both tyrosinase and dopa oxidase activity. No other activity of either classification was found in the preparation. Oxygen consumption by tyrosinase during oxidation of the monohydric phenol substrates tyrosine and 4-hydroxyanisole (4HA) was monitored by oximetry in order to determine the stoichiometry of the reactions. For complete oxidation, the molar ratio of oxygen:4HA was 1:1. Under identical conditions, oxidation of tyrosine required 1.5 mol of oxygen/mol of tyrosine. The additional oxygen uptake during tyrosine oxidation is due to the internal cyclization of dopaquinone to form cyclodopa, which undergoes a redox reaction with dopaquinone to form dopachrome and dopa, which is then oxidized by the enzyme, leading to an additional 0.5 mol of oxygen/mol of original substrate. Oxygen consumption for complete oxidation of 200 nmol of 4HA was constant over a range of concentrations of tyrosinase of 33–330 units/ml of substrate. The maximum rate of reaction was directly proportional to the concentration of tyrosinase, whereas the length of the lag phase decreased non-linearly with increasing tyrosinase concentration. Activation of the enzyme by exposure to citrate was not seen, nor was the lag phase abolished by exposure of the enzyme to low pH. Michaelis–Menten analysis of tyrosinase in which the lag phase is abolished by pre-exposure of the enzyme to a low concentration of dithiothreitol gave K_m values for tyrosine and 4HA of 153 and 20 μM respectively.

INTRODUCTION

Tyrosinase (monophenol,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 *o*-hydroxylation followed by oxidation of the dihydric phenol so formed to the corresponding *o*-quinone in a single step without release of the dihydric phenol intermediate [3].

The active site contains a binuclear copper cluster in tyrosinase from mushroom (*Agaricus bisporus*) and from human malignant melanoma [4,5], hence the commercially available mushroom enzyme has been widely used as a model for mammalian tyrosinase. Most of the enzyme in a fresh preparation is in the met-tyrosinase form, in which the active site is bicupric and unable to bind oxygen. Only a relatively small proportion of the enzyme is present in the active monophenolase or oxy-form. This is produced when the met-form undergoes a two-electron reduction to the bicuprous state, which binds oxygen to form a bicupric–peroxide complex [6]. The initial rate of monohydric phenol oxidation is therefore slow unless a suitable two-electron reducing agent (e.g. dihydroxyphenylalanine (dopa) [7] or a thiol compound [8]) is supplied exogenously. Generation of dihydric phenols from the *o*-quinone oxidation products (e.g. dopa from dopaquinone) provides an auto-activating mechanism, as shown in Scheme 1, which results in a gradually accelerating rate of reaction.

The instability of the quinonoid oxidation products of tyrosinase substrates renders spectrophotometric estimations of the reaction kinetics by measurement of product chromophore

accumulation unreliable. This is especially relevant to studies involving investigation of adduct formation by quinone products, since the absorption spectrum of the adduct is frequently markedly different from that of the original product [8]. This problem may be avoided by use of an oximetric technique to measure directly the progress of the reaction by means of the oxygen consumption.

In this study we have re-examined some aspects of monohydric phenol oxidation by tyrosinase, using an oximetric technique. The purity and substrate specificity of the mushroom tyrosinase preparation was also investigated by non-denaturing PAGE followed by staining both for protein and for enzyme activity.

MATERIALS AND METHODS

General

Mushroom (*Agaricus bisporus*) tyrosinase [sp. activity 2200 units/mg of protein (manufacturer's estimate; 1 unit = $\Delta A_{280}/\text{min}$ of 0.001)], L-tyrosine, L-dopa, Coomassie Blue R250 and dithiothreitol (DTT) were obtained from Sigma, Poole, Dorset, U.K. 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 phosphate-buffered saline (Dulbecco's A) (PBSA) Oxoid, Basingstoke, Hants., U.K.), pH 7.4, which was treated with Chelex-100 chelating resin (Bio-Rad Laboratories). Sodium dithionite was obtained from BDH, Poole, Dorset, U.K.

