Inactivation Mechanism of Tyrosinase

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SHIMAO, K., SEIJI, M. and FUKUZAWA, H. Inactivation Mechanism of Tyrosinase. Tohoku J. exp. Med., 1974, 114 (3), 263-272 — Soluble tyrosinase isolated from the Harding-Passey mouse melanoma was incubated with 14C-dopa and the time course of disappearance of dopa was measured by radioactivity assay at various tyrosinase concentrations. At the end of certain incubation periods, the tyrosinase activity was lost and the dopa concentration reached at stationary levels depending on the initial tyrosinase concentrations. The kinetic studies were carried out in order to analyze the relationship between the amount of tyrosinase incubated and the amount of dopa disappeared at various incubation periods. The results of the kinetic treatments indicated that inactivation of tyrosinase occurred mainly in proportion to the total concentration of tyrosinase present and the inactivation due to the binding with reaction products did not seem to play an important role in the mechanism. In other words, the inactivation mechanism involved appears to be similar to the reaction inactivation, which is known to occur in the reaction of plant tyrosinase.

In mammals, melanin formation and deposition occur on cytoplasmic particles of the melanocyte because tyrosinase is bound to specialized organelle melanosomes found in the melanocyte (Seiji 1967). In the process of melanization, melanosomes are gradually transformed into mature melanosomes with the deposition of melanin on their internal structure and in the mature melanosome tyrosinase is no longer active. In other words, tyrosinase is known to be inactivated during melanin formation (Seiji and Fitzpatrick 1960; Seiji and Miyazaki 1971). With respect to the inactivation of tyrosinase, it has been well known that the pronounced inactivation or destruction of plant catecholase occurs during the course of the reaction. This reaction inactivation does not appear to be due to products known to be formed during the oxidation of catechol, but occurs at the time when catechol is oxidized (Nelson and Dawson 1944). On the other hand, in preparations of mammalian tyrosinase, this sort of effect has not been known with the diphenolic substrate, dopa. The decrease in activity following incubation with dopa of melanosomes isolated from mouse melanoma appears to be related to the degree of in vitro melanization of melanosomes by incubation in dopa (Seiji and Fitzpatrick 1960). When purified soluble tyrosinase isolated from mouse melanoma was incubated with L-dopa in a similar manner as melanosomes were treated, the

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activity of soluble tyrosinase incubated decreased inversely with the amount of
dopa in the preincubation and it was also shown that the soluble tyrosinase thus
incubated combined with the products of the dopa-tyrosinase reaction (Seiji and
Miyazaki 1971). From these observations the reduction in activity has been
assumed to result from a blocking of the active centers of the enzyme with the
quininoid intermediates.

Kinetic studies of various substrates including L- and D-tyrosine and L- and
d-dopa and of a variety of inhibitor compounds have been carried out on the early
phase of the enzymatic oxidation of tyrosinase (Nelson and Dawson 1944; Fox and
Burnett 1958; Shimao 1962; Osaki 1963; Pomerantz 1963, 1966; Long et al.
1971). But there seems to be no report on kinetic studies of the mechanisms of
total inactivation which occurs in tyrosinase reaction in mammalians.

The experiment described in the present paper was performed in an effort to
clarify the relationship between the amount of tyrosinase used and the amount of
dopa disappeared during the dopa-tyrosinase reaction. The kinetic studies were
carried out in order to further clarify the mechanism of inactivation of tyrosinase
in the course of oxidation of dopa.

MATERIALS AND METHODS

Materials. Harding-Passey mouse melanomas were serially transplanted in a strain of
Swiss mice. L-Dopa was purchased from the Sigma Chemical Co., St. Louis, and 14C-DL-
dopa (2.46 mCi/m mole) was obtained from the Radiochemical Centre, Amersham. Soluble
tyrosinase was purified by the method of Miyazaki and Seiji (1971).

Determination of tyrosinase activity. Tyrosinase activity was estimated by the colorimet-
ric method of Shimao (1962). The optical density was determined with the Klett-
Summerson photoelectric colorimeter (\(\Delta E\) = scale reading).

Analytical procedure. Protein was determined according to the method of Lowry et
al. (1951).

