REVERSIBLE OXYGENATION OF TYROSINASE

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Summary. When mushroom tyrosinase reacts aerobically with molar equivalents of H_2O_2, a hitherto unobserved absorption spectrum develops, apparently according to first order kinetics, and then decays. The spectrum has peaks, $\varepsilon 345$ nm = 10 x 10^3 M_{Cu}^{-1} cm^{-1} and $\varepsilon 600$ nm = 7 x 10^2 M_{Cu}^{-1} cm^{-1}. Both bands disappear upon deoxygenation, and reappear upon reoxygenation, in a cyclic manner. The reaction system can be interpreted in terms of four states of the enzyme in the reaction: $T_r + H_2O_2 \rightleftarrows T' \rightleftarrows T'' \rightleftarrows T''' + O_2$, where T_r is the resting state, T' a kinetically necessary intermediate, T'' an oxygenated state, and T''' the cuprous enzyme.

Materials and Methods. Delta-tyrosinase prepared according to the procedure of Nelson and Mason (1) had: catecholase activity, 4200 Miller-Dawson units (2) per mg protein; cresolase activity, 47 manometric units per mg protein (2); total Cu, 0.20%, ESR-detectable Cu, 0.01%; laccase and catalase activity, not detectable. The enzyme was employed in 0.026 M sodium borate solution, pH 8.6. Apotyrosinase was prepared from delta-tyrosinase by a modification of the method of Kubowitz (3). It contained 0.01% Cu, and had no detectable enzymic activity. Reagents: catechol, m.p. 103-4°; tert-butyl catechol, 55°; p-cresol, vacuum redistilled; H_2O_2, Merck Superoxol, 30%, suitably diluted in H_2O and standardized against Na oxalate, MCB analytical grade; catalase, crystalline beef liver, Sigma; L(+) ascorbic acid, Mallinkrodt; N_2, prepurified. All other reagents were of the best grade commerically available. Copper was estimated with a Techtron Type AA-5 atomic absorption spectrophotometer. ESR-detectable Cu was determined with a Varian N 4500 spectrometer at -175°, with Cu(II)-EDTA standards. Protein was estimated by the method of Lowry and others (4), using bovine serum albumin standard (Labtrol).

Results. Resting tyrosinase had no absorption bands in the visible or near ultraviolet regions. However, upon addition of H_2O_2 in the presence of air, in ratios of 1 to 10 moles per atom of enzyme Cu, absorption bands

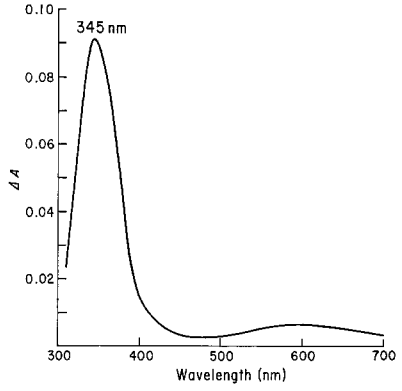

Fig. 1. The difference spectrum, H_2O_2-treated minus untreated mushroom tyrosinase, observed when δ-tyrosinase (0.43 mg/ml) in 0.026M sodium borate buffer, pH 8.6, total Cu, 13.5 μM, ESR-detectable Cu, 0.7 μM) was treated with H_2O_2, 23.8 μM final concentration, and allowed to stand at 25° for 18 minutes (for maximum intensity of spectrum). Light path, 1.00 cm.

at 345 nm and 600 nm developed (Fig. 1). Apotyrosinase did not yield this spectrum; however, when it was reconstituted with stoichiometric amounts of $CuSO_4$ enzymic activity was regained, together with the capacity to react with H_2O_2. The ESR spectrum of tyrosinase, at -180°C, was unchanged by the H_2O_2 reaction.

Development of the 345 nm absorption band followed apparent first order kinetics (Fig. 2), but the rate constant depended upon the initial concentration of H_2O_2. The double reciprocal plot, $1/k_{obs}$ vs $1/[H_2O_2]_{initial}$, was a straight line with a limiting value of $1/k$ = approximately 1 sec (Fig. 3). This result is compatible with a reaction mechanism in which an initial rapid pre-equilibrium precedes a rate-determining slow formation of the substance, T'', absorbing at 345 nm: $T_r + H_2O_2 \overset{Keq}{\rightleftharpoons} T' \overset{k_1}{\rightleftharpoons} T''$, where the intermediate T' forms rapidly and then undergoes a first order transformation to the absorbing compound, T'', and $K_{eq} = [T']/[T_r][H_2O_2]$. The value of k_1 obtained from the plot in Fig. 3 is of the order of 1 sec^{-1}, and the value of K determined from the expression derived by Portsmouth and Beal (5) is about 500 M^{-1}.

