Reactivity of Oxytyrosinase toward Substrates*

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SUMMARY
An oxygenated tyrosinase (T") is formed by addition of H2O2 to resting mushroom enzyme (Jolley, R. L., Jr., Evans, L. H. & Mason, H. S. (1971) The Second International Symposium on Oxidases and Redox Systems, Memphis, Tennessee, in press; (1972) Biochem. Biophys. Res. Commun. 46, 878). The reactivity of T" toward monophenolic tyrosinase substrates was investigated by the stopped flow method. The first-order rate constants for T" disappearance in the presence of p-hydroxybenzaldehyde and p-hydroxybenzoate were about 0.5 and 0.3 s⁻¹, respectively. These rates were sufficiently large to support the over-all enzyme-catalyzed reactions and were not affected by H2O2 concentrations within the concentration ranges examined. T" appears to be the first oxygenated intermediate detected with a copper oxidase.

Reagents—Stock solutions of H2O2 (Merek Superxol, 30%) were diluted with distilled water and titrated with standard KMnO4 solution. p-HB⁺ (Eastman) and 3,4-dihydroxybenzoic acid (Eastman) were recrystallized from ethanol and water to m.p. 212-214° and 108-109°, respectively. The reagents purified by sublimation in vacuo were 4-tert-butylcatechol (Aldrich), m.p. 154-155°; 4-tert-butylphenol (Matheson Coleman & Bell), m.p. 99-100°; p-HBA (Eastman), m.p. 116-117°; and 3,4-dihydroxybenzoic acid (Aldrich), decomposed at 154-155°.

Flow Experiments—Absorbance changes of oxygenated tyrosinase in the presence of substrates were observed with a Durrum-Gibson stopped flow spectrophotometer (light path, 2 cm) with a Bausch & Lomb tungsten lamp and a grating monochrometer. The temperature was maintained at 25°. A Bausch & Lomb tungsten lamp and a grating monochrometer were used as a light source. The wave length was set at 345 nm (band width, 10 nm), a maximum in the absorption spectrum of T".

In all the flow experiments the reagents were dissolved in 0.1 M potassium phosphate buffer, pH 7.0. An aerobic solution (O2) was used as a light source. The wave length was set at 345 nm (band width, 10 nm), a maximum in the absorption spectrum of T".

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† L. H. Evans, T. Wagner, and H. S. Mason, unpublished results.

‡ The abbreviations used are: p-HB, p-hydroxybenzoic acid; p-HBA, p-hydroxybenzaldehyde.
The tyrosinase substrates p-cresol and tert-butylcatechol abolished the 345-nm absorption band of T\(^{-}\), but the rates were too fast to be followed with a Cary model 14 spectrophotometer. We found that these substrates also reacted with T\(^{-}\) too rapidly to be followed with a Durrum-Gibson stopped flow spectrophotometer, and in this study the reaction rates of T\(^{-}\) with monophenols which give rise to "poor" catecholic substrates (9) were examined.

