Biochemistry (Moscow), Vol. 67, No. 2, 2002, pp. 277-280. Translated from Biokhimiya, Vol. 67, No. 2, 2002, pp. 332-336.

Study of the Kinetics of Oxidation of Monophenols by Tyrosinase. The Effect of Reducers

G. S. Gukasyan

Armenian Scientific Research Institute of Applied Chemistry (ARIAC), ul. Bagratuniants 70, Yerevan, 375029 Armenia; fax: (3741) 562-465; E-mail: bio@arminco.com

Received July 5, 2000
Revision received May 29, 2001

Abstract—Kinetics of oxidation of monophenols by tyrosinase from the fungus *Aspergillus flavipes* 56003 and the effect of Fe^{2+}, serine, and ascorbic acid on this reaction were studied. The effectors were shown to accelerate the oxidation of monophenols, decreasing the lag-time of the reaction. It is assumed that the activation of the tyrosinase in the presence of Fe^{2+} is due to a direct reduction of the active site copper ions. Serine and ascorbic acid are supposed to affect the reaction of quinone transformation. The activation of the enzyme in the presence of Fe^{2+} suggests that the oxidation of monophenols is an autocatalytic process.

Key words: tyrosinase, *Aspergillus*, kinetics

Tyrosinase (EC 1.14.18.1) is an enzyme exhibiting two catalytic activities: hydroxylase and dehydrogenase [1]. Due to this feature, the enzyme catalyzes oxidation of monophenols, yielding quinones, which are further transformed spontaneously into polymeric pigments. Synthesis of melanin from tyrosine is an example of such a reaction [2, 3].

In both reactions catalyzed by tyrosinase, molecular oxygen is used as the electron acceptor, being reduced to water without hydrogen peroxide formation [1]. The hydroxylase reaction results in cleaving of an oxygen molecule, one of the atoms being included into the substrate molecule. The reduction of the other oxygen atom is coupled to oxidation of the cosubstrate [4]. The peculiarity of the tyrosinase reaction is that the cosubstrate is diphenol, a product of monophenol hydroxylation [4].

To date, numerous investigations have been performed to elucidate the mechanism of oxidation of monophenols by tyrosinase. Summarizing the results available, the following basic characteristics of the enzyme can be outlined. Tyrosinase is a copper-containing protein, its active site containing two copper ions. Depending on the valence of the copper ions, the enzyme can exist in two catalytically different forms, a met-form and an oxy-form. In the oxy-form monovalent copper ions interact with an oxygen molecule, yielding peroxide. This form of the enzyme catalyzes hydroxylation of monophenols. The met-tyrosinase contains bivalent copper ions and catalyzes dehydrogenation of diphenols. During the reaction, the copper ions are reduced to the monovalent state, yielding a deoxy-tyrosinase. The deoxy-form easily binds an oxygen molecule, being transformed into the oxy-form. A full cycle of the oxidation of monophenols includes their transformation into quinones. Preparations of tyrosinase contain mostly the met-form, and only a small part of the enzyme is in the oxy-form.

The process of oxidation of monophenols includes both enzymatic and nonenzymatic reactions. Quinones, being the products of enzymatic reactions, are labile compounds that can be further subjected to spontaneous chemical transformations. Depending on the structure of monophenols used, two ways of quinone stabilization are possible. If a quinone molecule contains a substituent (tyrosine), the stabilization proceeds through intramolecular cyclization. In the case of unsubstituted substrates (*p*-cresol, phenol), the quinone is stabilized by reacting with water or another nucleophile. Chemical transformations of quinones influence the enzymatic reactions (Fig. 1) [5].

In spite of numerous investigations, the catalytic mechanism of the oxidation of monophenols has not been established yet. In particular, the mechanism of tyrosinase activation during the reaction remains unclear. In the present work, the kinetics of the oxidation of monophenols is considered in terms of the kinetics of autocatalytic processes. The effect of the enzyme activation in the presence of Fe^{2+} was used to study the mechanism of the reaction.

