Phytochemistry, Vol. 26, No. 4, pp. 917–919, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

L-MIMOSINE, A SLOW-BINDING INHIBITOR OF MUSHROOM TYROSINASE

JUANA CABANES, FRANCISCO GARCÍA-CÁNOVAS, JOSÉ TUDELA, JOSÉ A. LOZANO and FRANCISCO GARCÍA-CARMONA*

Departamento de Bioquímica, Universidad de Murcia, Spain

(*Revised received* 8 *August* 1986)

Key Word Index: Mushroom tyrosinase; L-minosine; slow-binding inhibitor.

Abstract—It was found that L-mimosine is a slow-binding inhibitor of L-DOPA oxidation by mushroom tyrosinase. This inhibition is characterized by a prolonged transient phase. A mechanism is postulated according to the kinetic data.

INTRODUCTION

Tyrosinase, or polyphenoloxidase (EC 1.14.18.1) is a bifunctional copper protein widely distributed on the phylogenetic scale and responsible for melanization in animals and browning in plants. The enzyme catalyses two different reactions: (1) cresolase activity, or hydroxylation of monophenols to *o*-diphenols using oxygen; and (2) catecholase activity, or oxidation of *o*-diphenols to *o*-quinone using oxygen. L-Mimosine is a toxic amino acid isolated from legumes belonging to the genera *Leucaena* and *Mimosa* which are indigenous in tropical and subtropical areas. Hair and weight loss, cataracts and infertility have been observed in experimental animals when fed with a diet containing L-mimosine. It has been suggested that L-mimosine influences tyrosine metabolism, possibly as a weak inhibitor of several metal-containing enzymes. The interaction of L-mimosine on binuclear copper active sites of tryosinase was investigated by chemical and spectroscopic studies [1] and by X-ray absorption fine structure studies [2]. Previous kinetic studies carried out by steady-state measurements established that L-mimosine was a classical† competitive inhibitor of tyrosinase [3, 4].

We study in this paper the time-dependent inhibition of L-mimosine on DOPA-oxidase activity of mushroom tyrosinase. It was concluded that L-mimosine is a competitive slow-binding inhibitor of tyrosinase according to Williams and Morrison's classification [5, 6], the rate constant being evaluated as the constant of interaction equilibrium between L-mimosine and tyrosinase.

RESULTS

When mushroom tyrosinase was assayed in the presence of L-DOPA, a steady-state rate was immediately attained; the absorbance changes were linear at least for 0.3–0.4 *A* units. When the reaction was started by addition of the enzyme in the presence of L-mimosine, there was initially little effect on the reaction rate but a marked time-dependent decrease was observed which varied as a function of the inhibitor concentration. Preincubation of L-mimosine with the enzyme in the absence of L-DOPA resulted in no loss of enzymatic activity and, after addition of the substrate, results similar to those previously described were also obtained. These findings indicate that enzymatic catalysis is necessary in order for slow binding of L-mimosine to the enzyme to take place. The progress curves obtained can be described by the integrated form of Frieden's equation [7] for a first-order process.

$$P = v_s t + (v_0 - v_s)(1 - e^{-k_{app} t})/k_{app} \quad (1)$$

where v_0, v_s and k_{app} represent, respectively, the initial and the steady-state rates and the apparent first order rate constant (the meaning of which depends on the mechanism). Calculated values of these constants from data fitting are shown in Table 1.

If the initial velocities (v_0) were plotted by the Dixon equation, we observed that these decreased when the inhibitor concentration were increased (see Table 1). These results suggest the presence of a rapidly formed enzyme inhibitor complex. When the initial velocities were plotted at four different substrate concentrations, a value of 0.08 mM was obtained for the apparent dissociation constant (K_i) for the E'I complex. The value of the overall

Table 1. Variation of v_0, v_s, k_{app} and k_{-6} with L-mimosine concentration, using 4.47 mM of L-DOPA as substrate. The value of k_6 was calculated as 10/min from the intercept with the ordinate axis (Fig. 1)

[I] (mM)	v_S (μM/min)	v_0 (μM/min)	k_{app} (/min)	k_{-6} (/min)
0.178	17.5	69.5	1.80	0.45
0.237	15.2	63.6	2.16	0.51
0.297	12.2	50.5	2.34	0.56
0.416	10.6	49.4	2.94	0.63
0.475	10.2	45.9	2.98	0.66
0.590	8.1	38.3	3.22	0.68

*Author to whom correspondence should be addressed.

