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FORMATION OF MELANIN-TYROSINASE COMPLEX AND ITS POSSIBLE SIGNIFICANCE AS A MODEL FOR CONTROL OF MELANIN SYNTHESIS

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Abstract. Previous reports have shown that the tyrosinase activities of melanosomes and soluble tyrosinase isolated from melanoma were diminished when these preparations were incubated with 3,4-dihydroxyphenylalanine (dopa). It was concluded from these results that the tyrosinase activities of these preparations decreased when melanin was synthesized in these systems. The present investigation has revealed that melanin synthesized in vitro from dopa formed a complex with purified mushroom tyrosinase. The addition of melanin diminished the tyrosinase activity of the sample. These results show that the formation of the melanin–tyrosinase complex results in a decrease in the activity of the tyrosinase solution. The tyrosinase activity of the melanin–tyrosinase complex could not be increased by certain procedures which were previously found to enhance the tyrosinase activity of isolated melanosomes.

KEY words: Melanoma; Melanosome; Dopa

It has been reported that the tyrosinase activities of isolated melanosomes and soluble tyrosinase are diminished when these preparations are incubated with dopa (6, 7). Under these conditions dopa was incorporated into insoluble melanin. When ^{131}I-labelled albumin was added to the system containing soluble tyrosinase and dopa, most of the radioactivity was found to be associated with the insoluble melanin. It was assumed from these studies that as melanin formation proceeds, tyrosinase molecules combine with melanin and intermediates such as dopaquinone or indole-5, 6-quinone and the active centres of tyrosinase are blocked during this process.

The present studies were undertaken in order to find out whether tyrosinase forms a complex with melanin and, if so, whether the tyrosinase activity of this complex can be increased by the procedures known to activate tyrosinase in melanosomes (2–5, 9–11). Our preliminary studies showed that when tyrosinase was incubated with L-dopa the enzyme disappeared from the supernatant. We felt that the physico-chemical processes taking place during the formation of insoluble melanin are rather complex and it is possible that the tyrosinase molecules may be physically trapped within the melanin particles. Hence the disappearance of tyrosinase in the above experiment and the sedimentation of labelled albumin along with melanin as reported by Seiji et al. (6, 7) may not necessarily be due to the complex formation with melanin. Consequently, we investigated whether tyrosinase would form a complex with melanin already synthesized. The results of these studies are reported in this communication.

MATERIALS AND METHODS

Purified mushroom tyrosinase (polyphenoloxidase) (specific activity 900–1 500 units/mg) was obtained from Sigma Chemical Company.

Melanin was synthesized by the action of purified mushroom tyrosinase on dopa, as described previously (1, 8).

Tyrosinase activity was determined as previously described (5).

The various treatments of the melanin–tyrosinase complex were carried out under the conditions employed for previous studies with melanosomes (5).

RESULTS

Effects of adding varying amounts of melanin to mushroom tyrosinase upon the tyrosinase activity. Mixtures containing varying amounts of melanin

Table I. *Effects upon the tyrosinase activity of adding various amounts of melantin to mushroom tyrosinase*

Varying amounts of melanin were added to 4.0 mg mushroom tyrosinase in a total volume of 1.0 ml 0.01 M PO_4 buffer, pH 6.8. The mixture was incubated at 37° for 2 hours. One set of the suspension was employed for the determination of tyrosinase activity. Another set was centrifuged at 100 000 *g* for 30 min. The sediment was suspended in 1.0 ml 0.01 M PO_4 buffer, pH 7.2. For tyrosinase assay the suspension and supernatant were diluted in such a manner that 10 μg tyrosinase was added to each reaction tube. 0.25 ml sediment was employed for the tyrosinase assay in each case

	nmoles of tyrosine oxidized		
Melanin (mg)	Suspension	Sediment	Supernatant
Nil	5 640		5 640
0.5	3 880	65	4 600
1.0	3 960	126	4 560
3.0	3 440	394	2 840
4.0	2 520	458	2 880

and a constant amount of tyrosinase were incubated at 37° for 2 hours and the tyrosinase activity was determined. The results are shown in Table I. Addition of melanin was found to reduce the tyrosinase activity. The suspension was centrifuged and the tyrosinase activities of the sediment and supernatant were determined separately. The sediment had considerable tyrosinase activity, which increased with increasing amounts of melanin added to the system. The tyrosinase activity of the supernatant fractions followed a pattern very similar to that observed with the whole suspension.

