The process for the activation of frog epidermis pro-tyrosinase

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1. Purified pro-tyrosinase from epidermis of the frog Rana esculenta ridibunda can be activated in vitro by several proteinases (trypsin, α-chymotrypsin, Pronase) and by light. 2. Both pro-tyrosinase and tyrosinase are composed of a single type of subunit having pI 7.2 and approximate molecular weights 68000 and 62000 respectively. A peptide of low molecular weight is released as a consequence of the proteolytic activation. Pro-tyrosinase and tyrosinase have different quaternary structures, the proenzyme being a dimer of Mr approx. 115000 and the enzyme a tetramer of Mr approx. 210000. 3. The activation process was affected by several agents (L-3,4-dihydroxyphenylalanine, urea, formamide) that prevented, partially or totally, the activation of pro-tyrosinase. 4. The activation of pro-tyrosinase seems to be the result of a cleavage of the polypeptide chain that determines changes in tertiary or quaternary structure.

Many proteins are synthesized as inactive precursors that can be converted into the active forms by a selective enzymic cleavage of peptide bonds. Typical examples are the processes of hormone production, blood clotting, fibrinolysis, complement activation, fertilization, metamorphosis, supramolecular assembly and digestion. Tyrosinase (EC 1.14.18.1) has been found as an inactive precursor in the epidermis of amphibians (Barisas & McGuire, 1974; Mikkelsen et al., 1975) and in the haemolymph of some insects (Ashida & Ohnishi, 1967). The pro-enzyme can be activated in vitro by proteinases (Barisas & McGuire, 1974; Lozano et al., 1975) and other agents such as light (Mikkelsen et al., 1975), heat (Chen, 1975) and immobilization on a solid support (Iborra et al., 1979). Tyrosinase is the only known enzyme participating in the process of melanin biosynthesis. It catalyses the oxidation of L-tyrosine to L-dopa (hydroxylase activity) and of L-dopa to dopaquinone (oxidase activity), oxygen being the oxidant in both processes.

Numerous examples of zymogen activation have been described and studied in detail (Neurath, 1957; Steiner et al., 1975), but few studies have been devoted to the investigation of the activation of pro-tyrosinase. This enzyme presents two aspects that make it a very suitable system for the study of the activation process: the oligomeric structure of the active enzyme (Barisas & McGuire, 1974; Mikkelsen & Triplett, 1975) and the possibility of obtaining a continuous spectrophotometric record of the activation process. In the present work we have studied the process of activation and the differences between pro-tyrosinase and tyrosinase.

Experimental

Materials

Frogs (Rana esculenta ridibunda) were obtained from local suppliers. Proteinases were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Sepharose 6B, CM-Sephadex and phenyl-Sepharose were obtained from Pharmacia (Uppsala, Sweden). Acrylamide and bisacrylamide were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Ampholines were from LKB Produkter (Bromma, Sweden). L-Dopa and all the other reagents were analytical grade from Sigma Chemical Co.

Enzyme preparation

Frog epidermis pro-tyrosinase was extracted and purified by the procedure of Lozano et al. (1975), with two additional steps, involving CM-Sephadex and phenyl-Sepharose chromatography, as described by Iborra et al. (1981). Epidermis was separated from dermis after incubation with a 2M-NaBr solution for 24 h at 0–4°C. After several washings with double-distilled water a negative
bromide reaction was achieved, and the epidermis was freeze-dried and kept at 0–4°C until used.

A 1g portion of freeze-dried epidermis was extracted with 20 ml of double-distilled water. After centrifugation at 15000 g (rsv, 7.9 cm) for 20 min, the supernatant was discarded and the sediment was extracted with 20 ml of 0.1M-sodium phosphate buffer, pH 7, and centrifuged at 18000 g (rsv, 7.9 cm) for 30 min. A saturated solution of (NH₄)₂SO₄ (neutralized at pH 7 with NaOH) was added until 40% saturation was reached, and the solution was kept overnight. After centrifugation at 15000 g (rsv, 7.9 cm) for 30 min, sediment was removed and a saturated solution of (NH₄)₂SO₄ was added again until 55% saturation was reached, and the solution was kept overnight. The solution was centrifuged at 15000 g and the precipitate was redissolved with 5 ml of 0.1M-sodium phosphate buffer, pH 7.