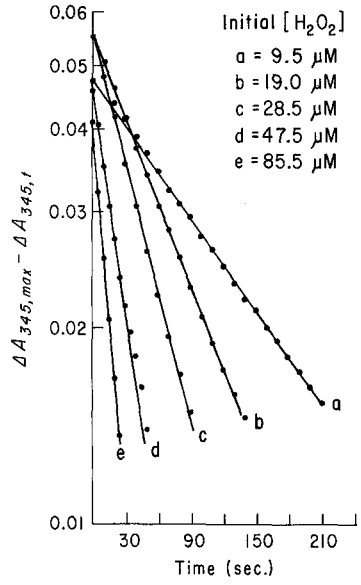

Fig. 2. The influence of initial [H_2O_2] on the rate of formation of the 345 nm absorption band in the difference spectrum, H_2O_2-treated minus untreated tyrosinase. δ-tyrosinase, 0.28 mg/ml in 0.026 M sodium borate buffer, pH 8.6; total Cu, 8.8 μM; ESR-detectable Cu, 0.4 μM; T = 25°.

From a preliminary estimate of the H_2O_2 concentration present at equilibrium in the presence of air (6) the overall equilibrium constant, $[T'']/[T_r][H_2O_2]$ is of the order of $10^5 M^{-1}$, and greatly favors the formation of T'' in the system. The numerical values for these kinetic and equilibrium constants are being further refined by work currently in progress.

An estimate of the extinction coefficient of the absorbing compound at 345 nm, with respect to enzymic (not ESR detectable) Cu was made by adding a large excess of H_2O_2 (5.7 moles per atom Cu) in the presence of air, in order to force the equilibrium toward the absorbing compound (Fig. 3). The extinction coefficient at 345 nm was approximately 10 x 10^3 $M_{Cu}^{-1}cm^{-1}$, and at 600 nm, about 700 $M_{Cu}^{-1}cm^{-1}$. In order to determine the extinction coefficient at 345 nm with respect to the H_2O_2 reacted, small aliquots of H_2O_2 were added to concentrated enzyme (enzyme Cu, 79 μM) in order to utilize the added H_2O_2 completely, and the average absorbancy increment per mole of H_2O_2 added was found to be 18 x 10^3 $M_{H_2O_2}^{-1}cm^{-1}$. From these values, it was calculated that 1.8 atoms of enzyme copper apparently reacted with each H_2O_2

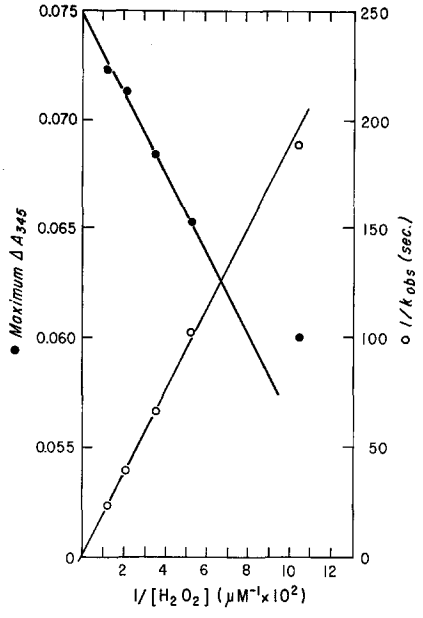

Fig. 3. (a) Double reciprocal plot of initial $[H_2O_2]$ and the corresponding rate constants, k, derived from Fig. 2, -o-o; (b) plot of maximum values of ΔA_{345} obtained at each initial $[H_2O_2]$, -●-●. Conditions as in Fig. 2.

molecule, which is consistent with a reaction ratio of 2 Cu/H_2O_2.

Formation of the 345 nm absorption band was not accompanied by appreciable changes in enzyme activity at H_2O_2/Cu ratios not exceeding 1.0. Larger ratios gave rise to losses in both catecholase and cresolase activities, and a sufficiently large excess of H_2O_2 (100-fold) resulted in virtually complete inactivation.

The 345 nm and 600 nm bands underwent slow decay with apparent first order kinetics, via an unknown pathway. After complete decay the spectrum could be fully regenerated by further addition of H_2O_2. Catalase markedly increased the rate of decay of the 345 nm band, presumably by a shift of the equilibrium involving H_2O_2. It is therefore possible that the decay may involve more than one process. The tyrosinase substrates, catechol, tert-butylcatechol, and p-cresol, rapidly abolished the 345 nm band, while the non-substrate reductant, ascorbate, did not. The substrate reactions indicate that the absorbing compound is a catalytic intermediate or is in rapid equilibrium with one. 5M guanidine hydrochloride also abolished the spectrum.