0006-2979/02/6702-0277$27.00 ©2002 MAIK "Nauka/Interperiodica"

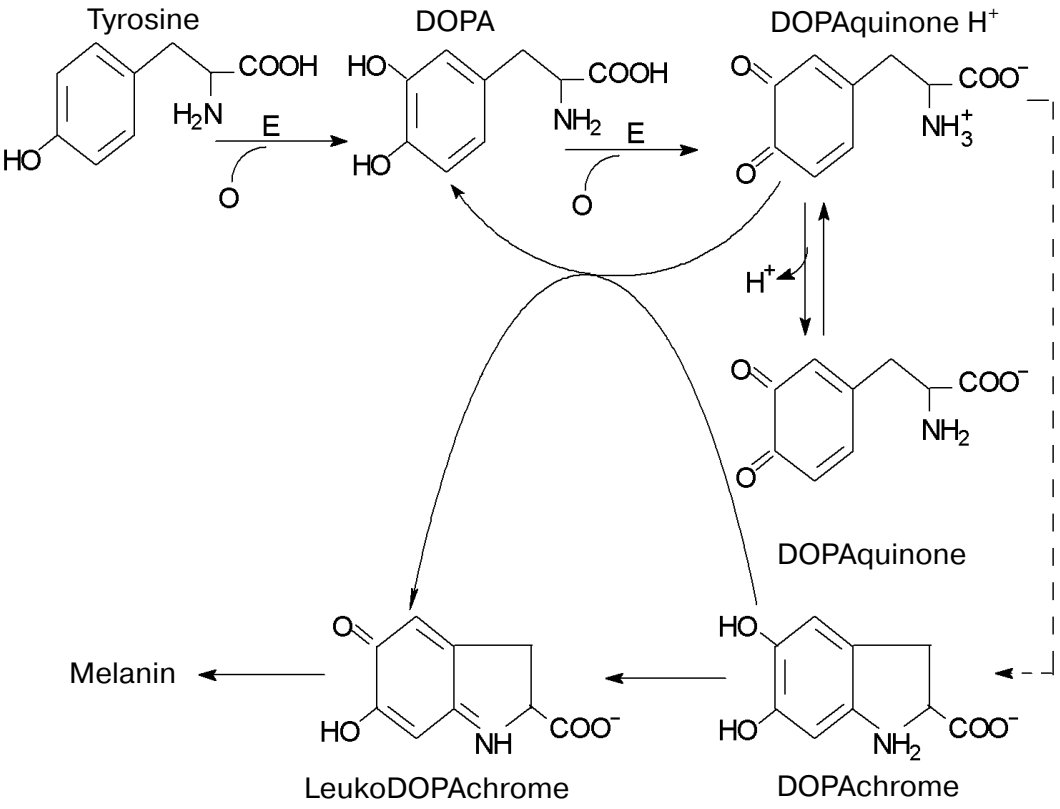

Fig. 1. Enzymatic and chemical reactions of tyrosine oxidation [5].

MATERIALS AND METHODS

A homogeneous preparation of tyrosinase was isolated from biomass of the fungus *Aspergillus flavipes* 56003 [6]. The tyrosinase activity was determined spectrophotometrically at 280 [7] and at 475 nm [8], as well as amperometrically, using a Clark electrode to measure the concentration of dissolved oxygen [8]. Dihydroxyphenylalanine (DOPA) was determined by the method of Arnow [9] and by HPLC [10]. The lag-time of the oxidation of monophenols was determined from the curves of the reaction product accumulation. The curve of monophenol oxidation has a sigmoidal form. The linear part of the curve was extrapolated to the X-axis, yielding a section corresponding to the lag-time of the reaction [11]. Ferrous ion was added to the reaction mixture as $FeSO_4$. All chemicals used were of analytical grade. All spectral experiments were performed using a Specord M 40 spectrophotometer.