†A classical inhibitor of an enzyme-catalysed reaction is one which, on a steady-state time-scale, undergoes rapid interaction with the enzyme and which inhibits at concs that are much greater than the concs of the enzyme.

Abbreviations: L-DOPA: 3,4-dihydroxy-L-phenylalanine; L-mimosine: β-[*N*-(3-hydroxypiridone-4)]-α-aminopropionic acid.

dissociation constant (K_i') may, however, be calculated by plotting the values of steady-state velocities (v_s) vs the concentration of L-mimosine (see Table 1), giving a value of 0.015 mM.

Evaluation of the slow-transition constant

According to Cha [8] and with the following assumptions:

(1) Steady-state conditions are reached instantaneously between E, ED, E', E'D, and E'I.
(2) A prolonged non-steady-state conditions exist between E'I and E'I*.
(3) The substrate concentration is much greater than the enzyme concentration; thus the depletion of free substrate by binding to the enzyme is negligible.
(4) The inhibitor concentration is much greater than the enzyme concentration; thus the depletion of free inhibitor by binding to the enzyme is negligible.
(5) Experimental observations are made only while the effect of substrate depletion (by conversion to the product) and the effects of product inhibition on the reaction velocity are negligible.
(6) The reaction is started by the addition of the enzyme or the substrate.

The mechanism proposed in Scheme 1 can be represented by:

$$E_s \underset{k_{-6}}{\overset{k_6 f_{E'I}}{\rightleftharpoons}} E'I^*$$

where $f_{E'I}$ represents the factor of Cha for the enzymatic species E'I, the following equation being obtained:

$$f_{E'I} = \frac{\frac{k_2(k_{-3}+k_4)}{k_3}|I|}{K_i\frac{k_4(k_{-1}+k_2)}{k_1} + K_i|D|(k_2+k_4) + K_i\frac{k_2(k_{-3}+k_4)}{k_3} + \frac{k_2(k_{-3}+k_4)}{k_3}|I|} \quad (2)$$

As,

$$-\frac{d|E_s|}{dt} = \frac{d|E'I^*|}{dt} = k_6 f_{E'I}|E_s| - k_{-6}|E'I^*| \quad (3)$$

by integration of this equation, knowing that $|E_T| = |E_s| + |E'I^*|$ and substituting the value of $f_{E'I}$ we can obtain:

$$v = \frac{k_{-6}}{k_{app}} v_0 \left(1 + \frac{k_{app} - k_{-6}}{k_{-6}} e^{-k_{app}t}\right) \quad (4)$$

where

$$k_{app} = \frac{k_6|I|}{K_i(1+|D|/K_m)+I} + k_{-6} \quad (5)$$

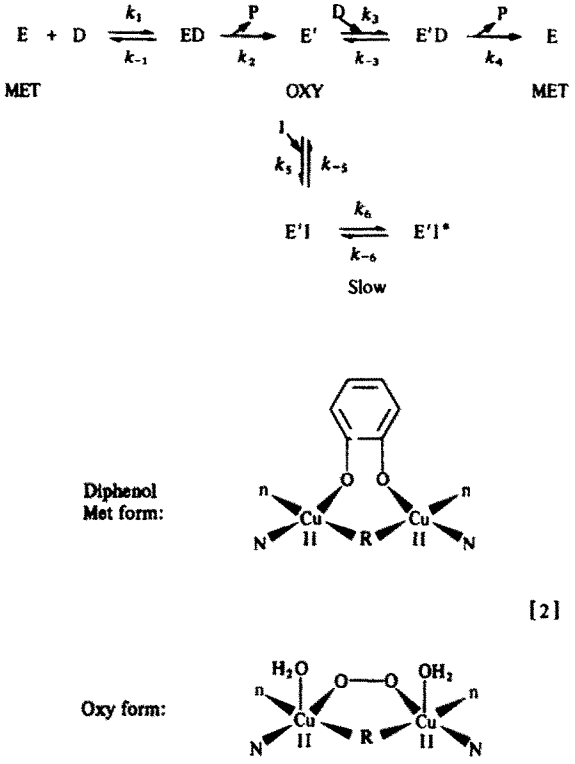
K_m is the Michaelis apparent constant for DOPA in the mechanism proposed and is equal to:

$$K_m = \frac{k_3k_4(k_{-1}+k_2)+k_1k_2(k_{-3}+k_4)}{k_1k_3(k_4+k_2)} \quad (6)$$