PAGE

PAGE under non-denaturing conditions was performed on 10% gels in a continuous buffer system of final concentration

Abbreviations used: dopa, dihydroxyphenylalanine; DTT, dithiothreitol; 4-HA, 4-hydroxyanisole; PBSA, phosphate-buffered saline (Dulbecco's A).

† To whom correspondence should be addressed.

reaction mixture is achieved], maximum rate of reaction (nmol of oxygen utilized/min; $R_{\max}$) and total oxygen consumption by each reaction mixture were measured.

(c) **Effect on the kinetics of 4HA oxidation of addition of citrate to the reaction mixture.** The effect on a standard reaction mixture of the addition of a range of concentrations of trisodium citrate was investigated. The total oxygen consumption by the system, lag length and $R_{\max}$ for a range of concentrations of citrate of 0.5–3.0 mM was recorded.

(d) **Effect on the kinetics of 4HA oxidation of low pH.** The effect on the kinetics of a standard reaction mixture in which a range of formate buffers from pH 5.1 to 7.1 was substituted for PBSA was measured. Total oxygen consumption, lag length and $R_{\max}$ were recorded at each pH.

(e) **Michaelis–Menten kinetic analysis.** Tyrosinase (330 units in 200 μ l of PBSA) was preincubated with 10 μ l of a solution of 1 mM-DTT in PBSA (10 nmol of DTT), which has been shown previously to abolish the lag phase of monohydric phenol oxidation [8], so that the initial velocity of a given reaction corresponds to $R_{\max}$ and is directly proportional to the concentration of substrate. The resulting mixture, after 5 min incubation at 25 °C, was added to 2 ml of tyrosine or 4HA solution in PBSA in the oxygen-electrode chamber, and the initial velocity (v_0) of the reaction was measured in terms of the rate of oxygen consumption. A range of concentrations of tyrosine of 50–333 μ M and of 4HA of 12.5–500 μ M was used. Lineweaver–Burk double-reciprocal plots of $1/v_0$ versus $1/[S]$ were used to determine the K_m for both substrates.

RESULTS AND DISCUSSION

PAGE under non-denaturing conditions was performed in order to examine the protein content and enzymic activity of mushroom tyrosinase, as the purity and specificity of the enzyme have frequently been called into question in criticism of the use of the mushroom preparation as a model of the mammalian enzyme. On Coomassie Blue staining, it was found that the preparation ran as a single major protein band of molecular mass 101 kDa (Fig. 1), by comparison with non-denatured standards over a series of gels of different polyacrylamide concentration. This finding suggests either that there is one protein present or that there is a family of several proteins of identical charge-to-mass ratio, the latter explanation being the less likely. No effect of DTT was seen on the migration of the active enzyme on the gel. Coomassie Blue staining detects 0.2–0.5 μ g of protein in a sharp band (2–5 % of the total protein applied).

The production of a melanoid pigment by the enzyme on exposure to phenolic substrates allows direct detection of tyrosinase activity in the native gel. Staining with either tyrosine or dopa revealed a single identical band corresponding to the protein band revealed by Coomassie Blue. Tyrosine proved to be the more effective stain, resulting in intense black staining with no background or precipitate formation. Dopa, however, produced a less intense grey–brown stain, with much precipitation of products of dopa autoxidation of the gel surface.

The total oxygen consumption for complete oxidation of 200 nmol of 4HA was always within 5 % of 200 nmol of oxygen. The stoichiometry of complete tyrosinase-catalysed oxidation of 4HA therefore indicated a 1:1 molar ratio of oxygen to 4HA. The plot of oxygen utilization versus 4HA concentration gave a gradient of 0.96; correlation coefficient = 1 (Fig. 2). This stoichiometry reflects the utilization of one atom of oxygen in hydroxylation of the monohydric phenol and the reduction of the second oxygen atom to water by the two-electron oxidation of the resulting dihydric phenol to water. The stoichiometry observed confirms that found previously by using the oxygen-

sensitive spin label 3-carbamoyl-2,5-dihydro-2,2,5,5-tetra-methyl-1H-pyridoyl-1-yloxy ('CTPO') [9]. In addition, data are provided to show that this stoichiometry is retained at concentrations of 4HA below 50 μ M, at which point the CTPO study implied deviations from a 1:1 ratio. The proposition that a *p*-quinone species may form due to nucleophilic addition of water to the *o*-quinone product of 4HA oxidation, followed by oxidation of the trihydric phenol so formed [10], is not supported by these data, since this further oxidative step would require an additional oxygen atom, resulting in a stoichiometry of 1.5 oxygen to 1 4HA.

