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# Dithiothreitol Inactivation of Frog Epidermis Tyrosinase*

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Frog epidermis tyrosinase inactivation by dithiothreitol (DTT), both in the proenzyme and active forms, have been studied.

Upon increasing DTT:enzyme-up to $10^6$:1 ratios and depending on the incubation period, two inactivation steps both in proenzyme and enzyme were observed. Enzyme lost its activity faster than proenzyme. Oxygen favoured inactivation.

After dialysis of the DTT:protein ($10^6$:1) incubation medium, 20 % of the original enzyme activity was recovered. However it decreased to 15 % if the enzyme had been incubated with substrate. Conformational changes due to loss of activity were not shown on the fluorescence spectra.

Tyrosinase (E.C. 1.14.18.1) (5) is a copper enzyme having two differents catalytic activities: *a*) hydroxylation of tyrosine producing L-3,4 dihydroxy phenylalanine (L-dopa) and *b*) oxidation of dopa into dopa-quinone. This work is related with the last reaction in which oxygen is the oxidant.

The enzyme participates in melanogenesis and is found, in some tissues, as an inactive molecule or proenzyme (2, 4, 14). Some proteinases catalyzes the conversion of the inactive enzyme from frog epidermis into the active form (12).

On the other hand the reductive effect obtained by agents as 2-mercaptoethanol, dithiothreitol (DTT), glutathione, etc., on some disulfide bridges in proteins is a well known phenomenum and different circumstances can produce a certain degree of reversibility (10, 17).

DTT is a good reductor of disulfide bridges and has no action on free thiol groups (3). Its actuation can be carried out in other additional ways: *a*) DTT auto-oxidation produces hydrogen peroxide which can oxidize certain specific residues (16); *b*) DTT can act as a chelating agent of metalic ions (7).

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The purpose of this work is to study the effect of DTT on the active and inactive forms of frog epidermis tyrosinase, and to discuss the possible mechanisms of the inactivation of the enzyme.

## Materials and Methods

Trypsin type III (E.C. 3.4.4.4), 12,500 BAEE U/mg, dithiothreitol and bovine serum albumin (type V) were supplied by Sigma. L-dopa and the other chemicals (analytical grade) were from Merck. Frogs *(Rana esculenta ridibunda)* were obtained from local suppliers.

*Enzyme preparation.* Extraction and subsequent purification of the enzyme was carried out by affinity chromatography on p-amino benzoic acid, as previously described (9).

*Dopa-oxidase activity measurements.* Activation of the enzyme was obtained with soluble trypsin acting on the proenzyme for 5 min at 37° C or with insoluble trypsin linked to Sepharose.

0.5 ml of activated enzyme was added at zero time to 2.5 ml of substrate solution containing 7 nmol/mg of L-dopa in buffer phosphate pH 7. The dopachrome formed was spectrophotometrically followed at 475 nm and slopes at zero time were calculated in order to know the initial velocities.

One enzyme unit transforms 1 $\mu$mol of L-dopa per minute at 20° C and pH 7. $\epsilon$ value for dopachrome is $3.7 \times 10^4$ l/mol per centimeter.

*Protein determination.* Protein was determined using the HARTREE modification of the Lowry method (8) with bovine serum albumin as standard.

*Proenzyme and enzyme inactivation.* Proenzyme or enzyme solutions, approximately $0.5 \times 10^{-5}$ M were incubated at 4° C in 0.1 M phosphate buffer pH 7.0 and DTT with variable molar ratios rangin from 1:1 to $10^6$:1 of DTT: enzyme. Aliquots were removed every 10 minutes during a 60 min period and their activities determined.

The removal of air in the medium was effected by means of nitrogen bubbling for 30 min through the solution of 0.1 M phosphate buffer pH 7.

The recovery of the activity, after treatment of DTT, was attempted by an 8 h dialysis at 4° C against 0.1 M phosphate buffer.

*Fluorescence measurements.* The fluorescence measurements were made using the same molar ratios and incubation periods as those of activity measurements. Non corrected spectra were obtained in a Perkin-Elmer spectrofluorimeter, mod. 24, with a Xenon lamp. Light excitations were performed at 280 nm in order to determine the contribution of tyrosine and tryptophane residues to the fluorescence, or at 290 nm for the participation of only tryptophane residues (15).

## Results

Two different steps can be distinguished in the inactivation of the proenzyme produce by incubation with DTT, using ration ranging from 1:1 to $10^6$:1 for DTT: protein. In the first step which lasted less than 10 minutes, activity was lost rapidly. In the second, the activity diminished very slowly (fig. 1, curve 1).

When DTT:proenzyme ratio was as high as $10^6$:1, the activity dropped very quickly and before 10 minutes was practically zero (fig. 1, curve 3). A striking situation was observed at $10^3$:1 ratio, in which during 1 hour of incubation, activity was disminishing at less velocity than in the other cases (fig. 1, curve 2). The behaviour obtained when active enzyme was incubated with DTT, can be observed in the same figure, curves 4-6. Results very similar to those of the proenzyme, but with the active enzyme residual activities were always lower than with the proenzyme.

