Effect of metal ions on the kinetics of tyrosine oxidation catalysed by tyrosinase

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The conversion of tyrosine into dopa [3-(3,4-dihydroxyphenyl)alanine] is the rate limiting step in the biosynthesis of melanins catalysed by tyrosinase. This hydroxylation reaction is characterized by a lag period, the extent of which depends on various parameters, notably the presence of a suitable hydrogen donor such as dopa or tetrahydropterin. We have now found that catalytic amounts of Fe^{2+} ions have the same effect as dopa in stimulating the tyrosine hydroxylase activity of the enzyme. Kinetic experiments showed that the shortening of the induction time depends on the concentration of the added metal and the nature of the buffer system used and is not suppressed by superoxide dismutase, catalase, formate or mannitol. Notably, Fe^{3+} ions showed only a small delaying effect on tyrosinase activity. Among the other metals which were tested, Zn^{2+} , Co^{2+} , Cd^{2+} and Ni^{2+} had no detectable influence, whereas Cu^{2+} and Mn^{2+} exhibited a marked inhibitory effect on the kinetics of tyrosine oxidation. These findings are discussed in the light of the commonly accepted mechanism of action of tyrosinase.

Tyrosinase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase; EC 1.14.18.1) is a copper-containing enzyme responsible for melanogenesis in plants and animals, which catalyses both hydroxylation of tyrosine to dopa and its subsequent oxidation to dopaquinone (Hearing *et al.*, 1980; Lerch, 1981). The first reaction, which represents the rate-limiting step in melanin biosynthesis (Lerner *et al.*, 1949), is characterized by a lag period that has subsequently been explained in terms of a hysteretic process of the enzyme (García Carmona *et al.*, 1980). The extent of this induction time depends on various parameters including, besides pH and both substrate and enzyme concentration, the presence of a suitable hydrogen donor.

Kinetic studies carried out on tyrosinases from various sources (Pomerantz, 1966; Pomerantz & Murthy, 1974; Hearing & Ekel, 1976; Prota *et al.*,

1981) have shown that dopa, in very low concentration, is the most effective reducing agent in eliminating the lag period, whereas other catechols, such as dopamine, adrenaline and noradrenaline, behave similarly to ascorbate and NADH and NADPH in only shortening it, even at high concentration. Tetrahydropterin, a well-known specific cofactor of other aromatic hydroxylases (Lerner *et al.*, 1977; Marota & Shiman, 1984), is also effective in stimulating tyrosinase activity, although to a lesser extent than dopa. At present, no other organic or inorganic substances have been reported to shorten or lengthen the lag period of tyrosine oxidation.

Although metal ions are known to play a role in many biological processes, little attention has been directed to their possible involvement in melanogenesis, particularly in the early enzymic stages.

As a part of our continuing studies on the chemistry of melanin pigmentation (Prota, 1980; Sealey *et al.*, 1982; Palumbo *et al.*, 1983), we report the results of a survey on the effect of metal ions on the activity of purified *Sepia* tyrosinase, readily available in large amounts from the ink of the cephalopod *Sepia officinalis* (Prota *et al.*, 1981).

Abbreviations used: dopa, 3-(3,4-dihydroxyphenyl)-alanine; SOD, superoxide dismutase.

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Materials and methods

Chemicals

L-Tyrosine was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and purified from possible dopa contamination by passage through a column of alumina gel (300mg) in 0.1 M- Na_2HPO_4 , pH 8.5. The solution so obtained was subsequently adjusted to a suitable pH value with 0.1 M- NaH_2PO_4 and further purified by Chelex 100 chromatography (100–200 mesh; Na^+ form; Bio-Rad) to remove traces of metal ions (Poyer & McCay, 1971; Harris *et al.*, 1972). NaH_2PO_4 , Na_2HPO_4 , EDTA and Tris were purchased from Merck (Darmstadt, Germany). Hepes was obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). Catalase (2500 units/mg) from bovine liver, SOD (3000 units/mg) from bovine blood, sodium formate, D-mannitol and L-dopa were products from Sigma. All other chemicals were of the highest purity available. Distilled water, buffers, catalase and SOD solutions were passed over Chelex 100 resin before use. Glassware was washed with 1 M-HCl, 0.01 M-EDTA and 10–15 rinses of deionized water with specific resistance $> 10^8 \Omega$ at 25°C.