Figs. 1 and 2 depict the time course of absorbance changes at 345 nm of T\(^{-}\) maximum upon addition of the monophenolic substrates, p-hydroxybenzoate and p-hydroxybenzaldehyde, respectively. It should be noted that tyrosinases as prepared ("resting tyrosinase") contain variable amounts of an endogenous oxygenated form characterized by an absorption maximum at 345 nm, in addition to the species converted to T\(^{-}\) by H\(_2\)O\(_2\). In this study no distinction was made between the reactivity of endogenous and H\(_2\)O\(_2\)-generated T\(^{-}\). In all experiments, T\(^{-}\) was generated with H\(_2\)O\(_2\) to a constant absorbance increment at 345 nm, representing maximal T\(^{-}\) formation (3). The H\(_2\)O\(_2\)-induced absorbance changes were then corrected to take account of the absorbance of endogenous T\(^{-}\) for calculations of rate constants. The final values were 0.024 ± 0.001 for enzyme preparation HA 5/10 II, and 0.034 ± 0.001 and 0.042 ± 0.001 for preparations HA 5/22 II and HA 8/19 I, respectively, at the concentrations used in the experiments. k\(_{obs}\) for the disappearance of T\(^{-}\) was then measured from the initial slopes of the oscilloscope traces. Not all of the T\(^{-}\) disappeared upon addition of substrates; the maximum decreases in absorbance at 345 nm estimated from Figs. 1 and 2 represented less than 70% and 35% of the original values, respectively. This was because the conditions employed in these experiments permitted turnover of the enzyme, since both oxygen and monophenolic substrates were in substantial excess of enzyme. The rates of T\(^{-}\) disappearance markedly slowed down with time (Figs. 1 and 2), probably because of the establishment of steady state conditions. The final absorbance changes throughout the spectrum of T\(^{-}\) after reaction with p-HBA is depicted in Fig. 3. The loss of absorbance at each wave length was proportional to the absorbance of T\(^{-}\) at that wave length (3), and no deviations were observed which could indicate the formation of some other enzyme complexes, or of quinone interfering with kinetic meas-

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**TABLE I**

Properties of tyrosinase preparations

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>HA 5/10 II</th>
<th>HA 5/22 II</th>
<th>HA 8/19 I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isozymes*</td>
<td>α</td>
<td>β</td>
<td>γ</td>
</tr>
<tr>
<td>Total copper (percentage of protein)</td>
<td>0.19</td>
<td>0.22</td>
<td>0.21</td>
</tr>
<tr>
<td>EPR-detectable copper (percentage of total copper)</td>
<td>3.1</td>
<td>3.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Δ(_{245}) by H(_2)O(_2)(^{b}) (MCu(^{2+}) cm(^{-2}) 10(^{-5}))</td>
<td>6.17</td>
<td>6.74</td>
<td>7.67</td>
</tr>
<tr>
<td>Δ(_{245}) by direct oxygenation (MCu(^{2+}) cm(^{-2}) 10(^{-5}))</td>
<td>0.0</td>
<td>1.11</td>
<td>0.77</td>
</tr>
<tr>
<td>Catecholase activity (units per mg)*(^{d})</td>
<td>1943</td>
<td>1510</td>
<td>252</td>
</tr>
<tr>
<td>Cresolase activity (units per mg)*(^{d})</td>
<td>262</td>
<td>112</td>
<td>214</td>
</tr>
</tbody>
</table>

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* Notation of Bouchilloux et al. (7)

* Extrapolated to infinite H\(_2\)O\(_2\) concentration.

* Calculated from the difference spectrum (aerobic — anaerobic enzyme).

* Units as defined by Dawson and Magee (6), except that rates were determined with a Clark electrode.

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**Fig. 1 (left).** Time course of absorbance change at 345 nm during the reaction of T\(^{-}\) with p-HB. Tyrosinase (preparation HA 5/10 II, 3.60 μg copper, final concentration) was incubated with 10 μM H\(_2\)O\(_2\) for 5 to 15 min in 0.1 M phosphate buffer, pH 7.0, then mixed with p-HBA solution at 25°C. Initial concentrations of p-HBA are indicated in the figure. Initial absorbance increment at 345 nm due to total T\(^{-}\) formation, ΔA = 0.024 ± 0.001.

**Fig. 2 (right).** Time course of absorbance change at 345 nm during the reaction of T\(^{-}\) with p-HBA. Tyrosinase (preparation HA 5/22 II, 3.08 μg copper, final concentration) was incubated with 10 μM H\(_2\)O\(_2\) for 7 to 17 min in 0.1 M phosphate buffer, pH 7.0, and mixed with p-HBA solution at 25°C. Initial concentrations of p-HBA are indicated in the figure. Initial absorbance increment due to T\(^{-}\) formation, ΔA = 0.037 ± 0.001, K\(_{obs}\) (p-hydroxybenzaldehyde) = 11 μM; clear distinctions of initial slopes were difficult in the original traces for experiments at 50 μM substrate and higher.
Fig. 3. The spectral changes observed by the stopped flow method. Conditions were the same as those in Fig. 1 except that the p-HB concentration was fixed at 50 μM, and ΔA at different wave lengths was read at 100 s. The results of flow experiments (●) are compared with the spectrum of T" (— — —) (cf. Ref. 3) in arbitrary units.