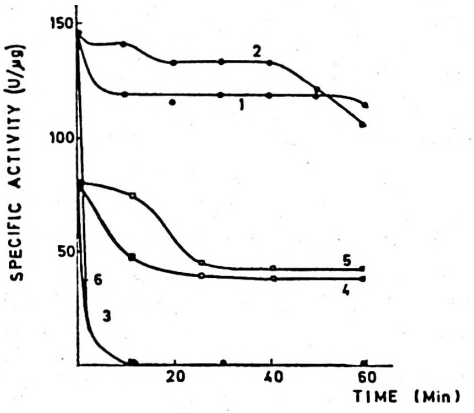


Fig. 1. *Enzymatic activity variation of proenzyme (●-●) and enzyme (□-□) forms of tyrosinase with incubation time at differents DTT : protein ratios.*
1, 4, Molar ratios ranging between 1:1 and $10^5$:1 (except $10^3$:1); 2, 5, Molar ratio $10^3$:1; 3, 6, Molar ratio $10^6$:1.

Figure 2 represents the relative residual activities of both forms of the enzyme after 60 min of incubation in the presence and abscence of DTT, which was used at several concentrations. It seems that both forms had intermediate states in their inactivation having 60-75 % and 45-55 % of their initial activities respectively. Inactivation was completely achieved at a molar ratio DTT:protein of $10^6$:1.

In the first minutes of incubation, in-

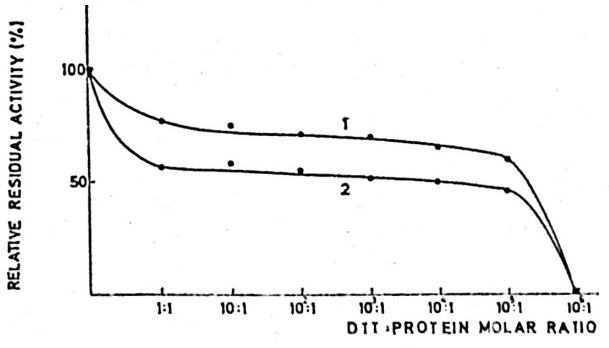


Fig. 2. *Plot of relative residual activity versus DTT : protein molar ratio.*
Incubation time: 60 minutes. The residual activity was referred to the protein activity without DTT. 1, Proenzyme; 2, Enzyme.

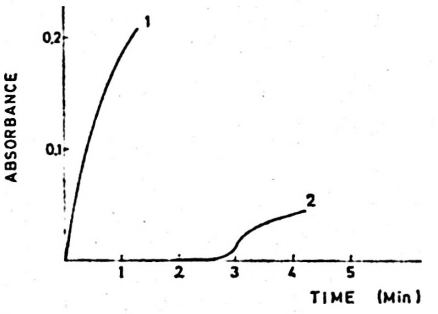


Fig. 3. *Reactivatory effect of substrate (L-dopa) to the inhibitory action of DTT on the enzyme activity.*
1, Enzyme without DTT; 2, $10^6$:1 molar ratio DTT: protein.

activation always proceeded quickly, independent of DTT concentration. When the enzyme was incubated at 25° C with DTT (ratio $10^6$:1) and L-dopa, a lag period of 3 min was observed after which there was a partial recovering of activity until it reached 20 % of that of the non-treated enzyme (fig. 3). Nitrogen bubbling through the incubation medium protected the enzyme against inactivation. Using the $10^6$:1 ratio of DTT:enzyme, at the end of a 60 min of incubation 15 % of the initial activity remained.

Table I shows the fluorescence measurements obtained at 280 and 290 nm excitation wavelenghts on similar concentrations of proenzyme or enzyme.

Excitations carried out at 280 and 290 nm at several DTT:E ratios showed only minor displacements in the fluorescence ma-

Table I. *Wavelengths and relative intensities of the fluorescence maximum for proenzyme and active forms at the same concentrations, ($0.5 \times 10^{-6}$ M), when excitation was carried out to 280 and 290 nm respectively.*

| | Proenzyme | Enzyme |
|---|---|---|
| $\lambda^{em}_{280}$ | 337.0 | 328 |
| $I^{280}_{R}$ | 81.2 | 100 |
| $\lambda^{em}_{290}$ | 333.0 | 328 |
| $I^{290}_{R}$ | 81.7 | 100 |

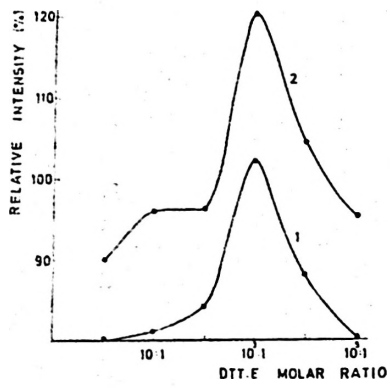


Fig. 4. *Fluorescence relative intensity percentage versus DTT: protein molar ratio for the enzyme form.*
1, At $\lambda_{exc.} = 280$ nm; 2, At $\lambda_{exc.} = 290$ nm.

xima as compared to those obtained without the participation of DTT. Incubation with DTT produced displacements of $\pm$3-4 nm or lower (if the excitation was at 290 nm).

