REVISTA ESPAÑOLA DE FISIOLOGIA, 35, 209-214, 1979

# Hydroxylating Activity of Frog Epidermis Tyrosinase

F. García-Cánovas, F. García-Carmona, J. D. Galindo, E. Pedreño and J. A. Lozano *

Departamento de Bioquímica
Facultad de Medicina
Murcia (España)

(Received on July 27, 1978)

F. GARCIA-CANOVAS, F. GARCIA-CARMONA, J. D. GALINDO, E. PEDREÑO and J. A. LOZANO. *Hydroxylating Activity of Frog Epidermis Tyrosinase.* Rev. esp. Fisiol., 35, 209-214. 1979.

Trypsin activated in a similar way both the tyrosine hydroxylase and the dopa-oxidasa activities of frog epidermis tyrosinase. Several electron donors reduced or eliminated the lag period for the hydroxylating enzyme. $4 \times 10^{-6}$ M dopa was particularly effective, but without affecting the stationary activity after lag period.

Tyrosine hydroxylase had $K_M = 2.6 \times 10^{-3}$ M for tyrosine and $2 \times 10^{-3}$ M dopa was a competitive inhibitor with $K_I = 5 \times 10^{-4}$ M. The enzyme was inactivated during its actuation. Data on thermal denaturation were similar to other obtained from dopa oxidase. Our results tend to confirm our previous hypothesis that the activatory process of the enzyme is accompanied by a spatial unfolding of the enzyme molecule.

Tyrosinase (E.C. 1.14.18.1) (4) is a copper-protein catalyzing two different transformation: (a) the hydroxylation of tyrosine producing 3.4-dihydroxyphenylalanine (dopa); (b) the oxidation of dopa obtaining dopaquinone and dopachrome, with atmospheric oxygen as the oxidant in both processes. Tyrosinase is the only known enzyme which participates in the process of melanogenesis and it is found in an inactive form in several sources (2, 3, 13). The inactive enzyme in frog epidermis is easily activated by trypsin (1). This report describes some properties of the hydroxylating activity of the enzyme.

## Materials and Methods

The frogs *(Rana esculenta ridibunda)* were obtained locally from November to March. Epidermis separation from dermis, and partially purified enzyme preparation have been previously described (8).

Commercial chemicals were of reagent grade or highest purity available and were employed without further purification. Norit A was obtained from Serlabo

* To whom all correspondence should be addressed.

(Paris) and Celite 535 was purchased from Koch-Light Laboratories Ltd.

L-(3.5$^3$-H)-tyrosine (Radiochemical Centre), 42 Ci/mmol, was purified by thin layer chromatography, on cellulose, at 4° C, using the eluant butanol: acetic acid: water (12:3:5). The tyrosine fraction was evaporated to dryness under nitrogen stream and adjusted to a known volume immediately before the beginning of the assays. To check the correct position of tyrosine labelling and the minimal significance of intramolecular migrations, some reactions were carried out until exhaustion of the substrate. In agreement with POMERANTZ (12) the tritium isotope effect was considered as irrelevant.

The enzyme was activated with soluble trypsin or by using an immobilized trypsin column (8). The assay of tyrosine hydroxylase activity was performed using a simplified POMERANTZ's method (9, 11). Approximately 15 milliunits of the enzyme were added, at zero time, to a reaction medium (1.25 ml) consisting of 0.1 M sodium phosphate buffer pH 7.0, 0.8 mM L-tyrosine, 40 $\mu$M L-dopa and L-(3.5-$^3$H)-tyrosine (2-4 $\times$ $10^6$ d.p.m.). In some cases ascorbic acid was added. 200 $\mu$l of 20 % trichloroacetic acid were added at the end of the normal incubation period (5 min), then a mixture of 0.1 g of Norit A and 0.5 g of celite 535 was added to a total volume of 10 ml (completed with bidistilled water). The medium was vigorously shaken and the mixture centrifuged at 5,000 $\times$ *g* for 15 min. 2 ml aliquots of the supernatant samples were counted using 10 ml of the scintillation solution (300 mg dimethyl POPOP; 4 g PPO; 100 g naphthalene, and dioxane up to 1,000 ml), in a SL-30 Intertechnique scintillation counter, correcting the quenching of the samples with an external ratio device.