The spectrum of the hydrogen peroxide reaction product could be reversibly lost and regained by decreasing and then increasing the oxygen tension. Thus, when delta-tyrosinase was treated with H_2O_2 in the presence of air, the 345 nm and 600 nm bands were generated as expected. When the system was deoxygenated by flushing with N_2, the 345 nm and 600 nm absorption

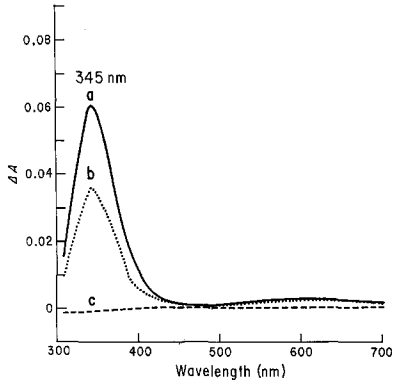

Fig. 4. Oxygenation of δ-tyrosinase. Sample and reference cuvettes contained 2 ml each of δ-tyrosinase, 0.37 mg protein/ml, 11.65 μM Cu, 0.58 μM ESR detectable Cu, in 0.026 M borate buffer at pH 8.6 at ambient temperature. The H_2O_2-induced difference spectrum (a) was obtained by addition of H_2O_2 to the sample cuvette to a final concentration of 9.7 μM. The deoxygenated difference spectrum (c) was obtained by repeated flushing of the H_2O_2-treated sample with N_2 alternated with shaking on a tonometer for a total of 5 minutes. The oxygenated difference spectrum (b) was obtained by flushing the deoxygenated sample with O_2 followed by shaking on a tonometer for 40 seconds. The cuvettes were 1 cm pathlength cuvettes equipped with an inlet and outlet for gas flow over the solution. The spectrum was swept at 1 nm sec^{-1}.

bands were lost and were regained upon reoxygenation (Fig. 4), although with reduced amplitude. This reduction in height of the regenerated 345 nm band compared to its original height is probably due to the apparent first-order decay of the spectrum already mentioned, the product not having the capacity for reoxygenation. The cycle of deoxygenation and oxygenation with loss and regain of the 345 nm band was repeated three times after the initial addition of H_2O_2, but each time with a reduced intensity of the recovered band. When H_2O_2 was added to resting tyrosinase in the absence of O_2, no detectable 345 nm band was generated, but it appeared after oxygenation of this system.

Discussion. The following properties of the H_2O_2-tyrosinase reaction have now been observed: (1) the development of a spectrum with maxima at 345 nm and 600 nm, (2) the apparent first order development of this spectrum, (3) the promotion of the rate of its decay by catalase, (4) the rapid disappearance of the spectrum in the presence of substrates of tyrosinase, and (5) the reversible loss and regain of the spectrum upon deoxygenation and reoxygenation. The two absorption peaks in the absorption spectrum are apparently coherent; we assume that they are characteristics of a single substance, T''. They resemble oxyhemocyanin peaks at 340-347 nm (depending upon species) ε = 8,900 - 10,000 $M_{Cu}^{-1}cm^{-1}$, and at 570-580 nm, ε = 370 - 500 $M_{Cu}^{-1}cm^{-1}$ (7). The two substances probably have similar structures, since both are capable of reversible oxygenation, and contain copper at their active sites. They may therefore have an evolutionary relationship in which tyrosinase (which occurs throughout biology) presumably preceded hemocyanin (which occurs in molluscs and arthropods). In aggregate, our evidence suggests that we have detected four states of tyrosinase: $T_r + H_2O_2 \rightleftharpoons T' \rightleftharpoons T'' \rightleftharpoons T''' + O_2$, in which T_r = resting enzyme, T' = a kinetically required intermediate, T'' = a spectroscopically observed form, and T''' = an oxygenatable form. We have no proofs of structure of any of these various states in hand, but we propose that these forms would fit the mechanism proposed by Mason (8) and extended by Orgel (9) and Mason (10) if T_r were protein-$Cu(II)_2$, T'' were protein-$Cu(I)_2O_2$ or an equivalent oxidation level containing oxygen derived from the atmosphere, and T''' were protein-$Cu(I)_2$.

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References

1. Nelson, R. M., and Mason, H. S., in H. Tabor and C. W. Tabor, Editors, Methods of Enzymology, Vol. XVII, Academic Press, N.Y., 1970, p. 626.
2. Dawson, C. R., and Magee, R. J., in S. P. Colowick and N. O. Kaplan, Editors, Methods in Enzymology, Vol. II, Academic Press, N.Y., 1955, p. 817.
3. Kubowitz, F., Biochem. Z., 299, 32 (1938).

4. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
5. Portsmouth, D., and Beal, E. A., Eur. J. Biochem., 19, 483 (1971).
6. Vanneste, W., Evans, L. H., and Mason, H. S., unpublished results.
7. Van Holde, K. E., Biochemistry, 6, 93 (1967).
8. Mason, H. S., Nature, 177, 79 (1956).
9. Orgel, L., Biochem. Soc. Symposium 15, 8 (1956).
10. Mason, H. S., Adv. Enzymol., 19, 79 (1957).