Enzyme preparation

Specimens of *Sepia officinalis* (cuttlefish), collected in the Bay of Naples, were killed by decapitation and the ink was obtained by cutting and gently squeezing the ink sacs. Tyrosinase was then isolated from the ink and purified by chromatography on DEAE-cellulose as previously described (Protà *et al.*, 1981). The enzyme so obtained (0.50 unit/mg of protein) was freed from metal impurities by chromatography on Chelex 100 resin.

Spectrophotometric assays

The effect of metal ions on the lag period in the *Sepia*-tyrosinase-catalysed oxidation of tyrosine was studied spectrophotometrically by the dopachrome method (Mason, 1948). The standard reaction mixture contained 0.5 mM-L-tyrosine and 0.01–0.03 unit of *Sepia* tyrosinase/ml in 0.1 M-sodium phosphate, pH 6.8. Aqueous solutions of the following metal salts were used: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $(\text{NH}_4)_2\text{SO}_4$, $\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{Zn}(\text{CH}_3\text{CO}_2)_2 \cdot 2\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$. All the kinetic experiments were performed on a Perkin-Elmer model 552 spectrophotometer with circulating water at 37°C. In calculating the enzyme units, the molar absorption coefficient of dopachrome at 475 nm was taken as 3600 (Mason, 1948). The enzyme unit was defined as the amount of enzyme required to produce 1 μmol of

dopachrome/min under the above conditions. One unit of SOD was defined as the amount of the enzyme that causes a 50% decrease in the rate of autoxidation of adrenaline at pH 10.2 (Misra & Fridovich, 1972). The catalase unit was taken as the amount that decomposes 1 μmol of H_2O_2 /min at pH 7.0, at 25°C.

All experiments were run at least two times to check reproducibility of the results.

H.p.l.c. analyses

H.p.l.c. was carried out on a μ -Bondapak C-18 column (3.9 mm $\times$ 30 cm) with a Waters Associates apparatus equipped with a u.v.-absorbance detector (model 480). The column was eluted with 0.05 M-ammonium acetate, pH 4.5, containing 4 mM-pentane-1-sulphonic acid.

Results

The influence of metal ions on the lag period in the tyrosinase-catalysed oxidation of tyrosine was evident from preliminary experiments in which the activity of the enzyme was assayed before and after careful purification of the reagents by Chelex 100 chromatography (Fig. 1). After this treatment, a

Fig. 1. Lag period in the oxidation of L-tyrosine catalysed by *Sepia* tyrosinase before (○) and after (△) purification of the reagents by Chelex-100 chromatography

L-Tyrosine (0.5 mM) was incubated at 37°C in the presence of *Sepia* tyrosinase (0.012 unit/ml) in 0.1 M-phosphate buffer, pH 6.8. The reaction course was followed spectrophotometrically by measuring the A_{475} , as described in the Materials and methods section.

more than 2-fold increase in the induction time was observed, as a consequence of the removal of metal impurities. Following this observation, the effect of transition metals on the kinetics of tyrosine oxidation catalysed by *Sepia* tyrosinase was systematically examined.

Fig. 2 shows the enzyme activity in the presence of various metal ions, all at the same concentration in phosphate buffer: Zn^{2+} , Cd^{2+} , Ni^{2+} and Co^{2+} have no influence on the typical kinetics of the reaction, Cu^{2+} and Mn^{2+} exhibit a marked inhibitory effect resulting in a 2-fold increase in the induction time, whereas Fe^{2+} has the unique property of stimulating the tyrosinase activity. It is noteworthy that this activation is highly specific for the reduced form of iron, since Fe^{3+} ions have only a small and opposite influence.

