PSEUDOPEROXIDASE ACTIVITY OF MUSHROOM TYROSINASE

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Summary

Under anaerobic conditions, ethyl hydroperoxide functions as a two-electron acceptor in the tyrosinase-catalyzed oxidation of 4-*tert*-butylcatechol to 4-*tert*-butyl-*o*-benzoquinone, apparently by the following mechanism:

$$T\text{-}[Cu(II)]_2 + TBC = T\text{-}[Cu(I)]_2 + TB\text{-}o\text{-}BQ + 2H^+$$

$$T\text{-}[Cu(I)]_2 + EtOOH + 2H^+ = T\text{-}[Cu(II)]_2 + EtOH + H_2O$$

This is a direct demonstration of the pseudoperoxidase activity of tyrosinase. Ethyl hydroperoxide failed to oxidize either oxy- or deoxyhemocyanin.

INTRODUCTION

Tyrosinase, a copper protein found throughout the phylogenetic scale, catalyzes the o-hydroxylation of phenols and the oxidation of catechols to o-quionones (1). The enzyme from mushroom *(Agaricus bisporus)* has a molecular weight of 120,000 and contains four copper atoms which, in the resting, oxidized state, occur in antiferromagnetically coupled pairs (2,3). Reaction of resting enzyme (T_r) with H_2O_2 yields oxytyrosinase (T"), which may be reversibly deoxygenated to give T"' (4), according to the equation:

$$\underset{T_r}{[Cu(II)]_2} + H_2O_2 \rightleftarrows \underset{T"}{\text{oxytyrosinase}} \rightleftarrows \underset{T'"}{[Cu(I)]_2} + O_2$$

In the present study the reactions between the alkyl hydroperoxide, ethyl hydroperoxide, and various oxidation states of tyrosinase and hemocyanin were examined. A peroxidase activity of tyrosinase in the presence of ethyl hydroxperoxide and a catechol was observed.

MATERIALS AND METHODS

Tyrosinase was prepared from mushrooms *(Agaricus bispora)* by a modification of the method of Nelson and Mason (5). A sample of the β-isozyme having a catecholase activity of 2400 units per mg, a catecholase: cresolase ratio,

with 4-*tert*-butylcatechol and p-cresol respectively as substrates, of 18 (5), and a copper content of 0.18%, was used throughout. *Cancer magister* hemocyanin was prepared according to the procedure of Thomson et al. (6). Ethyl hydroperoxide (EtOOH) was synthesized by the procedure of Schonbaum and Lo (7). The H_2O_2-free product was obtained in an aqueous solution and was assayed by iodometry (8). Oxytyrosinase was prepared and deoxygenated as described previously (4).

REACTION OF TYROSINASE AND HEMOCYANIN WITH EtOOH

The reaction between tyrosinase and EtOOH was carried out in 26 mM sodium borate, pH 8.6. Anaerobic experiments were performed as described earlier (4). Hemocyanin experiments were performed similarly in 0.01 M sodium phosphate, pH 7.0, containing 10 mM $MgCl_2$. Spectra were recorded on a Cary model 14 spectrophotometer using 1.0 cm path length cuvettes. All experiments were performed at room temperature.

Experiments utilizing EtOOH as the electron acceptor in the catecholase reaction were carried out by adding a small volume of oxygen-free enzyme solution to 3.0 ml of deoxygenated substrate solution (6 mM 4-*tert*-butylcatechol in citrate phosphate buffer, pH 5.1) (5). The reaction was begun by adding EtOOH, and product (quinone) formation was followed at 400 nm.

RESULTS

Under aerobic conditions, the addition of 22 μM EtOOH to a tyrosinase (T_r) solution 13 μM in EPR-nondetectable copper resulted in the difference spectrum shown in Figure 1. The spectrum indicates a loss of intrinsic oxytyrosinase (T_i")(4). Addition of 22 μM EtOOH to a solution of oxytyrosinase (T") 13 μM in EPR-nondetectable copper, gave a rapid loss of absorbance at 345 nm which could be reversed by subsequent addition of H_2O_2. T"', prepared by deoxygenation of oxytyrosinase, did not undergo any spectral change in the visible region when treated with EtOOH. However, subsequent attempts to reoxygenate the sample did not produce any absorbance increase at 345 nm. EtOOH-treated T"' could be converted to T" only by the addition of H_2O_2.