**Table II**

Extinction coefficients of monophenols and their oxidation products at 345 nm

<table>
<thead>
<tr>
<th>Compounds</th>
<th>ε_{345} × 10^{-4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxybenzoate</td>
<td>0.0</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoate</td>
<td>0.0</td>
</tr>
<tr>
<td>4-Carboxy-o-benzoquinone</td>
<td>1.07</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>1.16</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzaldehyde</td>
<td>8.57</td>
</tr>
<tr>
<td>4-Formyl-o-benzoquinone</td>
<td>3.41</td>
</tr>
</tbody>
</table>

* In 0.1 M potassium phosphate buffer, pH 7.0.
* Obtained by oxidation of 3,4-dihydroxybenzoate by tyrosinase, at pH 7.0, in potassium phosphate buffer.
* Obtained by oxidation of 3,4-dihydroxybenzaldehyde by tyrosinase, at pH 7.0, in potassium phosphate buffer.

Measurements at 345 nm (ε_{345 nm} = 1.24 × 10^{4} M^{-1} cm^{-1}). These results show that T" is present at the steady state which occurs after initiation of the T" monophenol reaction in the presence of O₂, but they do not establish that T" is catalytically functional during the turnover. Since detailed and exact analyses of the over-all reactions were difficult in this situation, the apparent first order rate constants (k_{obs}) of the reaction between T" and the substrates were taken to be the initial rates of the absorbance changes at 345 nm divided by the absorbance of T" at the initial state, on the assumption that all T" is reactive with the monophenolic substrates.

The contribution of absorbance changes due to the conversion of substrate to products must also be considered in the estimation of reaction rates from the rates of absorbance changes at 345 nm. The extinction coefficients of the monophenols used in these experiments and their oxidation products, at 345 nm, were measured and are shown in Table II. Since the extinction coefficients of p-HB and its oxidation products are far smaller than that of T" (Table I), their contributions at 345 nm can be neglected. How ever, in the case of 3,4-dihydroxybenzaldehyde, the primary product from p-HBA, and 4-formyl-o-benzoquinone, the expected absorbances are of the same order of magnitude as that of T" and could cause errors in estimation of the rates from absorbance changes. In any case, since product formation would diminish the actual absorbance change at 345 nm due to disappearance of T", the calculated rate constants are probably minimum values for the T" reaction. Attempts to determine the amounts of 3,4-dihydroxybenzaldehyde and the corresponding quinone

**Fig. 4 (left).** Double reciprocal plots of k_{obs} against concentration of p-HB. k_{obs} values were obtained from initial rates in the stopped flow experiments (see text). Conditions were the same as those in Fig. 1 except for varying H₂O₂ concentrations (●; 10 μM; O; 0.5 μM).

**Fig. 5 (right).** Double reciprocal plots of k_{obs} against concentration of p-HBA. Conditions are the same as those in Fig. 3.

**Table III**

Rate constants for T"-monophenol reactions, and over-all turnover numbers of tyrosinase with monophenolic substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>k_{max}</th>
<th>K_{m}</th>
<th>Turnover number</th>
<th>Enzyme preparation used</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxybenzoate</td>
<td>0.3</td>
<td>50</td>
<td>0.15</td>
<td>HA 5/10 II</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>0.5</td>
<td>28</td>
<td>0.022</td>
<td>HA 5/22 II</td>
</tr>
<tr>
<td>p-Hydroxybenzoquinone</td>
<td>0.5</td>
<td>11</td>
<td>0.004</td>
<td>HA 8/19 I</td>
</tr>
</tbody>
</table>

* In 0.1 M potassium phosphate buffer, pH 7.0, at 25°C.
* Obtained from Fig. 1.
* Measured at 1 to 8 mM substrate and extrapolated to infinite substrate concentration. Total copper, 3.2 μM.
* Obtained from Fig. 2.
* Minimum values based on the assumption that products make no contribution at 345 nm.
* Measured at 2 to 4 mM substrate and extrapolated to infinite concentration. Total enzymic copper, 4.9 μM.
* Measured at 5 mM substrate. Total enzymic copper, 4.7 μM.

formed at the initial stage of the reaction were unsuccessful, because their absorption bands were close to that of T".