Fig. 2. Effect of various metal ions on the lag period in the tyrosinase-catalysed oxidation of tyrosine

Sepia tyrosinase concentration was 0.015 unit/ml, all the metal ions were at the same concentration ($50\mu\text{M}$), and the conditions were as described for Fig. 1. ----, Control.

The inhibitory effect of both Cu^{2+} and Mn^{2+} on tyrosinase was dependent on the concentration of the metals up to the limiting value of $50\mu\text{M}$, beyond which no further lengthening of the lag period was observed. Complete suppression of the inhibition occurred in the presence of chelating agents such as EDTA.

A similar trend with respect to the concentration-dependence was observed when the effect of Fe^{2+} on tyrosinase activity was examined (Fig. 3). The minimum amount of Fe^{2+} inducing a detectable stimulation on 0.027 unit of tyrosinase/ml is about $1\mu\text{M}$ and, as with Cu^{2+} and Mn^{2+} , no significant enhancement of the effect occurred at concentrations higher than $50\mu\text{M}$. The activating effect was dependent on the nature of the buffer system used, being maximum in phosphate and remarkably lower in HEPES or Tris. EDTA magnifies the stimulation by Fe^{2+} in HEPES or Tris, but is quite ineffective in phosphate, a buffer with

Fig. 3. Effect of various concentrations of Fe^{2+} on the lag period in the oxidation of L-tyrosine catalysed by tyrosinase *Sepia* tyrosinase concentration was 0.027 unit/ml and all conditions were as described for Fig. 1: —, control; Δ , $1\mu\text{M-Fe}^{2+}$; $\blacktriangle$, $5\mu\text{M-Fe}^{2+}$; $\bullet$, $50\mu\text{M-Fe}^{2+}$; $\circ$, $100\mu\text{M-Fe}^{2+}$; ----, $5\mu\text{M-Fe}^{2+} + 25\mu\text{M-EDTA}$.

well-known chelating properties towards Fe^{2+} (Nofre *et al.*, 1961).

Subsequent experiments were aimed at ascertaining the possible involvement of active oxygen species such as $\text{O}_2^{\cdot-}$, H_2O_2 and $\text{OH}^\cdot$ in the Fe^{2+} -induced activation of tyrosinase. Addition of H_2O_2 up to a 10-fold excess with respect to the metal, as in the Fenton reaction conditions, proved to be completely ineffective in enhancing the stimulation of tyrosine oxidation induced by Fe^{2+} alone. Moreover, catalase, formate and mannitol did not influence tyrosinase activation by Fe^{2+} . In this connection, it is noteworthy (Nofre *et al.*, 1961; Halliwell, 1978) that, on autoxidation, Fe^{2+} does produce small amounts of $\text{O}_2^{\cdot-}$, which are scavenged by SOD. Nevertheless, when the effect of Fe^{2+} (e.g. $25\mu\text{M}$) on tyrosinase was assayed in the presence of SOD (130 units/ml), no removal of the stimulation was observed.

As an additional control in the absence of metal ions, SOD, catalase, formate and mannitol, even at high concentration, also had no detectable effect on the tyrosinase activity.

In another series of experiments, the possible role of Fe^{2+} in catalysing the non-enzymic hydroxylation of tyrosine and subsequent oxidation of dopa to dopachrome was examined. When 0.5 mM-tyrosine or -dopa was incubated at 37°C for 30 min with Fe^{2+} (e.g. $10\mu\text{M}$), in the absence of tyrosinase, no detectable reaction was observed by u.v. spectroscopy and h.p.l.c. analysis of the mixture. As an additional control, the effect of preincubation of tyrosine in the presence of Fe^{2+} was examined. When the enzyme was added to a mixture of the reagents left for, e.g., 4 h at 37°C , the kinetic course of the reaction was similar to that observed under the usual conditions.

Discussion

The results reported in this paper provide evidence that some metal ions of wide occurrence in biological systems, i.e. Fe^{2+} , Cu^{2+} and Mn^{2+} , have a marked effect on tyrosinase activity. In the present study most of the experiments were carried out with purified *Sepia* tyrosinase, available in large amounts from the ink of the cuttlefish *Sepia officinalis* (Prota *et al.*, 1981). However, preliminary experiments indicated that the results can be extended to tyrosinases from other sources, notably mushrooms.