Dopa oxidase activity was spectrophotometrically measured as previously described (8).

## Results

Fig. 1 shows the ability of dopa as a hydrogen donor. Dopa at 40 $\times$ $10^{-6}$ M completely eliminated the lag period. However, ascorbic acid up to 1 $\times$ $10^{-3}$ M did not eliminate it entirely. This behaviour is similar to that observed in enzymes from other sources (7, 11). In a similar way to that of dopa oxidase activity (8) the total activation of the proenzyme was reached in the presence of trypsin (3 units/ml) in the medium of activation, but the presence of trypsin during a long time period did affect the stability of the enzyme. For this reason activation was normally obtained running the proenzyme through a column of insoluble trypsin-linked to Sepharose 6B. Carboxypeptidase A also activated the proenzyme, but was less effective as compared with its effect on dopa oxidase proenzyme. The percentages of activations were 54 and 73 % respectively.

The study of the thermal denaturation of the enzyme was carried out placing the preparations of enzyme and proenzymes in thermostatic baths. Aliquots were taken out at different times and their activity measured by the radiometric method, after activation if necessary. Inactivation was substantial only at tem-

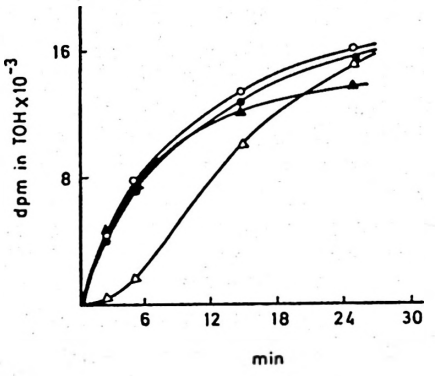


Fig. 1. *Action of L-dopa on the lag period.* ▲—▲ 0.16 mM L-dopa; ●—● 0.08 mM L-dopa; O—O 0.04 mM L-dopa; Δ—Δ no L-dopa.

Table I. *Thermodynamic characteristics of thermal denaturation of tyrosinase hydroxylanting activity.*

| Enzyme | (°C) | Velocity[a] constant $k_i$ | Activation[b] energy $E_a$ | Δ H[b] | Δ G[b] | Δ S[c] | $Q_{10}$[d] |
|---|---|---|---|---|---|---|---|
| | 70 | 1,656 | | 52.57 | 24.42 | 82.07 | |
| Proenzyme | | | 53.2 | | | | 7.9 |
| | 80 | 13,133 | | 52.57 | 23.77 | 81.52 | |
| | 70 | 517 | | 31.31 | 25.21 | 17.4 | |
| Active | | | 32.0 | | | | 4.4 |
| | 80 | 2.262 | | 31.29 | 24.94 | 18.0 | |

a. $10^6 \times s^{-1}$. b. kcal × $mol^{-1}$. c. cal × $mol^{-1}$ × $°K^{-1}$. d. $k_{25}o/k_{70}o$.

peratures above 60° C, presenting first order kinetics within determined time intervals. In each case log activity was plotted against time, and velocity constants for inactivation ($k_i$) were deduced from the respective slopes. Active enzyme was more sensible to thermal denaturation than the proenzyme which, furthermore, presented a biphasic behaviour. In this case $k_i$ was calculated only from the first portion of the graph. Log $k_i$ was plotted against temperature$^{-1}$, and from the slopes of the corresponding straight lines an activation energy of 52.3 kcal × $mol^{-1}$ and 32 kcal × $mol^{-1}$ was calculated for the denaturation of the proenzyme and enzyme, respectively. At each temperature, enthalpy (ΔH), free energy (ΔG) and entropy (ΔS) changes were obtained from the equations:

$$\Delta G = R.T.\ (\ln \frac{KT}{h} - \ln k_i);$$

$$\Delta H = E_a - R.T.$$

$$T.\ \Delta S = \Delta H - \Delta G \text{ and}$$

$$Q_{10} = \frac{k_t + 10}{k_t}, \text{ in which}$$

K = Boltzmann's constant; h = Planck's constant; $k_i$ = velocity constant for inactivation; T = °K. In table I it may be observed that $k_i$ values are higher for the proenzyme than for the activated enzyme. A similar situation is found for entropy change and $Q_{10}$ values. In the absence of trypsin, thermal treatments from 25 to 85° C, lasting from 5 to 60 minutes, were unable to activate the proenzyme partially or totally.