In vitro incorporation of 14C-dopa by tyrosinase for the kinetic study. Various concentra-
tions of tyrosinase were incubated at 37\(^\circ\) with 3.6 mM 14C-DL-dopa (8 \(\mu\)Ci 14C-DL-dopa + 2.5
mg L-dopa), 1000 units of penicillin G potassium, M/15 phosphate buffer, pH 6.8, in a total
volume of 3.5 ml. At the end of various incubation times, 3.5 ml of 10\% trichloroacetic
acid solution was added to the reaction mixture, then centrifuged at 20,000 \(\times g\) for 20 min.
The radioactivity of an aliquot of the supernatant thus obtained was determined.

Measurement of radioactivity. The radioactivity of an aliquot of the supernatant was
measured in glass vials in a Packard TRI-CARB model 3380 spectrometer.

RESULTS AND DISCUSSION

Fig. 1 shows the time courses of the precipitation of the melanin formed
with 14C-DL-dopa and soluble tyrosinase. The radioactivity of the 14C-dopa left
in the supernatant decreased as melanin formation proceeded, while that of the
control, in which tyrosinase was omitted, did not show any change during the
incubation period tested. The rate of decrease in the radioactivity of 14C-dopa
appeared to depend on the amount of tyrosinase employed. The larger the amount
of tyrosinase used, the faster and greater the decrease in radioactivity of 14C-dopa.
The decrease in 14C-dopa reached a final value at 8 hr of incubation in all experi-
mental groups. In other words, melanin formation no longer seemed to take place even though the substrate, l-dopa, still remained in the reaction medium. There seemed to exist some kind of mechanism which inactivated the enzyme. In this particular experiment, the amounts of radioactivity of 14C-dopa remained in the supernatant at 8 hr incubation could be used as a parameter of the melanin formation, because the recovery of radioactivities in the supernatant and the precipitate was over 95% in the preliminary experiments. The relationship between the amount of tyrosinase used and the amount of melanin formed at the end of 8 hr incubation was not linear but upward convex curve.

Fig. 1. Time courses of the precipitation of the melanin formed with 14C-dopa and soluble tyrosinase. Various amounts of soluble tyrosinase (JE=100: o, 200: x, 400: +, 600: *) were employed (+: without tyrosinase).

In the previous experiments (Seiji et al. 1973), in which melanosomes were incubated with 14C-dopa instead of soluble tyrosinase, similar upward convex curves were obtained and an attempt was made to interprete inactivation of tyrosinase during oxidation of dopa by kinetic approach. However, the postulated ratios of enzyme and substrate concentrations used were found to be far from the actual value so that the conclusions derived were found to be misguided. A revised approach has been made with the soluble tyrosinase in this report to the same problem, inactivation of tyrosinase during oxidation of dopa, based on reasonable values of the enzyme and substrate concentrations.

From the experimental results obtained previously (Seiji and Fitzpatrick 1960; Seiji and Miyazaki 1971), it is quite reasonable, at the present time, to assume that the reaction product, P, reacts with the enzyme, E, to form an inactive E-P
complex. On the basis of this assumption, kinetic studies were carried out in order to interpret the experimental results obtained.

Time course of disappearance of the substrate, dopa, was calculated for the following three types of mechanism of inactivation and compared with the experimental results:

I.\[D + E \overset{k_1}{\underset{u}{\rightleftharpoons}} DE \rightarrow E + P\]
\[E + P \overset{k_3}{\underset{v}{\rightarrow}} EP\]

II.\[D + E \overset{k_1}{\underset{u}{\rightleftharpoons}} DE \rightarrow E + P\]
\[E + 2P \overset{k_3'}{\underset{v}{\rightarrow}} EP_2\]

III.\[D + E \overset{k_1}{\underset{u}{\rightleftharpoons}} DE \rightarrow E + P\]
\[E \overset{k_3''}{\underset{v}{\rightarrow}} E^*\]
\[DE \overset{k_4''}{\underset{w}{\rightarrow}} E^*\]

where D, dopa; E, tyrosinase; DE, enzyme-substrate complex; EP, inactive enzyme-product complex; E*, inactivated enzyme; and small letters under the symbols stand for molar concentration of the molecules expressed by the respective symbols.