CM-Sephadex C-50 was swollen in 50 mM-sodium phosphate buffer, pH 7, and poured on to a glass filter. A 5 ml volume of pro-enzyme solution was added to the gel with stirring. Pro-tyrosinase was desorbed with 0.1M-sodium phosphate buffer, pH 7, and 2 ml fractions were collected. The pro-enzyme solution obtained in the CM-Sephadex step, previously adjusted to 0.5M-sodium phosphate buffer, was passed through a phenyl-Sepharose CL-4B column (1.2 cm × 20 cm) equilibrated with 0.5M-sodium phosphate buffer, pH 7, and washed with the same buffer until no protein was detected in the eluates. Then 0.1M-sodium phosphate buffer, pH 7, was used, and 2 ml fractions were collected. The purified pro-enzyme solution was concentrated by ultrafiltration on an Aminco CF-25 filter.

**Pro-enzyme activation**

Activation of the pro-enzyme was accomplished either by incubation with soluble proteinases or by using a trypsin–Sepharose column (Iborra et al., 1977). Activation by light was achieved by exposure to white light (300 W lamp, 30 cm distance) at a constant temperature of 25°C.

**Enzyme activity**

Enzyme activity was measured spectrophotometrically at 475 nm. One enzyme unit was defined as the amount of enzyme transforming 1 μmol of L-dopa/min at 25°C, with 7.6 mM-L-dopa in 0.1M-sodium phosphate buffer, pH 7. ε₄₇₅ for the conversion of L-dopa into dopachrome was taken as 3600 M⁻¹ cm⁻¹.

Kinetics of the activation process were investigated in an Aminco DW2 spectrophotometer, equipped with a cuvette, magnetic stirrer and a Hewlett-Packard recorder with a kinetic response that allows the dead recording time to be minimized. To 2 ml of 7.6 mM-L-dopa in 0.1M-sodium phosphate buffer, pH 7, 5 μl of pro-tyrosinase (1.4 mg/ml) and 0.5 ml of trypsin (0.4 mg/ml) in 0.1M-sodium phosphate buffer, pH 7, were added. Dopachrome formation was followed at 475 nm.

The protein content of pro-enzyme and enzyme preparations was determined by a modified Lowry method (Hartree, 1972), with bovine serum albumin as standard.

**Gel electrophoresis**

Electrophoresis in SDS/polyacrylamide gels was performed as described by Weber & Osborn (1969). Slab-gel electrophoresis was performed with the discontinuous system described by Laemmli (1970). The 15%-acrylamide gels (acrylamide/bisacrylamide, 30:0.8, w/w) were used under the conditions described by Douglas et al. (1979). Determination of dopa oxidase activity on the gels was achieved by incubating the gels (previously treated several times with a solution of 3M-urea and 2% Triton X-100 in 0.1M-Tris/HCl buffer, pH 7.4) with 7.6 mM-L-dopa.

Electrophoresis of the peptide produced in the activation process was performed in SDS/22.5%-polyacrylamide gels; 150 μg portions of protein were applied and the gels were run at 7mA/gel for 2 h.

**High-voltage electrophoresis**

High-voltage electrophoresis was performed in a Shandon Southern L-24 apparatus with cellulose acetate as support and 50 mM-phosphate buffer, pH 6.5, at 200 V/cm for 3 h, with a water cooling device.

**Isoelectrofocusing**

The isoelectrofocusing gels (0.25 cm × 12 cm) were prepared and run as described by Cabral & Schatz (1979). The pH range of the isoelectrofocusing gels was 4–9.5.

