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 68 000 and 62 000 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 M_r approx. 115 000 and the enzyme a tetramer of M_r approx. 210 000. 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

Abbreviations used: dopa, 3,4-dihydroxyphenylalanine; SDS, sodium dodecyl sulphate.

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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 20ml of double-distilled water. After centrifugation at 15000g (r_{av} , 7.9cm) for 20min, the supernatant was discarded and the sediment was extracted with 20ml of 0.1M-sodium phosphate buffer, pH 7, and centrifuged at 18000g (r_{av} , 7.9cm) for 30min. A saturated solution of $(\text{NH}_4)_2\text{SO}_4$ (neutralized at pH 7 with NaOH) was added until 40% saturation was reached, and the solution was kept overnight. After centrifugation at 15000g (r_{av} , 7.9cm) for 30min, sediment was removed and a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was added again until 55% saturation was reached, and the solution was kept overnight. The solution was centrifuged at 15000g and the precipitate was redissolved with 5ml of 0.1M-sodium phosphate buffer, pH 7.

CM-Sephadex C-50 was swollen in 50mM-sodium phosphate buffer, pH 7, and poured on to a glass filter. A 5ml 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 2ml 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 (1.2cm × 20cm) 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 2ml 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 (300W lamp, 30cm distance) at a constant temperature of 25°C.

Enzyme activity

Enzyme activity was measured spectrophotometrically at 475nm. One enzyme unit was defined as the amount of enzyme transforming 1μmol of L-dopa/min at 25°C, with 7.6mM-L-dopa in 0.1M-sodium phosphate buffer, pH 7. ϵ_{475} for the conversion of L-dopa into dopachrome was taken as 3600M⁻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 2ml of 7.6mM-L-dopa in 0.1M-sodium phosphate buffer,

pH 7, 5μl of pro-tyrosinase (1.4mg/ml) and 0.5ml of trypsin (0.4mg/ml) in 0.1M-sodium phosphate buffer, pH 7, were added. Dopachrome formation was followed at 475nm.

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 7.6mM-L-dopa.

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

High-voltage electrophoresis

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

Isoelectrofocusing

The isoelectrofocusing gels (0.25cm × 12cm) 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 10min. The isoelectrofocusing dimension was run at 260V for 14h. The second dimension was in SDS/15%-polyacrylamide gels (40mA, 6h). Pro-tyrosinase and tyrosinase (20μg portion of each) were applied on to the isoelectrofocusing gel.

Gel filtration

Gel filtration was performed on a Sepharose 6B column (0.27cm × 60cm) equilibrated with 0.1M-sodium phosphate buffer, pH 7. The volume of the fractions applied to the column was 0.75ml. The molecular-weight standards used were chymotrypsinogen (M_r , 25000), ovalbumin (M_r , 45000),

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 barbarta* (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:

by coupling this reaction to the oxidation:

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. 68000 and 62000 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 1. *Purification of frog epidermis pro-tyrosinase*
For experimental details see the text.

Purification step	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	3.3	(100)	(1.0)
55%-satn. (NH ₄) ₂ SO ₄	13.8	68	4.2
CM-Sephadex	48.1	57	14.6
Phenyl-Sepharose	62.4	48.5	19

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 proteinases 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.

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

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

To 2ml of 7.6mM-L-dopa, 5μl of pro-tyrosinase (1.4mg/ml) and 0.5ml of trypsin (0.4mg/ml) in 0.1M-sodium phosphate buffer, pH 7, were added. Dopachrome formation was followed at 475nm. 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 $\tau = 1/k$.

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

Proteinase	Lag (τ) (s)	<i>k</i> (s ⁻¹)	Slope (ΔA_{475} /min)
Pronase	6	0.166	0.22
α -Chymotrypsin	6.2	0.161	0.22
Trypsin	6.2	0.161	0.22

(proteinase/pro-tyrosinase ratio 50:1)

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

Incubation time (min)	Trypsin/pro-tyrosinase ratio (w/w)	Lag (τ) (s)	<i>k</i> (s ⁻¹)	Slope (ΔA_{475} /min)
0	50:1	6.2	0.161	0.22
0	100:1	6.2	0.161	0.22
1	50:1	6.1	0.164	0.17
1	100:1	6.1	0.164	0.17

Fig. 3. Activation of pro-tyrosinase by light. Pro-tyrosinase (1.5 mg/ml) was incubated at 25°C. Samples (15 μ l) were removed after various periods of time, and were added to 2 ml of 7.6 mM-L-dopa and 0.5 ml of 0.1 M-sodium phosphate buffer, pH 7. Δ , Pro-tyrosinase exposed to light; $\bullet$, pro-tyrosinase not exposed to light; $\circ$, pro-tyrosinase exposed to light and activated by trypsin. Portions (15 μ l) of the sample exposed to light were incubated for 5 min with 0.5 ml of trypsin (2 mg/ml) in 0.1 M-sodium phosphate buffer, pH 7, at 37°C, before addition of 2 ml of 7.6 mM-L-dopa.

