THE INACTIVATION OF INVERTASSE BY TYROSINASE

II. THE INFLUENCE OF COPPER AND GOLD ON THE OXIDATION OF INVERTASE AND PEP SIN

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Previous studies from this laboratory have demonstrated the oxidation of certain proteins by tyrosinase (1, 2) and the inactivation of invertase by tyrosinase (3, 4). Different tyrosinase preparations vary greatly in their ability to inactivate invertase; in particular, certain dialyzed or very highly purified tyrosinase solutions seem to have lost their ability to oxidize invertase. In Paper I of this series (4), it was emphasized previously that small amounts of a phenolic activator, e.g. catechol or tyramine, might play a critical rôle in the inactivation of invertase and other proteins by tyrosinase. In the present study, the possible rôle of metal ions as activators for the oxidation of invertase and pepsin has been studied.

Methods

Commercial tyrosinase preparations (Treemond, Syn-Zyme, and Worthington) were used in this study, but in a few experiments a catecholase fraction prepared according to Mallette et al. (5) was used. Tyrosinase activity of the Treemond stock solution was 4000 Miller-Dawson units per ml. or 2000 units per mg. of dry weight. The other tyrosinase preparations were similar in activity.

Yeast invertase of high purity¹ (6) was employed in all experiments. Except in the spectroscopic studies in which stock invertase was used, it was diluted 250 times with water before use. The stock invertase had a time value of 0.30 minute and contained 0.20 mg. of nitrogen and 0.4 mg. of carbohydrates per ml.

In the standard procedure, 0.2 ml. of tyrosinase (stock solution diluted twelve times) is incubated with 0.5 ml. of 0.05 M phosphate buffer, pH 6.0, plus metal salt solution and water to make a final volume of 1.6 ml. Toluene (0.1 ml.) is added as a preservative during the 18 hours incubation at 37°. Two types of controls were used: the first was identical with the experimental except that the tyrosinase had been boiled; the second was

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¹ Supplied by Dr. H. Dieu, University of Liège.
identical with the experimental except that it was run anaerobically. Oxygen was excluded from the control by flushing out the oxygen with nitrogen, and then removing the nitrogen and traces of oxygen with a high vacuum pump. Good agreement between the two types of controls is obtained except at very high metal ion concentration, when results with the boiled tyrosinase are unreliable.

Results

Results when the system contains \(1 \times 10^{-6}\) mole of \(\text{CuCl}_2\) per ml. are shown in Fig. 1. After incubation for 18 hours, the invertase activity of the experimental and control solutions was measured by adding 5 ml. of 12 per cent sucrose plus 3.4 ml. of buffer (0.05 M phosphate buffer, pH 6.0) at 37°. 1 ml. samples of the digests were taken at 3 minute intervals and the amount of invert sugar determined (3) with the dinitrosalicylic acid reagent. In all experiments the hydrolysis of sucrose follows zero order kinetics (Fig. 1). Rates of hydrolysis measured from the slopes of the straight lines are accurate to ±5 per cent. In the typical experiment shown in Fig. 1, the invertase inactivated by treatment with active tyrosinase plus \(\text{Cu}^{++}\) has only 55 per cent of the activity of the controls. Identical results were obtained when spectroscopically pure \(\text{CuCl}_2\) was used instead of the c.p. chemical.

The acceleration by \(\text{Cu}^{++}\) of the destruction of invertase by tyrosinase
is greatly complicated by the fact that the Cu\(^{++}\) itself may partially inhibit the invertase irreversibly over the same concentration range in which it is effective in facilitating the inactivation of invertase by tyrosinase.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Invertase activity measured as in Fig. 1 after treatment with either boiled or active tyrosinase is plotted as a function of the concentration of added copper ions. The upper curve presents, in per cent, the ratio of the experimental invertase activity to the control invertase activity.