The double reciprocal plots, k_{obs} against substrate concentration, were straight lines (Figs. 4 and 5), and k_{max} (k_{obs} extrapolated to infinite substrate concentration) and K_{m} (substrate concentrations giving half-maximum reaction rates) derived from these plots are listed and compared with the maximum rates of over-all oxygen uptake at pH 7.0 and 25°C in Table III. Lag periods in oxygen consumption are generally observed when monophenolic substrates are oxidized by tyrosinase, but addition of small amounts of catechol shortens or abolishes the lag. Addition of catechol (up to 30 μM) did shorten lag times, but produced no effect on the maximum rates of oxygen consumption during oxidation of p-HB (5.9 mM) and p-HBA (5.3 mM) by tyrosinase, as given in Table III.

The first order rate constant, k_{max}, for T" disappearance can be compared with the over-all rate of oxygen consumption (turnover number) if the two are determined under conditions of identical or nearly identical enzyme concentration, and if the assumptions made are that there are two copper atoms at each active site, that each site binds one oxygen molecule, and that
TABLE IV

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Turnover number</th>
<th>$K_m$</th>
<th>Enzyme preparation used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles O$_2$ s$^{-1}$</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoate</td>
<td>9.8±</td>
<td>0.57±</td>
<td>HA 5/10 II</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzaldehyde</td>
<td>1.2±</td>
<td>0.33±</td>
<td>HA 5/22 II</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzaldehyde</td>
<td>1.5±</td>
<td>0.36±</td>
<td>HA 8/19 I</td>
</tr>
</tbody>
</table>

a In 0.1 M potassium phosphate buffer, pH 7.0, at 25°C.

b Measured at 0.2 to 15 mM substrate. Total enzymic copper, 0.16 μM.

c Measured at 0.2 to 1.3 mM substrate. Total enzymic copper, 1.6 μM.

d Measured at 0.3 to 3 mM substrate. Total enzymic copper, 0.93 μM.

The rates of these reactions are under study.

When p-HBA was used as tyrosinase substrate, the disappearance of T" was considerably faster than over-all O$_2$ consumption (Table III). This result indicates that the rate-limiting step is other than that observed spectrophotometrically. The rate-limiting step may be the hydroxylation of the monophenol, or there may be some intermediate between the monophenol and catechol, like an epoxide, as yet undetected. This is being examined. On the other hand, some over-all rate limitation may occur after hydroxylation, at least with some monophenolic substrates.

The results obtained in this study are all consistent with the participation of T" in the catalytic cycle of tyrosinase. Nevertheless, hydroxylation by O$_2$ transfer from T", and quinone formation have not yet been experimentally demonstrated in the reactions of T", and it remains possible that T" is outside the catalytic cycle, e.g., in equilibrium with another enzyme species that plays a catalytic role. Further studies are therefore required to establish fully the catalytic role of T" as well as to determine the full mechanism of tyrosinase action. If, as appears probable, T" is a catalytically functional oxytyrosinase, it is the first such copper enzyme intermediate to be observed, and it joins oxytryptophan oxygenase (15, 16), oxyproteoatechuate 3,4-dioxygenase (17), oxy-L-lysine mono-oxygenase (18) and oxy-p-hydroxybenzoate hydroxylase (19), and oxytocchorme P-450 (20, 21) as enzymic examples of catalytic states which may involve molecular oxygen.

REFERENCES

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