Figure 1 shows $1/(k_{app}-k_{-6})$ plotted against $1/|I|$ for four different substrate concentrations; the straight lines obtained coincided with eqn (5). From the intercept with the ordinate axis, the value of $k_{app}-k_{-6}$ can be obtained for the slow transition process. This value equals k_6 when the inhibitor concentrations tend to infinity, thus k_6 = 10/min. The steady-state rate (v_s) for this system would be obtained when $t \to \infty$ and therefore:

$$v_s = \frac{k_{-6}}{k_{app}} v_0. \quad (7)$$

Since v_s, k_{app} and v_0 can be obtained experimentally, k_{-6} can be evaluated (Table 1).



Scheme 1. Postulated mechanism for slow-binding inhibition of mushroom tyrosinase by L-mimosine.

DISCUSSION

A prolonged transient phase is observed for the inhibition of tyrosinase by L-mimosine, and thus L-mimosine presents the characteristics of a slow-binding inhibitor. L-Mimosine is different from other analogues of substrate inhibitors, previously described as slow-binding inhibitors to other enzymes [9–11], since L-mimosine requires enzymatic turnover to exhibit its inhibitory effect, and therefore it cannot be considered as a classical competitive inhibitor [3, 4]. The slow binding of L-mimosine to tyrosinase and the equation for the progress can fit the hysteretic enzyme concept introduced by Frieden [7]; thus we can assume that mushroom tyrosinase belongs to this category of enzymes, at least with regard to its interaction with L-mimosine. As discussed by Williams and Morrison [5] it is necessary to determine

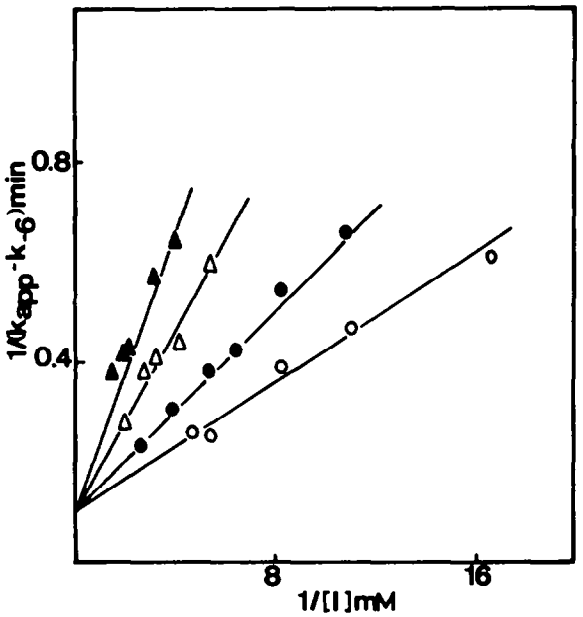


Fig. 1. Graphical calculation of k_6 for the inhibition of mushroom tyrosinase by L-mimosine. Reaction mixture at 25° contained buffer (pH 6.5), and L-DOPA at concentrations (mM) of: (▲) 6.08; (△) 4.56; (●) 3.04; and (○) 1.52.

whether the initial rates of the progress curves vary as a function of L-mimosine concentration in order to establish if the enzyme–inhibitor complexes are rapidly formed. Such evidence has now been obtained. Thus, the experimental data obtained in this paper are qualitatively consistent with the mechanism shown in Scheme 1, where the inhibitor binds to an intermediate form (oxytyrosinase) of the catalytic cycle which has been proposed for L-DOPA oxidation [12–15]. This mechanism postulates the successive binding of two diphenolic substrates for completion of the catalytic cycle with the occurrence of an enzymatic oxy form (E'), which, compared to the met form, has a greater affinity for the substrate [13]. Thus, the inhibitor will compete with the binding of the second molecule of L-DOPA. The slow development of the enzymatic inhibition may be explained by a fast formation of the complex E'I which slowly isomerizes into a second complex E'I*. The more enzyme drawn into E'I* the more pronounced the inhibition becomes. Therefore L-mimosine must be classified as a competitive slow-binding inhibitor, according to the classification of reversible enzyme inhibitors established by Morrison [6].