**Two-dimensional electrophoresis**

This procedure was based on that of Cabral & Schatz (1979), which is a modification of the original method of O'Farrell (1975). Triton X-100 was used instead of Nonidet P-40, and the samples, treated with the dissociating mixture, were heated at 70°C for 10 min. The isoelectrofocusing dimension was run at 260 V for 14 h. The second dimension was in SDS/15%-polyacrylamide gels (40 mA, 6 h). Pro-tyrosinase and tyrosinase (20 μg portion of each) were applied on to the isoelectrofocusing gel.

**Gel filtration**

Gel filtration was performed on a Sepharose 6B column (0.27 cm × 60 cm) equilibrated with 0.1M-sodium phosphate buffer, pH 7. The volume of the fractions applied to the column was 0.75 ml. The molecular-weight standards used were chymotrypsinogen (Mr, 25000), ovalbumin (Mr, 45000), 1982.
bovine serum albumin ($M_r$ 67000), collagenase ($M_r$ 109000), catalase ($M_r$ 240000) and ferritin ($M_r$ 540000).

**Results**

**Activation process**

Table 1 shows the steps in the purification procedure and the specific activity of the enzyme at each stage. Electrophoresis on 10%-polyacrylamide gels of samples of pro-enzyme purified 19-fold and containing 20 μg of protein showed a single band when stained with Coomassie Brilliant Blue. Pro-enzyme with this grade of purification was used in all the experiments. The extraction of pro-enzyme from epidermis by phosphate buffer plus detergents (Triton X-100, cholate and deoxycholate) did not increase the amount of pro-enzyme. Proteinases with different specificities (trypsin, chymotrypsin, thermolysin, Pronase) activated the pro-enzyme. Fig. 1 shows the time course of pro-enzyme activation by proteinases. The pro-enzyme/proteinase weight ratio was varied in the range 75–150:1 and the efficiency for activating the pro-enzyme was similar with all the proteinases tested. Addition of crude preparations of epidermis pro-tyrosinase extracted with phosphate buffer to purified enzyme activated by immobilized trypsin did not alter its activity, thus excluding the possibility of the existence of a free tyrosinase inhibitor of peptidic nature, such as the one described in cuticle extracts from larvae of the fleshfly Sarcophaga barbata (Hughes & Price, 1975).

Fast activation of pro-enzyme by high proteinase concentration was studied (Fig. 2). Under these conditions we determined the rate constant $k$ of the last step of the activation process:

$$\text{Pro-enzyme} + \text{proteinase} \rightarrow \text{pro-enzyme} - \text{proteinase} \xrightarrow{k} \text{enzyme} + \text{proteinase}$$

by coupling this reaction to the oxidation:

$$\text{L-Dopa} + \text{oxygen} \rightarrow \text{dopachrome}$$

by the active enzyme. The enzyme-catalysed oxidation of L-dopa itself showed no lag phase.

From the trace recording the lag period and the slope were calculated, by the procedure of Frieden (1970). For a fixed amount of pro-enzyme, all the proteinases gave the same slope, showing that all of them activate the pro-enzyme completely. An increase in the proteinase/pro-enzyme ratio did not produce any variation in the duration of the lags (Table 2).

It is noteworthy that the substrate L-dopa can affect the activation process. Incubation of the pro-enzyme with 7.6 mM L-dopa for a few seconds and further activation by trypsin gave lower values of enzymic activity than when trypsin and pro-enzyme were added simultaneously to L-dopa. This effect was dependent on time and on L-dopa and pro-enzyme concentrations. Tyrosine showed the same behaviour.

The purified pro-enzyme was also activated by light, as reported by Mikkelsen et al. (1975) for Rana pipiens pro-tyrosinase. However, the activation process was slower than that described by those authors. Fig. 3 shows the time course of activation of pro-tyrosinase by light. A sample of pro-tyrosinase not exposed to light did not develop tyrosinase activity. However, samples exposed to light and those not so exposed were activatable to the same degree by trypsin. The photochemically activated enzyme had kinetically similar behaviour to that of tyrosinase activated by proteinases.