RESULTS

The kinetics of tyrosine oxidation by tyrosinase was investigated. As seen from Fig. 2 (curve *1*), accumulation of the products of tyrosine oxidation is described by a sigmoidal curve with a lag-time of about 5 min. After the lag-time, activation of the enzyme is observed. To elucidate the mechanism of the activation of the enzyme towards monophenols during the reaction, the effect of various additions was studied. Bivalent iron ions [12, 13] and different reducers [11] have been known to activate tyrosinase. A solution of $FeSO_4$ was added to the reaction mixture to a final concentration 1-4 μg/ml. As seen from Fig. 2, an activation of tyrosine oxidation was observed: the lag-time decreased, the inflection point of the curve being shifted towards the zero point. As seen from the figure, the dependencies of the oxidation rate and the lag-time on Fe^{2+} concentration were characterized by a saturation region. Preincubation of the substrate in the presence of Fe^{2+} did not result in acceleration of the tyrosine oxidation by tyrosinase. These data suggest a direct interaction of the activator (Fe^{2+}) with the enzyme.

Study of the kinetics of the tyrosinase reaction in the presence of compounds affecting the nonenzymatic stages of the reaction can help in getting significant information on the mechanism of oxidation of monophenols by tyrosinase. We investigated the effect of ascorbic acid and serine on oxidation of tyrosine and *p*-cresol. Ascorbic acid, being a reducer, must promote the formation of

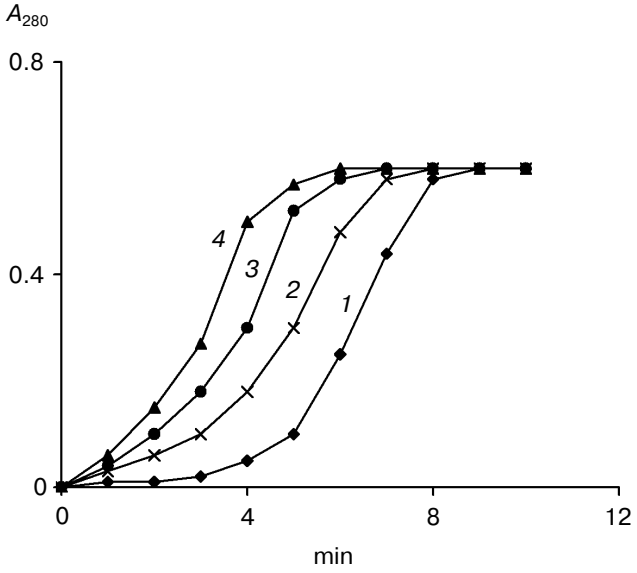

Fig. 2. Effect of different concentrations of $FeSO_4$ on tyrosine oxidation: *1*) no additions; *2*) 1.5 μg/ml; *3*) 2 μg/ml; *4*) 4 μg/ml.

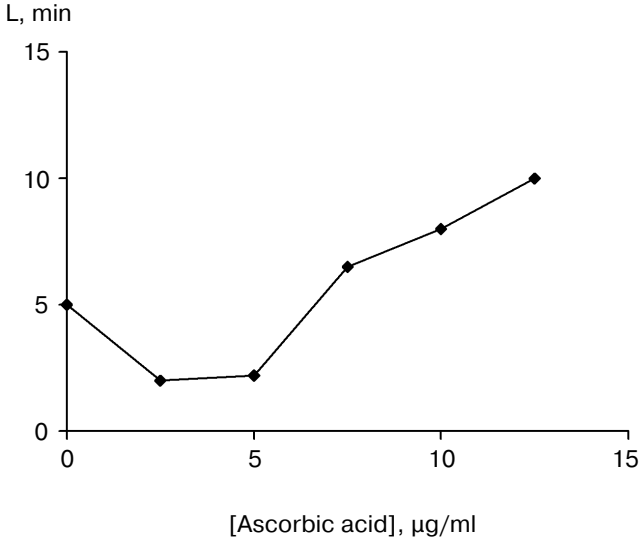

Fig. 3. Effect of ascorbic acid on the lag-time of tyrosine oxidation. The reaction was monitored by accumulation of colored products.

diphenols from quinones (Fig. 1). Studying the effect of different concentrations of ascorbic acid on the kinetics of the accumulation of the colored product of tyrosine oxidation, we found that relatively low concentrations of ascorbic acid decreased the lag-time of quinone formation. On further increasing of the concentration of the reducer, the lag-time lengthens (Fig. 3); however, the steady state rate of the reaction also increased.