The relative inactivation of invertase by the Cu\(^{++}\) plus boiled tyrosinase is compared with the inactivation in the presence of both Cu\(^{++}\) and active tyrosinase in Fig. 2. Except at copper concentrations above 1 \(\times\) 10\(^{-6}\) M
the inactivation is much more extensive for copper plus active tyrosinase than for the metal plus boiled tyrosinase and also occurs at a more rapid rate (see Fig. 3). In both instances the inactivation of invertase follows first order kinetics until about half the invertase is inactivated; it then proceeds much more slowly (Fig. 3).

![Figure 3](http://www.jbc.org/)

**Fig. 3.** The kinetics of the inactivation of invertase by copper and by copper plus boiled or active tyrosinase are plotted as a function of the time of exposure of the invertase to these substances.

Copper sulfate or acetate can replace the chloride in activating the tyrosinase-invertase system. Cu$^+$ is apparently as effective as Cu$^{++}$ because the monovalent ion rapidly oxidizes to the divalent form and because the Cu$_2$Cl$_2$ is contaminated with CuCl$_2$.

Dialysis of the copper-tyrosinase or the copper-invertase, before combining the components in the final system, results in the failure of invertase to be inactivated subsequently by the tyrosinase. Analyses of the copper firmly bound to the invertase are presented in a later section.

The effect of a number of different metal salts on the invertase-tyrosinase system has been studied over a wide range of salt concentrations up to

* The curves come together at very high copper concentrations, presumably because the boiled tyrosinase is less effective in binding free copper than is active tyrosinase.
concentrations which inhibit the invertase. Studies with AlCl₃, (NH₄)₂SO₄, NaCl, MnCl₂, CoCl₂, MgSO₄, NiCl₂, PbCl₂, FeCl₂, and AgNO₃ indicate that none of these was contributory and that, of all the metals used, only gold³ behaved like copper in the inactivation of invertase by tyrosinase. The results with gold are shown in Fig. 4. Both metals by themselves inhibit invertase, and at concentrations lower than those which produce partial inhibition of the invertase these metals do not facilitate the action of tyrosinase on invertase (see Figs. 2 and 4).

![Fig. 4. Invertase activity, after treatment of the enzyme with either boiled or active tyrosinase, is plotted as a function of the concentration of added gold chloride.](image)

The effects of cupric ions on the rate of oxidation by tyrosinase of other substrates were studied. Similar studies with gold were unsatisfactory because, at the high concentration⁴ required, the gold in the absence of protecting protein inactivated the tyrosinase. The standard manometric method of measuring oxygen consumption in the tyrosinase system was used (1, 7). The addition of 1 × 10⁻⁶ M CuCl₂ to the substrate in m/30 acetate buffer, pH 5.6, had no effect on the rate of oxidation of tyrosine, p-cresol, 3,4-dihydroxyphenyl-L-alanine, catechol, or glycyl-L-tyrosine.⁵

³ Silver may have a slight effect, but silver by itself is so toxic to invertase that any additional effect of tyrosinase is obscured.

⁴ Much lower concentrations of gold were used in the invertase and pepsin studies.

⁵ Delta Chemical Works.
On the other hand, the oxidation of 25 mg. of pepsin plus 0.5 ml. of tyrosinase (1300 units per ml.) in 4.5 ml. of M/30 acetate buffer, pH 5.6, is greatly accelerated by the addition of 1 mg. of CuCl₂ to the system (Fig. 5). The reaction was followed both manometrically by measuring oxygen consumption and colorimetrically by measuring the increase in absorption of light at λ 4000 Å. Peptic activity was measured by the standard method of Anson (8) with hemoglobin as a substrate. It was found that neither the tyrosinase alone (1) nor CuCl₂ alone nor CuCl₂ plus tyrosinase effected any inactivation of pepsin. The manometric and spectroscopic
data indicate that, while Cu\(^{++}\) increases the rate of pepsin oxidation by tyrosinase greatly, the end-point is about the same with or without the added copper. Unlike the situation with invertase, dialysis of pepsin against distilled water after the addition of copper does not prevent the usual acceleration of pepsin oxidation by tyrosinase. Copper analyses (according to the method of Cartwright et al. (9)) for the metal bound to pepsin showed that not all the copper was removed by dialysis for 4 hours against distilled water. The average of the data of ten experiments at pH 5.6 indicate that 9.9 atoms of copper were bound irreversibly by each
molecule of pepsin. Similar studies with invertase after removing the free copper by dialysis show 2.2 $\gamma$ of copper per mg. of dry weight of invertase.