**Subunit composition**

The subunit composition of pro-tyrosinase and tyrosinase was studied by electrophoresis in 15%-polyacrylamide gels containing SDS. Pro-enzyme and enzyme showed different mobilities, corresponding to chains of $M_r$ approx. 68 000 and 62 000 respectively (Fig. 4). Tyrosinase activated by different agents (trypsin, α-chymotrypsin, thermolysin, Pronase, light) showed the same mobility. Analysis of samples taken at intervals during the process of the activation of pro-tyrosinase by trypsin, and therefore with different proportions of active tyrosinase, showed a good correlation between the bands corresponding to pro-enzyme and enzyme and the

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>3.3</td>
<td>(100)</td>
<td>(1.0)</td>
</tr>
<tr>
<td>55%-satn. (NH₄)₂SO₄</td>
<td>13.8</td>
<td>68</td>
<td>4.2</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>48.1</td>
<td>57</td>
<td>14.6</td>
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<tr>
<td>Phenyl-Sepharose</td>
<td>62.4</td>
<td>48.5</td>
<td>19</td>
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percentage activity (Fig. 4). These results suggest that, in the activation process, a cleavage of the polypeptide chain of the pro-enzyme takes place. Since tyrosinases activated by proteases and light show the same mobility, it is suggested that the cleavage site, in all cases, occurs in a fixed region on the polypeptide chain.

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**Fig. 1. Activation of pro-tyrosinase by proteinases**

Pro-tyrosinase (2 mg/ml) was incubated with various amounts of proteinases. Samples (20 μl) were removed and activity was assayed on their addition to 2 ml of 7.6 mM-L-dopa and 0.5 ml of 0.1 M-sodium phosphate buffer, pH 7. △, Pro-tyrosinase/chymotrypsin ratio 150:1 (w/w); •, pro-tyrosinase/trypsin ratio 100:1 (w/w); ○, pro-tyrosinase/Pronase ratio 75:1 (w/w).

**Table 2. Kinetic parameters of activation of pro-tyrosinase by proteinases**

For experimental details see the text.

(a) Activation of pro-tyrosinase by several proteinases

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Lag (τ) (s)</th>
<th>k (s⁻¹)</th>
<th>Slope (ΔA₄₇₅/min)</th>
</tr>
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<tr>
<td>Pronase</td>
<td>6</td>
<td>0.166</td>
<td>0.22</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>6.2</td>
<td>0.161</td>
<td>0.22</td>
</tr>
<tr>
<td>Trypsin</td>
<td>6.2</td>
<td>0.161</td>
<td>0.22</td>
</tr>
</tbody>
</table>

(proteinase/pro-tyrosinase ratio 50:1)

(b) Effect of incubation of pro-tyrosinase with L-dopa

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Trypsin/pro-tyrosinase ratio (w/w)</th>
<th>Lag (τ) (s)</th>
<th>k (s⁻¹)</th>
<th>Slope (ΔA₄₇₅/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50:1</td>
<td>6.2</td>
<td>0.161</td>
<td>0.22</td>
</tr>
<tr>
<td>0</td>
<td>100:1</td>
<td>6.2</td>
<td>0.161</td>
<td>0.22</td>
</tr>
<tr>
<td>1</td>
<td>50:1</td>
<td>6.1</td>
<td>0.164</td>
<td>0.17</td>
</tr>
<tr>
<td>1</td>
<td>100:1</td>
<td>6.1</td>
<td>0.164</td>
<td>0.17</td>
</tr>
</tbody>
</table>

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**Fig. 2. Kinetics of the activation of pro-tyrosinase by trypsin**