EXPERIMENTAL

Mushroom tyrosinase (2230 units/mg), L-DOPA and L-mimosine were purchased from Sigma. All other chemicals used were analytical grade. Catecholase activity was determined spectrophotometrically with a Perkin–Elmer spectrophotometer model Lambda 3 on-line interfaced with a Perkin–Elmer computer model 3600 data-station, using L-DOPA (6.08 mM) as substrate in NaPi buffer (pH 6.5). One unit of enzyme is taken as the amount of enzyme that produces 0.5 μmol of dopachrome per min, since this implies the production of 1 μmol of dopaquinone [16, 17]. The dopachrome accumulation was measurement at λ = 475 nm (ε = 3700/M/cm). Protein concentration was determined by the method of ref. [18].

The progress curves were fitted by non-linear regression, using Marquardt's algorithm [19], to the equation:

$$P = c_2 t + (c_1 - c_2)(1 - e^{-c_3 t})/c_3 + c_4. \quad (8)$$

Equation (8) is equivalent to eqn (1), c_4 representing the experimental uncertainty on the zero time absorbance, caused by addition of enzyme to start the reaction.

Acknowledgement—This work was partially supported by a grant of Comisión Asesora de Investigación Científica y Técnica, Spain.

REFERENCES

1. Winkler, M. E., Lerch, K. and Solomon, E. I. (1981) *J. Am. Chem. Soc.* **103**, 7001.
2. Woolery, G. L., Powers, L., Winkler, M., Solomon, E. I., Lerch, K. and Spiro, T. G. (1984) *Biochim. Biophys. Acta* **788**, 155.
3. Hashiguchi, H. and Takahashi, H. (1976) *Molec. Pharmacol.* **13**, 362.
4. Kahn, V. and Andrawis, A. (1985) *Phytochemistry* **24**, 905.
5. Williams, J. W. and Morrison, J. E. (1979) *Methods Enzymol.* **63**, 437.
6. Morrison, J. E. (1982) *Trends Biochem. Sci.* **7**, 102.
7. Frieden, C. (1970) *J. Biol. Chem.* **245**, 5788.
8. Cha, S. (1968) *J. Biol. Chem.* **243**, 820.
9. Baici, A. and Gyper-Marazzi, M. (1982) *Eur. J. Biochem.* **129**, 33.
10. Duggleby, R. G., Attwood, P. V., Wallace, J. C. and Keeck, D. B. (1982) *Biochemistry* **21**, 3364.
11. Belda, F. J., García-Carmona, F., García-Cánovas, F., Gómez-Fernández, J. C. and Lozano, J. A. (1983) *Biochem. J.* **210**, 727.
12. Mason, H. S. (1957) *Adv. Enzymol.* **19**, 79.
13. Galindo, J. D., Pedreño, E., García-Carmona, F., García-Cánovas, F., Solano-Muñoz, F. and Lozano, J. A. (1983) *Int. J. Biochem.* **15**, 1455.
14. Lerch, K. (1981) in *Metal Ions in Biological Systems* (Sigel, H., ed.) pp. 143–186. Dekker, New York.
15. Lerch, K. (1983) *Molec. Cell. Biochem.* **52**, 125.
16. García-Carmona, F., García-Cánovas, F., Iborra, J. L. and Lozano, J. A. (1982) *Biochim. Biophys. Acta* **717**, 124.
17. García-Cánovas, F., García-Carmona, F., Vera, J., Iborra, J. L. and Lozano, J. A. (1982) *J. Biol. Chem.* **257**, 8738.
18. Lowry, O. H., Rosebrough, A. L., Farr, A. J. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
19 Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* **11**, 431.