The calculations were performed on the conventional assumption that temporary equilibrium is maintained for the dissociation of the enzyme-substrate complex, DE, with a dissociation constant (Michaelis’ constant), \(K_m\)
\[
\frac{[D][E]}{[DE]} = \frac{uv}{w} = \frac{k_2+k_3}{k_1} = K_m
\]
\[w = (v + w) \frac{u}{u+K_m},\]
\[v + w = e = \text{total enzyme concentration},\]

and the rate of product formation, which is equal to the rate of the substrate disappearance, is proportional to the equilibrium concentration of DE (\(e\)).

When the substrate concentration decreases as a result of the product formation and the active enzyme concentration decreases by the inactivation reaction,
the dissociation equilibrium shifts and \( w \) attains a new equilibrium values and
the product is formed at the corresponding rate.

Successive numerical solutions of the rate equations for the mechanisms I, II and III are as follows:

**I.**

\[
\begin{align*}
    u(t + \Delta t) &= u(t) - v(t) \frac{k_3}{k_4} \Delta t \\
    e(t + \Delta t) &= e(t) - v(t) x(t) \frac{k_3}{k_4} \Delta t \\
    v(t + \Delta t) &= v(t) + \frac{u(t + \Delta t)}{u(t + \Delta t) + K_m} \\
    x(t + \Delta t) &= x(t) + v(t) k_3 \Delta t - v(t) x(t) \frac{k_3}{k_4} \Delta t \\
    y(t + \Delta t) &= y(t) + v(t) x(t) \frac{k_3}{k_4} \Delta t
\end{align*}
\]

**II.**

\[
\begin{align*}
    u(t + \Delta t) &= u(t) - v(t) \frac{x^2(t)}{k_4'} \Delta t \\
    e(t + \Delta t) &= e(t) - v(t) x^2(t) \frac{k_3}{k_4'} \Delta t \\
    v(t + \Delta t) &= v(t) + \frac{u(t + \Delta t)}{u(t + \Delta t) + K_m} \\
    x(t + \Delta t) &= x(t) + v(t) k_3 \Delta t - v(t) x^2(t) \frac{k_3}{k_4} \Delta t \\
    y(t + \Delta t) &= y(t) + v(t) x^2(t) \frac{k_3}{k_4} \Delta t
\end{align*}
\]

Fig. 2. Decrease in the substrate concentration as calculated for the mechanism I. The
mechanism I is shown in the text. Numerical values of \( K_m, k_3, k_4 \) and \( e_0 \) (initial value of \( e \)) used for the calculation are shown in the figure. The curve for the smallest value of \( e_0 \) is essentially different from the experimental one (Fig. 1).
Underlined terms are much smaller than other terms and were neglected in the calculation.

Initial substrate and enzyme concentrations and $K_m$ which are necessary for numerical calculations were determined by the following procedures. Initial substrate concentration was 3.622 mM by the condition of the experiment. The enzyme concentration was estimated from the initial velocity of dopa oxidation for the highest enzyme concentration which was 0.04855 unit per ml of the reaction mixture. According to Burnett (1971) the molecular weight of pure tyrosinase is $6 \times 10^4$ and enzyme activity is 69–42 units/mg. Taking the median value, 50 units/mg, for enzyme activity, the enzyme concentration was calculated as 0.04855 unit/ml = $1.618 \times 10^{-8}$M.

\[
\begin{align*}
\text{III. } & \quad u(t + \Delta t) = u(t) - v(t) \frac{k_3}{K_m} \Delta t \\
& \quad e(t + \Delta t) = e(t) - e(t) \frac{k_4^*}{K_m} \Delta t \\
& \quad w(t + \Delta t) = e(t + \Delta t) \frac{u(t + \Delta t)}{u(t + \Delta t) + K_m} \\
& \quad x(t + \Delta t) = x(t) + v(t) \frac{k_3}{K_m} \Delta t \\
& \quad y(t + \Delta t) = y(t) + e(t) \frac{k_4^*}{K_m} \Delta t
\end{align*}
\]