To 2 ml of 7.6 mM-L-dopa, 5 μl of pro-tyrosinase (1.4 mg/ml) and 0.5 ml of trypsin (0.4 mg/ml) in 0.1 M-sodium phosphate buffer, pH 7, were added. Dopachrome formation was followed at 475 nm. Determination of the value of the first-order rate constant k for the degradation of the complex pro-tyrosinase–trypsin was made by extrapolation of the graph to [P] = 0. The lag period τ can be related to k as τ = 1/k.
Electrophoresis in SDS/22.5%-polyacrylamide gels overloaded with tyrosinase activated by trypsin showed a peptide of low molecular weight that did not appear when equivalent amounts of both heat-denatured pro-enzyme and trypsin were applied. This peptide is the result of the proteolytic cleavage of the pro-enzyme chain.

Isoelectrofocusing under disaggregating conditions showed that pro-enzyme and enzyme have a very sharp isoelectric point at about 7.2. Two-dimensional electrophoresis by O'Farrell's (1975) method of mixtures of pro-enzyme and enzyme showed two spots of pI 7.2 and Mₐ approx. 68000 and 62000. Thus pro-tyrosinase and tyrosinase each seem to be composed of a single kind of subunit.

Oligomeric structure

The molecular weights of native pro-tyrosinase and tyrosinase, activated by immobilized trypsin, determined by gel chromatography in Sepharose 6B were approx. 115000 and 210000 respectively (Fig. 5). This shows that pro-enzyme has a dimeric structure, whereas the active enzyme is tetrameric. On electrophoresis in 7.5%-polyacrylamide gels, performed at pH 4.6, no appreciable differences were observed between these forms. However, pro-tyrosinase had greater anodic mobility than tyrosinase in high-voltage electrophoresis on cellulose acetate at pH 6.5 and 200 V/cm.

These differences in quaternary structures of pro-enzyme and enzyme seem to be related to the behaviour of both proteins towards dissociating agents such as urea and formamide. Tyrosinase incubated with 8 M-urea for 5 h retained its activity (Fig. 6). However, when pro-tyrosinase was incubated with different concentrations of urea the pro-enzyme lost the ability to be converted by trypsin into its active form. The possibility that urea could affect the catalytic action of trypsin can be ruled out, since the pro-enzyme incubated with trypsin in the presence of amounts of urea equivalent to those introduced by the sample of the urea-incubated pro-enzyme was completely activated. The effect of urea on the activation process could perhaps be related to any of at least three events: (a) an alteration of the conformation of the polypeptide-chain region recognized by the proteinase, so that proteolytic attack is prevented; (b) any change in the tertiary structure of the pro-enzyme that can affect the active site (or the
domains of the tertiary structure involved in the production of an active site); (c) interference with a process of association that could be required to obtain an oligomeric active form of the enzyme, after the proteolytic cleavage. The first possibility can be ruled out, since the pro-enzyme inactivated by urea and incubated with trypsin, under conditions similar to those of the activation, gave a product that had an apparent molecular weight of about 62000 in SDS/polyacrylamide-gel electrophoresis. Incubation of urea-treated pro-enzyme with trypsin for longer periods of time produced a gradual degradation of the pro-enzyme chain.

The fact that the native enzyme, with or without treatment with urea, was resistant to the proteolytic action of trypsin, a-chymotrypsin and Pronase in a time scale in which the pro-enzyme incubated with urea was easily degraded suggests that the more compact structure of tyrosinase (tetrameric structure) protects more peptide bonds from the attack of proteinases. The pro-enzyme, probably dissociated into monomers by urea, is much more exposed to this attack.

Other evidence in favour of the aggregated state of tyrosinase is provided by SDS/polyacrylamide-gel electrophoresis with determination of activity after removal of SDS. Gels stained with Coomassie Blue showed a main band corresponding to the monomer. However, similar gels stained with L-dopa showed a pink band not at the monomeric peak but in a zone corresponding to a species of molecular weight higher than 100000. This could be the tetrameric form of tyrosinase, since this enzyme, like those obtained from other sources, seems to be only partially dissociated into subunits by SDS treatment (Jolley et al., 1969; Ashida, 1971).