In a previous report (10) we studied the inactivation of the oxidase activity of banana phenolase during its catalytic action. The same situation has been present in the hydroxylase activity of frog epidermis: the rate of product formation

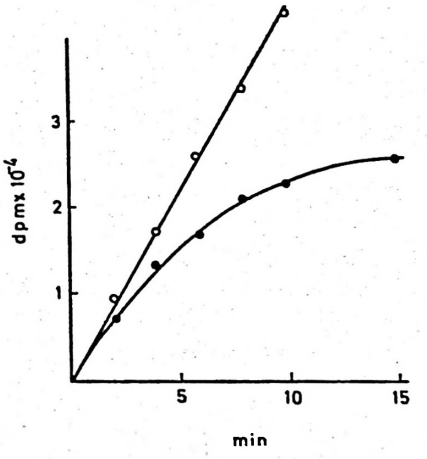


Fig. 2. *Time-course of the reaction in the presence of 40 μM L-dopa.* O—O with 1.16 mM ascorbic acid; ●—● ascorbic acid not added.

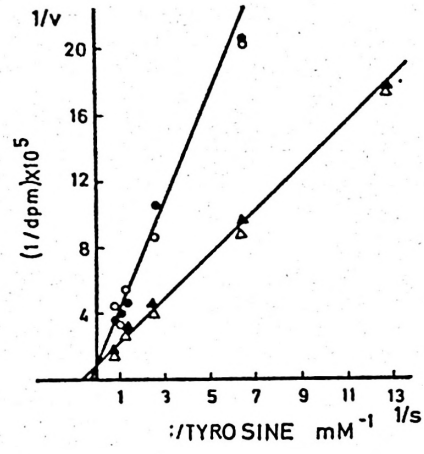


Fig. 3. *Competitive action of L-dopa versus L-tyrosine in presence of absence of ascorbic acid.*

Δ—Δ—40 μM L-dopa and ascorbic acid not added; ▲—▲ 40 μM L-dopa and 1.16 mM ascorbic acid; ●—● 2 mM L-dopa and ascorbic acid not added; O—O 2 mM L-dopa and 1.16 ascorbic acid.

was not proportional to, but diminished with the reaction time as seen in figure 2. The effect of ascorbic acid has been drastic on the hydroxylant activity, eliminating the inactivation reaction and confirming other data which indicated that the inactivation in the absence of ascorbic acid, was not due to the diminution of substrate concentration. The inhibitory action of L-dopa is shown in figure 3, with $K_i = 5 \times 10^{-4}$ M at $2 \times 10^{-3}$ M L-dopa concentration. The presence of ascorbic acid did not modify this inhibition, showing that dopachrome formation did not affect the inactivation reaction of the enzyme, which is in agreement with SHIMAO *et al.* (15).

## Discussion

Although many hypotheses exist to explain the mechanism of the *in vivo* activation of the enzymatic activities of tyrosinase, in melanomas the existence of an increase of the hydroxylating activity produced by the liberation of certain proteases has been demonstrated (6). It can be hypothesised, that in epidermis some proteases could act on the enzyme producing a limited proteolysis together with a conformational change. One of these two actuations, or both simultaneously, could have the responsability for the enzyme activation. On the other hand, our results on activation using carboxypeptidase A do not coincide with those described by BARISAS and MCGUIRE in dopa oxidase from *Rana pipiens* (1). We have verified the carboxypeptidase activity and the absence of tryptic activity in the preparation.