The effect of tyrosinase on pepsin and on invertase can also be studied by ultraviolet spectroscopy as previously described (1, 2). Incubation of both pepsin and undiluted stock invertase (Fig. 6) with tyrosinase results in an appreciable increase in absorption throughout the ultraviolet region. This increase in absorption is obtained both with and without copper ions added to the system.

DISCUSSION

Copper and gold, the only metals which activate the tyrosinase-protein system, exist in different valence forms and readily form complexes with many organic compounds. They belong to the same group in the periodic table and have low atomic volumes. Gold is much less satisfactory than copper in these studies, because gold strongly inhibits the tyrosinase. These metals are effective only in the oxidation of proteins by tyrosinase (e.g., invertase or pepsin) and have no effect on the rate of oxidation of substrates of low molecular weight, e.g., tyrosine, 3,4-dihydroxyphenyl-L-alanine, p-cresol, catechol, or glycyl-L-tyrosine.

Two theoretical interpretations are suggested which may not be mutually exclusive. In a system in which tyrosinase has a high affinity for its substrate, the addition of a metal ion is not required. On the other hand, when the substrate is a protein with which tyrosinase has little ability to combine, the metal ion, by virtue of its ability to be bound to proteins, may facilitate the union between tyrosinase and protein substrate. The metal ion by combining with the invertase or pepsin may render the enzyme unstable and more susceptible to oxidation by tyrosinase. Spectroscopic studies being carried out at the present time, as well as analytical data presented above, indicate that under the conditions of these experiments copper and gold ions are bound to invertase and pepsin and to typical amino acids and peptides as well. The results are quite similar to those of previous workers who have studied the binding of metal ions to amino acids, peptides, and proteins (10–14).

In some respects the activation by copper of the tyrosinase-invertase and tyrosinase-pepsin systems is similar to the recent results of Shacter (15), who found that copper activates serum catecholase action on catechol by abolishing the lag period and enhancing the catecholase activity. The mechanism of copper action may be different, however, for with serum catecholase the copper appears to act by counteracting the effects of a sulfhydryl inhibitor. No evidence of such an inhibitor of the mushroom tyrosinase-protein system has yet been found. Further studies will in-
dicate whether or not copper ions greatly accelerate the oxidation by tyrosinase of proteins other than invertase and pepsin.

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SUMMARY

The inactivation of invertase by tyrosinase is greatly accelerated by the presence in the system of $1 \times 10^{-6}$ M copper or gold, but not by other ions. The action of tyrosinase on invertase is also characterized by an increase in absorption of ultraviolet light with or without added metal ion.

Copper ions were found to accelerate the oxidation of pepsin by tyrosinase without change in peptic activity.

Evidence that the copper actually forms a complex with invertase or pepsin at pH 5.6 was obtained from copper analyses made on these proteins after dialysis. Chemical analysis indicated copper bound to both these proteins: 2.2 $\gamma$ of Cu per mg. of dry weight of invertase and 9.9 atoms of Cu per pepsin molecule.

Unlike its effect on invertase and pepsin, copper does not increase the rate at which tyrosinase oxidizes substrates of low molecular weight such as tyrosine, 3,4-dihydroxyphenyl-$L$-alanine, glycyl-$L$-tyrosine, $p$-cresol, and catechol. Apparently copper assists only in the oxidation of those substrates, such as proteins, for which tyrosinase has a relatively low affinity.

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