Interestingly, no increase in the lag-time was observed when the reaction rate was monitored by oxygen consumption. These data indicate that at high concentration of ascorbic acid, the formation of colored products is inhibited, but the reaction of monophenol hydroxylation is activated. Assay of dihydroxyphenylalanine in the reaction mixture within the lag-time of the reaction revealed accumulation of this intermediate product of tyrosine oxidation. After the exhaustion of ascorbic acid in the reaction mixture, the oxidation of dihydroxyphenylalanine accumulation starts. Thus, the increase in the steady state rate of the formation of colored products is observed.

The effect of serine on the rate of tyrosine oxidation is analogous to the effect of ascorbic acid. In this case, serine plays a role of a reducer. In the case of oxidation of *p*-cresol, the lag-time of the accumulation of the colored product was not affected (Fig. 4). This is explained by the reaction between serine and the quinone formed yielding a diphenol molecule and a compound of serine with quinone, which is further subjected to an intramolecular cyclization and finally transformed into a polymer pigment. In the case of tyrosine oxidation, the compound of serine with the corresponding quinone is not formed.

The analogous mechanism of the effect of serine on the oxidation of *p*-cresol was described earlier [11].

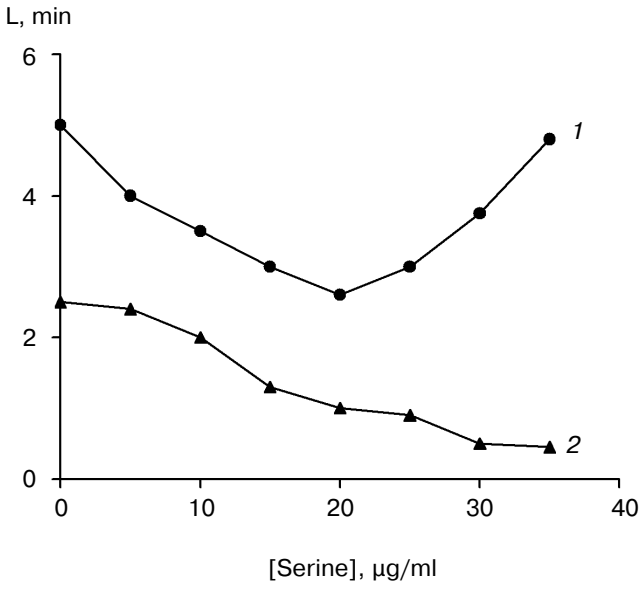

Fig. 4. Effect of serine on the lag-time of oxidation of tyrosine (*1*) and *p*-cresol (*2*).

DISCUSSION

The kinetic characteristics of the oxidation of monophenols by tyrosinase show that the amount of the enzyme form capable of oxidizing monophenols (oxy-tyrosinase) increases during the reaction. The scheme of tyrosine oxidation by tyrosinase (Fig. 1) shows that the increase in the amount of the oxy-tyrosinase can be due to the interaction of diphenol with the met-tyrosinase [5]. Diphenol can be formed in the course of spontaneous reduction of quinone. Thus, tyrosinase is activated by the products of its reaction, this being a characteristic of autocatalytic processes. Autocatalytic reactions are characterized by the following main features: the concentration of the active catalyst increases during the reaction; kinetics of the accumulation of the reaction product accumulation is described by a sigmoidal curve; the reaction starts in the case some amount of the active catalyst is present in the reaction mixture at zero time; the kinetics is characterized by the presence of a lag-time.