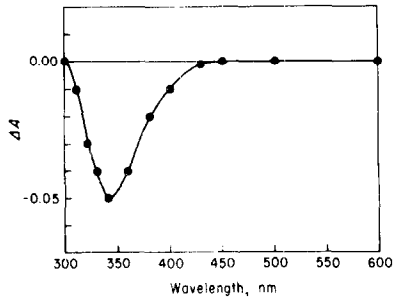

Figure 1. The difference spectrum, EtOOH-treated minus untreated resting tyrosinase (T_r). EPR-nondetectable copper, 13 μM; EtOOH, 22 μM.

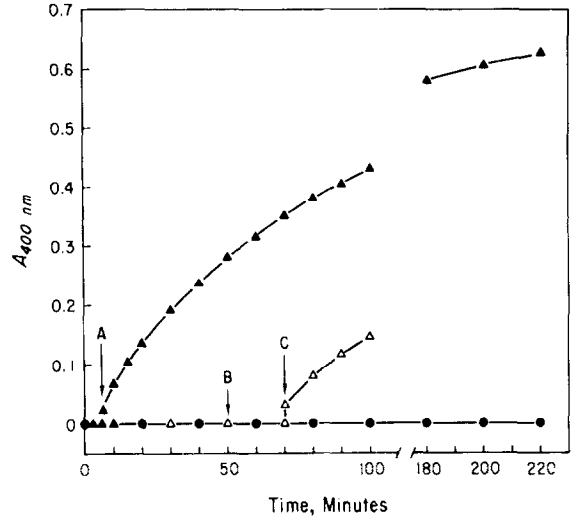

Figure 2. Demonstration of the pseudoperoxidase activity of tyrosinase. Samples I (▲) and II (●) contained 3.5 ml of substrate solution, 6 mM 4-*tert*-butylcatechol, plus 50λ enzyme. Total copper, 810 nM; EPR-nondetectable copper, 770 nM, under nitrogen. Sample III (Δ) contained 3.5 ml of anaerobic substrate solution plus 0.6 mM EtOOH. At point A, 0.6 mM EtOOH was added to sample I. At point B, 120 nM Cu(II) was added to sample III. At point C, 25λ of enzyme was added to sample III. Absorbance at 400 nm (4-*tert*-butyl-o-benzoquinone) was measured vs. a water blank. The initial absorbance increases at points A and C are caused by the addition of a small amount of O_2 to the system producing a burst of quinone formation. Addition of a small volume of air-saturated H_2O to an anaerobic enzyme/substrate mixture produced a similar, instantaneous increase in absorbance which then stopped. As judged from visible spectra, 4-*tert*-butyl-o-benzoquinone did not react with EtOOH. The theoretical final $A_{400\ nm}$ was 0.6.

These results suggest that EtOOH oxidizes T''' to T_r. Under anaerobic conditions, EtOOH should function as the electron acceptor in the catecholase

reaction of tyrosinase. Evidence of this is presented in Figure 2. The addition of EtOOH to an anaerobic mixture of 4-*tert*-butylcatechol and enzyme results in the slow formation of 4-*tert*-butyl-o-benzoquinone, monitored by its absorbance at 400 nm. Controls consisting of deoxygenated substrate solution plus enzyme and deoxygenated substrate plus EtOOH did not show any quinone formation. Based on the extinction coefficient of 4-*tert*-butyl-o-benzoquinone (unpublished data from this laboratory), each active site turned over 400 times in the first thirty minutes of the reaction. The total amount of quinone produced was approximately that expected from the amount of EtOOH added (the catechol being present in excess) but an exact determination was not possible because, in the several hours required for complete oxidation, the quinone underwent secondary, nonenzymatic reactions which altered its absorption spectrum. A study of initial rate of quinone formation as a function of initial EtOOH concentration indicated saturation kinetics and a K_m for EtOOH of 10 mM was estimated from the double reciprocal plot (Figure 3). It was difficult to obtain data for initial rates close to the maximum velocity because of nonlinearity in the initial rate of quinone formation. This may have been due to inactivation of the enzyme resulting from oxidation of amino acid residues at high concentrations of EtOOH. A partial loss of the characteristic aromatic