The total oxygen consumption for complete oxidation of 200 nmol of tyrosine was always within 5 % of 300 nmol of oxygen. The molar ratio for complete tyrosinase-catalysed oxidation of tyrosine was thus found to be 1.5 mol of oxygen/mol

Fig. 1. Non-denaturing PAGE of mushroom tyrosinase pre-exposed to a range of concentrations of DTT, stained with Coomassie Blue

Before electrophoresis (see the Materials and methods section), 200 μ l of tyrosinase containing 330 units of activity (150 μ g of protein) in 5-times-concentrated gel running buffer were exposed to 10 μ l portions of a range of concentrations of DTT (to give between 2 and 200 nmol of DTT per incubation) for 5 min at 25 °C. A 15 μ l sample of each incubation mixture was applied to the gel. Lanes, from left to right: empty; control, no DTT; 2 nmol of DTT; 20 nmol of DTT; 200 nmol of DTT; empty.

Fig. 2. Stoichiometry of oxidation of 4HA and tyrosine by mushroom tyrosinase

○, 4HA (stoichiometry 1 mol of 4HA:1 mol of O₂). ●, Tyrosine (stoichiometry 1 mol of tyrosine:1.5 mol of O₂).

of tyrosine; the gradient of the plot of oxygen utilization versus tyrosine concentration was 1.47; correlation coefficient = 1 (Fig. 2). The further oxidative step implied by utilization of the additional half mole of oxygen is consistent with the internal cyclization of the initial oxidation product, dopaquinone, to form the dihydric phenol, cyclodopa (leucodopachrome) [1], which rapidly undergoes a redox reaction with dopaquinone to form dopa and dopachrome [11,12] (see Scheme 1). The dopa so formed is then oxidized by tyrosinase, utilizing a further atom of oxygen per mol of tyrosine.

Further studies on the reaction kinetics of monophenol oxidation were undertaken with 4HA as a model monophenol, since the reaction involves solely the oxidation of 4HA to the respective *o*-quinone, 4-methoxy-*o*-benzoquinone [3]. The oxygen-utilization kinetics of the oxidation of tyrosine are complicated by the more rapid co-oxidation of dihydric phenol substrates formed after the initial *o*-quinone product, as reflected by the oxygen stoichiometry reported above, and hence do not reflect solely monophenolase activity.

The stoichiometry of the oxidation of a model monohydric phenol substrate, 4HA, is constant at 1 mol of oxygen/mol of substrate over a wide range of concentrations of tyrosinase. The total utilization of oxygen by each reaction mixture remained within 5% of 200 nmol for 200 nmol of 4HA (results not shown).

The length of the lag phase of monophenol oxidation was found to be inversely proportional to the enzyme concentration in a non-linear manner (Fig. 3). The curve appears to be asymptotic to the ordinate, suggesting infinite lag-phase length at infinitely low enzyme concentration, and to the abscissa, suggesting the persistence of a finite lag at infinitely high enzyme concentration. This is consistent with the presence of met-enzyme in any preparation which will not be immediately available for monohydric phenol oxidation and the need for recruitment of this met-enzyme into the reaction by dihydric phenol products of substrate oxidation by enzyme initially present in the monophenolase form. The non-linearity may reflect the contribution of the enzyme protein itself as a provider of nucleophilic groups which can increase the rate of production of the dihydric phenol, hence increasing the rate of recruitment of met-enzyme and therefore decreasing the length of the lag phase.