Comparing the kinetic characteristics of oxidation of monophenols by tyrosinase with the behavior of autocatalytic reactions, we assume that oxidation of monophenols by tyrosinase can be considered in terms of the theory of autocatalytic processes. A transformation of the met-tyrosinase into the oxy-tyrosinase during the reaction with diphenol is a key reaction in this process.

Autocatalytic character of reactions can be revealed by measuring the reaction rate in the presence of different starting concentrations of the active catalyst [14]. In the case of the tyrosinase reaction, the concentration of the oxy-form of the enzyme can be increased in the presence of bivalent iron ions, because a direct increase of the oxy-tyrosinase is impossible. Based on our own experiments and the literature data [12, 13], we assumed that the activation of tyrosinase in the presence of Fe^{2+} was due to the direct interaction of Fe^{2+} with copper ions in the active site of tyrosinase, this resulting in the reduction of Fe^{2+} and formation of oxy-tyrosinase. This effect was used in the present work to obtain different starting concentrations of oxy-tyrosinase. The results (Fig. 2) showed that the kinetics of tyrosine oxidation corresponded to the kinetics of autocatalytic processes. In the presence of different concentration of Fe^{2+}, a group of sigmoidal curves was obtained. The inflection points of these curves were shifted towards zero point on increasing the concentration of Fe^{2+}. It is noteworthy that the dependencies of the lag-time and the inflection point position on Fe^{2+} concentration exhibited a saturation region, this indicating a direct interaction of Fe^{2+} with the enzyme.

The activation of the reaction in the presence of ascorbic acid can also be explained by the effect of the reaction products on the catalyst. In this case, these compounds facilitate the reduction of quinones to diphenols. In the presence of diphenols, the met-form of tyrosinase is transformed into the oxy-form, which is active towards monophenols.

REFERENCES

1. Robb, D. A. (1983) in *Coord. Chem. Metalloenzymes. Proc. NATO Adv. Study Inst. San. Miniato*, Pisa, pp. 241-246.
2. Rodriguez-Lopez, J. N., Tudela, J., Varon, R., Garsia-Carmona, F., and Garsia-Canovas, F. (1992) *J. Biol. Chem.*, **267**, 3801-3810.
3. Del Marmol, V., and Beerman, F. (1996) *FEBS Lett.*, **381**, 165-168.
4. Ros, J. R., Rodriguez-Lopez, J. N., and Garsia-Canovas, F. (1994) *Biochim. Biophys. Acta*, **1204**, 33-42.
5. Juana Cabanes, Garsia-Canovas, F., Jose, A. Lozano, and Garsia-Carmona, F. (1987) *Biochim. Biophys. Acta*, **923**, 187-195.
6. Gukasyan, G. S. (1999) *Biochemistry (Moscow)*, **64**, 417-420.
7. Cory, J. R., and Frieden, E. (1967) *Biochemistry*, **6**, 116-119.
8. Konrad, L., and Ettlinger, L. (1972) *Eur. J. Biochem.*, **31**, 431-437.
9. Arnow, L. E. (1937) *J. Biol. Chem.*, **2**, 531-537.
10. Marumo, K., and Waike, J. N. (1986) *Biochim. Biophys. Acta*, **872**, 98-103.
11. Garsia-Carmona, F., Juana Cabanes, and Garsia-Canovas, F. (1987) *Biochim. Biophys. Acta*, **914**, 198-204.
12. Palumbo, A., Misuraca, G., Dischia, M., and Prota, G. (1985) *Biochem. J.*, **228**, 647-651.
13. Ros, J. R., Rodriguez-Lopez, J. N., and Garsia-Canovas, F. (1993) *Biochim. Biophys. Acta*, **1163**, 303-308.
14. Emmanuelle, N. M., and Knorre, D. G. (1969) *Course of Chemical Kinetics* [in Russian], Vysshaya Shkola, Moscow, pp. 261-264.