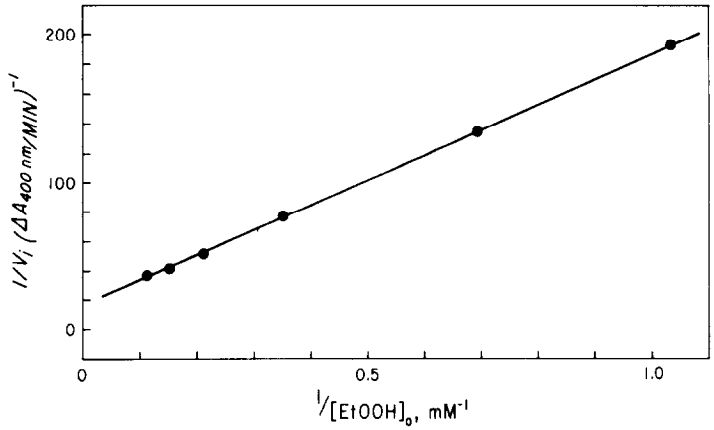

Figure 3. Double reciprocal plot of initial rate, Vi, $\Delta A_{400\ nm}$/min vs. $[EtOOH]_o$, mM. The apparent K_m for EtOOH is 10mM.

amino acid absorption spectrum was observed when tyrosinase was incubated with high concentrations of EtOOH. These results provide a direct confirmation of the pseudoperoxidase activity of tyrosinase described earlier (4).

Addition of 30 μM EtOOH to an oxyhemocyanin solution 16 μM in copper produced no change in the visible spectrum over a two hour period. Similarly, no spectral changes were observed on addition of EtOOH to deoxyhemocyanin and, on equilibration of the solution with air, full recovery of the oxyhemocyanin spectrum was observed.

DISCUSSION

The reaction between H_2O_2 and tyrosinase yields oxytyrosinase, which can be reversibly deoxygenated. EtOOH cannot form a similar series of compounds because of its inability to function as a reducing agent. EtOOH does oxidize T'" to T_r and, by removing T'" from the equilibrium between oxytyrosinase and T'", converts oxytyrosinase to T_r. The reaction between EtOOH and T'" is therefore:

$$T\text{-}[Cu(I)]_2 + EtOOH + 2H^+ \rightleftarrows T\text{-}[Cu(II)]_2 + EtOH + H_2O$$

Confirmation of this reaction is found in the ability of EtOOH to support catecholase (but not cresolase) activity under anaerobic conditions.

Hemocyanin possesses neither mono nor diphenolase activity, yet there are a number of similarities between oxyhemocyanin and oxytyrosinase (4). Contrary to expectations, EtOOH did not oxidize hemocyanin. Felsenfeld and Printz reported conversion of *Limulus polyphemus* hemocyanin to the met form using H_2O_2 as the oxidizing agent and partial oxidation of *Busycon canaliculatum* hemocyanin (9). The stability of *Cancer magister* hemocyanin to oxidation by EtOOH, in spite of the apparent similarity of active sites to tyrosinase, may result, at least in part, from a much more inaccessible oxygen binding site. If the hemocyanin site is small enough to be accessible only to molecules approximately the size of O_2, the EtOOH results may be reconciled with those of Felsenfeld and Printz. In any case, the peroxidatic and catalatic activities of copper

complexes and copper proteins (10,11) are well known, and tyrosinase appears to be no exception.

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