The finding that trace amounts of Fe^{2+} ions have the same effect as dopa in stimulating the hydroxylase activity of tyrosinase is of particular interest in relation to the molecular mechanisms underlying increased formation of melanin *in vivo*. Well framed in this context is the critical role

played by Fe^{2+} in certain processes, such as inflammation and lipid peroxidation, which are normally associated with melanin hyperpigmentation. Moreover, from the standpoint of comparative biochemistry, it is noteworthy that Fe^{2+} is required as cofactor for the activity of several aromatic hydroxylases, such as phenylalanine, tryptophan and tyrosine hydroxylases (Shiman *et al.*, 1971; Kuhn *et al.*, 1980).

With regard to a possible mechanism accounting for the observed effect of iron, available evidence indicates that the expression of the hydroxylase activity of tyrosinase is dependent on a pre-reduction step of the two Cu^{2+} ions at the active site of the enzyme (Healey & Strothkamp, 1981; Woolery *et al.*, 1984).

On this ground, the effect of Fe^{2+} on the kinetics of tyrosinase-catalysed oxidation of tyrosine can be envisaged in terms of two possible mechanisms: (1) aerobic hydroxylation of tyrosine, catalysed by Fe^{2+} , leading to trace amounts of dopa acting as hydrogen donor for the activation of the enzyme; (2) reduction of the Cu^{2+} ions of the enzyme to the Cu^+ state and consequent activation of the hydroxylase activity.

As far as the first mechanism is concerned, the hydroxylation of tyrosine should be reasonably brought about by $\text{OH}^\cdot$ radicals generated by autoxidation of Fe^{2+} by the intermediacy of $\text{O}_2^{\cdot-}$ and H_2O_2 , according to a Fenton-type reaction (Nofre *et al.*, 1961; Halliwell & Gutteridge, 1984). In contrast with this view is the fact that scavengers of all these oxygen species, such as formate, mannitol, SOD and catalase, as well as H_2O_2 , have no detectable effect on the Fe^{2+} -induced stimulation of the tyrosinase activity. Supporting evidence ruling out this hydroxylation mechanism came from the finding that prolonged preincubation of tyrosine with Fe^{2+} , aimed at favouring the possible formation of small amounts of dopa acting as a cofactor, does not result in any additional decrease of the lag period.

Consequently, Fe^{2+} -induced activation of tyrosinase could reasonably involve reduction of the Cu^{2+} ions at the active site of the enzyme, either directly or by interaction with oxygen reducing species as $\text{O}_2^{\cdot-}$. However, the lack of inhibitory effect by SOD and other scavengers on the activating properties of Fe^{2+} would lead one to rule out the implication of free oxygen species.

The direct reduction mechanism accounting for the role of Fe^{2+} is consistent with the observed dependence of the activation effect on the presence of chelating species, such as phosphate and EDTA, which are known to lower the redox potential of the couple $\text{Fe}^{2+}/\text{Fe}^{3+}$ from +0.78 V to +0.12 V (Nofre *et al.*, 1961). Additional support comes from the saturation effect (Fig. 3) with

respect to Fe^{2+} concentration, suggesting a direct interaction of the metal with the copper at the active site of the enzyme.

As far as the inhibitory effect of Cu^{2+} and Mn^{2+} on tyrosinase activity is concerned, the results so far available make it difficult to draw any definite conclusion about their mode of action. Their possible involvement as superoxide-dismutating agents (Halliwell, 1975; Lumsden & Hall, 1975) seems unlikely, since SOD, as well as other active oxygen scavengers, have no influence on the tyrosinase activity at all.

In conclusion, the results of this study, taken together, provide evidence for the critical role played by certain metal ions on the activity of the enzymic system involved in melanogenesis. Further work is now required to ascertain to what extent this finding, derived from experiments *in vitro*, can be extended to the situation *in vivo*.

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