**Discussion**

Our results show that pro-tyrosinase obtained from the epidermis from *Rana esculenta ridibunda* has a dimeric structure composed of two identical subunits of $M_r$ approx. 68000 and $pI$ 7.2. On the other hand, the active enzyme seems to be composed of four identical subunits of $M_r$ approx. 62000 and $pI$ 7.2. The activation process is related to the cleavage of the polypeptide chain with the release of a peptide of low molecular weight. Studies of second derivative fluorescence, performed in our labora-

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**Fig. 5. Determination of the molecular weights of pro-tyrosinase and tyrosinase by gel filtration**

Samples (0.75 ml) of pro-tyrosinase (P) (2 mg/ml) and of tyrosinase (T) (2 mg/ml) were applied on to a 2.7 cm (internal diam.) x 60 cm column of Sepharose 6B, equilibrated with 0.1M-sodium phosphate buffer, pH 7. Fractions of volume 3 ml were collected and enzyme activity was determined. The flow rate was approx. 24 ml/h. The column was calibrated with the proteins indicated in the Experimental section. Blue Dextran was used to determine the elution volumes of compounds that are completely excluded ($V_0 = 115$ ml; $V_t = 334$ ml).

**Fig. 6. Effects of urea on pro-tyrosinase and tyrosinase**

Pro-tyrosinase and tyrosinase (1 mg/ml) were incubated with different amounts of urea. Samples (100μl) were removed after various periods of time and added to 2 ml of 7.6 mM-L-dopa and 0.5 ml of 0.1M-sodium phosphate buffer, pH 7. The samples of pro-enzyme incubated with urea were activated in 400μl of 0.1M-sodium phosphate buffer, pH 7, containing 10μg of trypsin, before the addition of 2 ml of 7.6 mM-L-dopa. Δ, Tyrosinase; ○, tyrosinase incubated with 8M-urea; ▲, pro-tyrosinase; ▼, pro-tyrosinase incubated with 4M-urea; ●, pro-tyrosinase incubated with 8M-urea.
Process for the activation of pro-tyrosinase

I. Dimeric pro-tyrosinase; II. dimeric tyrosinase; III. tetrameric tyrosinase. Dark zones represent hydrophobic domains. □ represents peptide.

The catalytic form of the enzyme: (a) the behaviour of tyrosinase as a hysteretic enzyme; (b) the claim that a dimerization process depending on enzyme concentration, and influenced by tyrosine, is an obligatory step for hydroxylating activity to appear.

The structure of the tetrameric enzyme seems to be very stable, since it is highly resistant to inactivation by urea or formamide. In contrast, the pro-enzyme is clearly affected by these agents. The fact that urea does not prevent the proteolytic step, but affects the activation of pro-tyrosinase, tempts us to propose that urea could affect the association process of the enzyme.

Scheme 1 shows a tentative scheme for the process for the activation of pro-tyrosinase, based on the experimental results.

The fact that the proteolytic activation of soluble pro-tyrosinase in the epidermis of frogs is not demonstrable in other amphibians, nor in the avian and mammalian systems studied, suggested to Eppig & Hearing (1979) that post-transcriptional control mechanisms for melanogenesis vary widely between different pigment systems. However, as pointed out by Barisas & McGuire (1974), it is possible that tyrosinase is synthesized as zymogen in a variety of higher species, but that disruption of the tyrosinase-bearing tissue releases proteinases that activate the pro-enzyme and prevents its isolation. This last possibility would be consistent with both the studies of melanosome formation (Bagnara et al., 1979) and the post-translational modifications produced on secretory proteins. The translation of the mRNA for tyrosinase in a heterologous cell-free system could show the existence of a pre-tyrosinase, a prezymogen form found in many secretory proteins, that might be of significance in the study of chromatophore organellogenesis.

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References