The $R_{\max}$ increased in a linear manner with increasing enzyme concentration; correlation coefficient = 0.997. Hence the maximum rate of tyrosinase-catalysed reactions simply depends on the initial concentration of enzyme present in the reaction mixture, since all met-enzyme will be recruited into the monophenolase form during the lag phase.

The effect of citrate on the kinetics of monophenol oxidation was investigated, since it has been reported that citrate stimulates tyrosinase from mouse melanoma, human skin and mushroom [13]. However, the observations reported here were made in terms of direct oxygen utilization by the enzyme, in contrast with the previous report, in which estimates of tyrosinase activity were made by a fluorimetric assay of the products of the reaction [14], which may not reflect directly the activity of the enzyme. Addition of a range of concentrations of trisodium citrate to a standard reaction mixture had no significant effect on the pH of the reaction mixture and had no effect on the total oxygen utilization of the reaction system, which remained within 5% of 200 nmol for 200 nmol of 4HA. There was no evidence of abolition of the lag phase at any concentration of citrate tested, nor was there any effect on the maximum rate of reaction in any case. The addition of equivalent concentrations of citric acid had marked effects on the pH, which decreased to 5.1 at 3 mM-citric acid and which results in alteration of the kinetics of the enzyme.

The role of low pH in regulation of tyrosinase activity was also

Fig. 3. Plot of the length of the lag phase of 4HA oxidation by mushroom tyrosinase versus concentration of the enzyme

The reaction mixtures comprised 2 ml of 100 μ M-4HA in PBSA and 200 μ l of tyrosinase in PBSA to give the required number of units of activity per ml.

Fig. 4. Chart records of the oxygen utilization during the tyrosinase-catalysed oxidation of 4HA in sodium formate buffer over a range of pH

Traces: A, pH 7.1; B, pH 6.7; C, pH 6.05; D, pH 5.85; E, pH 5.55; F, pH 5.26; G, pH 5.1.

investigated in this study, as low pH has been reported to abolish the lag phase of monohydric phenol oxidation by tyrosinase [15,16]. In the present study, there was no effect of low pH on the total oxygen consumption of the standard reaction mixture, which remained within 5% of 200 nmol for 200 nmol of 4HA. The length of the lag phase appeared to be influenced markedly by low pH; at pH 5.1 the lag phase was diminished, but it increased to a maximum at pH 5.26 and remained at approximately that value up to pH 7.1. $R_{\max}$ increased with increasing pH in a near-linear fashion. These two effects resulted in a very slow, almost linear, rate of reaction at pH 5.1, which appeared to lack the accelerating component of typical tyrosinase-catalysed reactions at higher pH. At pH higher than 5.1, a more typical distinct lag, followed by acceleration to a higher $R_{\max}$, was seen (Fig. 4). Recruitment of met-enzyme by two-electron reduction by dihydric phenols involves the release of two protons [17] (see Scheme 1). Thus inhibition of recruitment at low pH may be explained in terms of mass action due to excess protons in the reaction mixture.

Michaelis–Menten kinetic analysis of tyrosinase under lag-free conditions gave a K_m for tyrosine of $153\ \mu\text{M}$ and for 4HA of $20\ \mu\text{M}$. These values are approx. 50% of those previously reported for the mammalian enzyme with tyrosine and for mushroom tyrosinase with 4HA [18]. These data suggest some masking of the real affinity of the enzyme for monohydric phenol substrates when a lag phase is present. This may be due to some consequence of the competition of the monohydric phenol for met-tyrosinase, thus decreasing the recruitment rate by dihydric phenols and also the apparent maximal rate of reaction. Previous K_m determinations have, by necessity, been extrapolated at high substrate concentration, owing to the pronounced lag phase at these concentrations which results in an extremely slow initial reaction velocity. Owing to the prior recruitment of the met-enzyme by DTT in this study, all of the enzyme is in the monohydric phenolase (cresolase) form on presentation of the substrate, so that the initial velocity of the reaction is directly proportional to the monohydric-phenol substrate concentration over the entire concentration range.

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