Fig. 3. Decrease in the substrate concentration as calculated for the mechanism II. The mechanism II is shown in the text. Numerical values of $K_m$, $k_3$, $k_4^*$ and $e_0$ used for the calculation are shown in the figure. Deviation from the experimental results is clearly seen for the smallest value of $e_0$. 

\[
\begin{align*}
\text{II} \\
K_m = 5 \times 10^{-4} \\
k_3 = 2.78 \times 10 \\
k_4^* = 8.89 \\
e_0 = 0.270 \times 10^{-8} \text{M}
\end{align*}
\]

\[
\begin{align*}
\text{M/ \text{L-O1 \times n}} \\
\text{0} \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \\
\text{Time (hr)}
\end{align*}
\]

\[
\begin{align*}
\text{M} \\
1.618 \times 10^{-8} \text{M} \\
0.270 \times 10^{-8} \text{M}
\end{align*}
\]
Michaelis' constant, $K_m$ for dopa oxidation has been determined by several authors (Pomerantz 1963; Burnett et al. 1967). Referring to these values $K_m = 5 \times 10^{-4}$ M was adopted.

To test which of the three mechanisms fits best to the experimental results, velocity constants $k_3$ and $k_4$ ($k'_4$ or $k''_4$) were first chosen so that the calculated values of the substrate concentration became nearly the same as the experimental data for the highest enzyme concentration. Then, calculations were performed for lower enzyme concentrations and the results were compared with the experimental data.

In the above mentioned procedures, the $k_3$ to $k_4$ ($k'_4$ or $k''_4$) ratio determines the final value of the substrate concentration and the absolute value of $k_3$ determines the rate of the reaction. The rate constants $k_3$ and $k_4$ ($k'_4$ or $k''_4$) can, therefore, be determined unequivocally by the procedure.

Results of the calculation for the mechanisms I and II at the highest and the lowest enzyme concentrations are shown in Figs. 2 and 3. As can be clearly seen from the figures, shape of the curves substantially deviated from those of the

![Graph](image-url)

Fig. 4. Decrease in the substrate concentration as calculated for the mechanism III. The mechanism III is shown in the text. Numerical values of $K_m$, $k_3$, $k'_4$ and $e_0$ for the calculation are shown in the figure. The results of calculation fit better to the experiments than those obtained in the other two mechanisms treated. Corresponding values obtained in the experiment (see Fig. 1) are inserted for comparison.
experiments. While, in the mechanism III, deviation from the experiments was much smaller as can be seen in Fig. 4. The reason for the systematic deviation from the experiments for the lower enzyme concentration is clear from the calculation procedure in which the velocity constants were determined only by the experiment for the highest enzyme concentration. Calculations were carried out for other values of the velocity constants in an attempt to get better fit to the experiments as a whole and one of the results is shown in Fig. 5.

Fig. 5. Decrease in the substrate concentration as calculated for the mechanism III. The values of the velocity constants shown in the figure are employed for the calculation. The results of calculation seem to fit best to those of the experiment. Corresponding values obtained in the experiment (see Fig. 1) are inserted for comparison.

It might be concluded that the mechanism III is the most probable one among the three mechanisms treated above. That is, the inactivation of tyrosinase occurred mainly in proportion to the total concentration of tyrosinase present and the inactivation due to binding with the reaction products did not seem to play an important role in the mechanisms. In other words, the inactivation mechanisms involved appear to be similar to the reaction inactivation which is known to occur in the reaction of plant tyrosinase.

According to the experimental results obtained so far (Seiji and Fitzpatrick 1960; Seiji and Miyazaki 1971), the decrease in activity following incubation with
dopa of melanosomes and soluble tyrosinase has been assumed to result from a blocking of the active centers of the enzyme with the quininoid intermediates. The kinetic studies performed here, however, indicated that the inactivation of tyrosinase occurred by the mechanism in which the velocity of inactivation was not dependent on the product concentration but on the total concentration of the enzyme in action. It has been known that in both in vitro and in vivo systems, the melanin formed during the dopa-tyrosinase and/or dopa-melanosomes reaction combines with the soluble tyrosinase and/or melanosomes (Seiji 1967; Seiji and Miyazaki 1971), therefore, the inactivation of tyrosinase due to the reaction inactivation is assumed to occur prior to the combination of the reaction products with tyrosinase.

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References