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 M_r approx. 68 000 and 62 000. 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. 115 000 and 210 000 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

Fig. 4. Electrophoresis in 15%-SDS/polyacrylamide gels of pro-tyrosinase and tyrosinase.

Pro-tyrosinase was incubated with trypsin under the conditions described in the legend to Fig. 1. Duplicate samples were removed after various periods of time. Dopa oxidase activity was measured in one sample, and the other was quickly mixed with 1 vol. of 0.1 M-sodium phosphate buffer, pH 7, containing 1% (w/v) SDS and 2% (v/v) 2-mercapto-ethanol, and boiled for 2 min. Samples containing about 30 μ g of protein were applied to the gel. The gel was run at a constant current of 40 mA for 6 h. (a) Pro-tyrosinase; (b) pro-tyrosinase activated 12% by trypsin; (c) pro-tyrosinase activated 55% by trypsin; (d) pro-tyrosinase activated 100% by trypsin (tyrosinase); (e) tyrosinase activated by light. Pro-tyrosinase was exposed to light, as described in the legend to Fig. 3, and a sample was withdrawn after complete activation.

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 protease, 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

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.) $\times$ 60 cm column of Sepharose 6B, equilibrated with 0.1 M-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 volume 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 (100 μ l) were removed after various periods of time and added to 2 ml of 7.6 mM-L-dopa and 0.5 ml of 0.1 M-sodium phosphate buffer, pH 7. The samples of pro-enzyme incubated with urea were activated in 400 μ l of 0.1 M-sodium phosphate buffer, pH 7, containing 10 μ g of trypsin, before the addition of 2 ml of 7.6 mM-L-dopa. Δ , Tyrosinase; O , tyrosinase incubated with 8 M-urea; $\blacktriangle$, pro-tyrosinase; $\blacktriangledown$, pro-tyrosinase incubated with 4 M-urea; $\bullet$, pro-tyrosinase incubated with 8 M-urea.

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 62 000 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, α -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 100 000. 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. 68 000 and pI 7.2. On the other hand, the active enzyme seems to be composed of four identical subunits of M_r approx. 62 000 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-

Scheme 1. *Scheme of the activatory process of pro-tyrosinase*

I, Dimeric pro-tyrosinase; II, dimeric tyrosinase; III, tetrameric tyrosinase. Dark zones represent hydrophobic domains. $\neg$ represents peptide.

tories by García-Borrón (1981) by the technique described by Balestrieri *et al.* (1978), showed that the pro-tyrosinase monomer has more tryptophan residues than has the enzyme monomer; this seems to indicate, in agreement with our results, that the activation peptide does not remain bound to the active enzyme. Since proteinases with different specificities can activate pro-tyrosinase and the resulting monomers are of a similar size, pro-tyrosinase subunit must present a highly exposed loop containing the peptide bonds that can be recognized by different proteinases. Thus the activation of pro-tyrosinase *in vivo* by limited proteolysis has no requirement for a highly specific proteinase.

The changes in oligomeric structures between pro-tyrosinase and tyrosinase could be mediated by modifications of the tertiary structure produced as a consequence of the proteolytic reaction. It seems likely that the proteolytic step could leave exposed to the solvent a region rich in residues with non-polar side chains. If this is so, the dimers would tend to aggregate, since the hydrophobic free energy gained from the decrease in solvent accessibility at the contact interface between the dimers is sufficient to compensate for the entropy loss due to the dimers' association itself (Chothia & Janin, 1975).

Some properties of tyrosinase from *Rana pipiens* have been described by Barisas & McGuire (1974) and Mikkelsen & Triplett (1975), although there exist some differences in subunit molecular weight and other discrepancies. Our results on tyrosinase from *Rana esculenta ridibunda* show that this enzyme is activated by light and proteinases, as is the enzyme from *Rana pipiens* reported by Mikkelsen & Triplett (1975). However, the subunit size, oligomeric structure and enzyme stability are closer to those described by Barisas & McGuire (1974).

It is unknown whether the proteolytically modified dimer possesses activity or the tetrameric structure is the only active form. However, some results (García Carmona *et al.*, 1980) suggest that the tetramer is

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 pre-zymogen form found in many secretory proteins, that might be of significance in the study of chromatophore organogenesis.

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