Data on thermal denaturation are in agreement with those previously obtained by us working with dopa oxidase (8), and indicate the possible existence of an enzyme unfolding during the activation process. In this manner the entropy change is positive and relatively higher for the proenzyme as compared with the active enzyme, as happens with the values of velocity constant and $Q_{10}$. It could be assumed that the transformation from proenzyme into enzyme is accompanied by an increase of disorder and certain unfolding of the enzyme molecule, thus adquiring a more stable energetic state.

With regard to the inactivation reaction of the enzyme during its actuation the process has been widely discussed (10, 14, 15) and some possible explanations proposed, some of them involving a rupture of the linkage existent between protein and copper. Nevertheless there is no satisfactory explanation for the ascorbic acid effect. GRISOLIA's concept of protein plasticity (5) could explain why the enzyme, during its catalytic activity, suffers a conformational change producing a state in which the tendency to recover its initial energetic structure would be attenuated, thereby favouring the denaturation process. The presence of ascorbic acid could reduce the tendency

towards the denaturated state by conversion of the enzyme into forms of lower energy.

*Acknowledgement*

This work was supported by a grant of Comisión Asesora Científica y Técnica de la Presidencia del Gobierno. Madrid. F. G. Carmona had a grant from Ministerio Educación of Spain.

## Resumen

Se utiliza un método radiométrico basado en la medida de $^3HOH$ liberado al medio por hidroxilación de L-(3,5-$^3H$) tirosina.

La tripsina activa en una forma similar ambas actividades, la tirosina hidroxilasa y la dopa oxidasa de tirosinasa de epidermis de rana.

La actividad tirosinasa hidroxilasa presenta un período de retardo eliminable en presencia de donadores de electrones. A concentraciones $4 \times 10^{-5}$ M dopa fue particularmente efectiva, sin afectar a la actividad, medida tras el período de retardo.

La tirosina hidroxilasa tiene una $K_M = 2{,}6 \times 10^{-3}$ M para tirosina y la dopa a $2 \times 10^{-3}$ M fue un inhibidor competitivo, con $K_I = 5 \times 10^{-4}$ M.

El enzima sufre un proceso de inactivación durante su actuación. Los datos sobre desnaturalización térmica fueron similares a otros obtenidos a partir de dopa-oxidasa.

## References

1. Barisas, B. G. and McGuire, J.: *J. Biol. Chem.*, 249, 3151-3156, 1974.
2. Burnett, J. B., Seiler, H. and Brown, I. V.: *Cancer Res.*, 27, 880-889, 1967.
3. Davidson, R. and Yamamoto, K.: *Proc. Nat. Acad. Sci. U. S.*, 60, 894-901, 1968.
4. Enzyme Nomenclature: *Commission on Biochemical Nomenclature*, Elsevier, Amsterdam, 1973.
5. Grisolía, S.: *Physiol. Chem. Physic.*, 4, 37-46, 1972.
6. Haberman, H. F. and Menou, I. A.: *Acta Dermatovener Stockholm*, 51, 407-412, 1971.
7. Lavollay, J., Legrand, G., Lehougre, G. and Neumann, J.: *Physiol. Veg.*, 13, 667-676, 1975.
8. Lozano, J. A., Montserrat, F., Galindo, J. D. and Pedreño, E.: *Rev. es. Fisiol.*, 31, 21-19, 1975.
9. Nagatsu, I., Levitt, M. and Udenfriend, S.: *Anal. Biochem.*, 9, 122-126, 1964.
10. Padrón, M. P., Lozano, J. A., González, A. G.: *Phytochem.*, 14, 1959-1963, 1975.
11. Pomerantz, S. H.: *Biochem. Res. Commun.*, 16, 188-194, 1964.
12. Pomerantz, S. H.: *J. Biol. Chem.*, 241, 161-168, 1966.
13. Satoh, G. J. Z. and Mishima, Y.: *Arch. Derm.*, 140, 9-14, 1970.
14. Seiji, M., Fukuzawa, H., Shimao, K. and Itakura, H.: In «Pigment Cell», vol. 1, Karger, Basel, 1973, p. 39-46.
15. Shimao, K., Seiji, M. and Fukuzaw, H.: *J. Exp. Med.*, 114, 263-272, 1974.