The relative fluorescence intensities for the DTT-treated enzyme were always lower than those of the normal enzyme. One exception (fig. 4), occurred if DTT: E ratio was $10^3$:1 in which case both relative intensities remained quite similar.

The type of inhibition produced by DTT did not correspond to a defined mechanism since kinetics of the process was dependent on DTT: E ratio. On the other hand, another reductive agent, 2-mercaptoethanol presents a well defined non-competitive mechanism (6), which is usual for substances which can be linked in a reversible way with some functional groups of proteins.

## Discussion

The action of a powerfull reductive agent like DTT on enzyme disulfides groups can result in their breakdown and consequently, induce profound conformational changes favouring the subsequent decrease of the catalytic activity of the enzyme (17). The degree of inactivation depends on the number of disulfide bridges needed for the activity.

Using DTT:protein ratios lower than $10^5$:1, DTT produced a partial inactivation of tyrosinase, possibly because not all the disulfide bridges involved in the maintenance of the enzyme conformation were reduced or modified at those DTT concentrations. Inactivation of the enzyme was more easily produced by DTT than the inactivation of the proenzyme, perhaps due to the enzyme being in a more unfolded conformation than the proenzyme (11) and in this way their disulfide bridges are more exposed to the attack of external agents.

The substrate, L-dopa, had a limited reactivatory effect. One possible explanation could be that the substrate induced a conformational change on the protein. In other types of experiments the substrate can induce the activation of the enzyme linked, as proenzyme, to an insoluble support and during this process seems to be some conformational changes. The presence of oxygen seems to favour the inactivation of the enzyme by DTT.

Since the fluorescence of tryptophane presents a higher quantum yield than of tyrosine, it could be deduced that tryptophane was more affected by the action of DTT, because: *a*) there were no significant batochromic displacements, and *b*) the relative intensities of fluorescence were generally lower in the presence of DTT.

The existence of a special relationship between activity and structure was not clear, since the activity varied in two steps as previously indicated, while wavelength displacements fluctuated greatly, without any connection with activities. In regards to relative intensities, only at DTT:E ratio of $10^4$:1 there was a similarity between intensities of the enzyme with and without DTT. This result may be a consequence of the good level of activity of the enzyme at that ratio as

compared to other different concentrations of DTT.

The data obtained seems to agree with one previously suggested mechanism (10) in which DTT produces inactive complex through the formation of mixed disulfide bridges DTT-protein that slowly and only in the presence of substrate, break down, leading to a certain degree of enzyme reactivation.

The formation of the mixed complex is more easily facilitated with the enzyme than with the proenzyme, because in the first case the protein is more unfolded. Another additional argument against the action of DTT as a reductive agent of the disulfide bridges of the enzyme is that in such case the fluorescence of the enzyme would be seriously affected in a way different from that observed, viz: *a*) an hypsochromic displacement was observed for the transition proenzyme → enzyme, supporting other data about the existence of a higher concentration of tyrosine than tryptophane in the enzyme (1, 13), and *b*) a high relative intensity of fluorescence suggesting that the disulfide bridges are modified in that transition. Furthemore, the proposed mechanism of DTT action and the partial recovering of activity by dialysis and by the presence of substrate is similar to that previously described by KIM and HOROWITZ (10) for the enzyme rhodanase.

## Resumen

Se estudia la inactivación de la proteína tirosinasa de epidermis de rana en las dos formas de proenzima y enzima por la acción de DTT. La inactivación tuvo lugar en dos etapas en función del tiempo de incubación con relaciones crecientes de DTT:proteína.

La pérdida de actividad en el enzima fue más acusada que en el proenzima, debido al posible mayor desplegamiento del primero.

Cuando la relación molar DTT:proteína alcanzó el valor $10^6$:1, la inactivación fue total. La ausencia de aire, conseguida mediante burbujeo de nitrógeno en el medio de incubación de la proteína, no consiguió la total inactivación de la misma.

La eliminación de DTT por diálisis de disoluciones DTT:enzima de $10^6$:1 condujo a una recuperación muy baja de actividad dopa-oxidasa de la proteína (20 %). La incubación del enzima en presencia de DTT en relación $10^6$:1 junto con L-dopa dio como resultado, tras un período *lag* de 3 minutos, una reactivación del 15 % respecto al enzima nativo.

No existieron cambios conformacionales medibles, por fluorescencia acompañando a la pérdida de actividad por la acción del DTT.

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