Table II. *Effects upon the tyrosinase activity of adding a constant amount of melanin to various amounts of mushroom tyrosinase*

Varying amounts of tyrosinase were added to 0.5 mg melanin in a total volume of 1.0 ml 0.01 M PO_4 buffer, pH 7.2 and incubated at 37° for 2 hours. Other conditions were as described under Table I

Tyrosinase (mg)	Suspension	Sediment	Supernatant
0.4	140	50	212
0.8	744	54	624
2.0	2 240	62	1 520
4.0	4 760	52	3 600
6.0	5 820	60	4 560
8.0	8 560	57	7 760

Table III. *Effects of various treatments on tyrosinase activity of melanin–tyrosinase complex*

30 mg melanin and 15 mg mushroom tyrosinase were mixed in a total volume of 6.0 ml 0.01 M PO_4 buffer, pH 6.8 and incubated at 37° for 2 hours. The suspension was centrifuged at 100 000 g for 30 min. The sediment was washed twice by suspending in 12 ml 0.01 M PO_4 buffer, pH 7.2 and centrifuged at 100 000 *g* for 30 min each time. The sediment was finally suspended in 12.0 ml PO_4 buffer. 1.0 ml portions of the suspension were subjected to the various treatments mentioned in the Table and 0.25 ml was employed for the tyrosinase assay

	nmoles of tyrosine oxidized		
Treatment	Suspension	Sediment	Supernatant
None	4.23	3.82	0.05
DOC	4.07	3.80	Nil
Triton X-100	4.17	4.05	0.06
Sonication	4.10	3.90	0.05

Effects of adding constant amounts of melanin to varying amounts of tyrosinase upon the tyrosinase activity. The effects of adding a constant amount of melanin to varying amounts of tyrosinase are shown in Table II. The tyrosinase activity was found to increase as the proportion of tyrosinase to melanin was increased. The tyrosinase activity of the sediment was roughly constant and independent of the amount of tyrosinase added. The tyrosinase activity of the supernatant was greater when larger amounts of tyrosinase were added.

Effects of various treatments of melanin–tyrosinase complex upon the tyrosinase activity. The above results indicate that tyrosinase forms a complex with melanin and that this product has tyrosinase activity. The possibility that the tyrosinase activity of this melanin–tyrosinase complex could be increased by procedures which have been reported to enhance the tyrosinase activity of melanosomes from B16 melanoma was investigated. A mixture of melanin and tyrosinase was incubated and the suspension centrifuged at 100 000 *g*. The sediment was washed and suspended in PO_4 buffer. This suspension was subjected to various procedures which have been reported to enhance the tyrosinase activity of melanosomes from B16 melanoma (2–5). The tyrosinase activity of the suspension after the various treatments was determined. The results are shown in Table III.

The tyrosinase activity of the melanin–tyrosinase complex was not increased by treatment with

sodium deoxycholate, Triton X-100, or by sonication (Table III). It was also observed that these treatments did not solubilize any significant amount of tyrosinase from the insoluble complex.

In another set of experiments, tyrosinase was incubated with dopa for approximately 18 hours. The melanin formed was centrifuged and washed as described previously (1, 8). This sample of melanin was subjected to the treatments mentioned above. No tyrosinase activity could be detected, either in the untreated or in the treated melanin.

DISCUSSION

The above results show that incubation of purified mushroom tyrosinase with melanin results in a decrease in the tyrosinase activity of the suspension. The melanin separated from this mixture contains tyrosinase activity. These findings show that tyrosinase forms a complex with melanin and that this complex has tyrosinase activity. It seems probable that the decrease in tyrosinase activity of the mixture of melanin and tyrosinase is due to the formation of this complex.

Experiments in which varying amounts of melanin and tyrosinase were mixed indicate that a constant amount of tyrosinase is bound to melanin, irrespective of the ratio of tyrosinase to melanin in the medium. It appears that even at very low concentrations of tyrosinase the melanin is saturated with tyrosinase and increasing the concentration of tyrosinase does not bring about any further increase in the amount of tyrosinase bound to melanin.

It seems possible that the decrease in the tyrosinase activity after the formation of melanin as reported by Seiji and associates (6, 7) may be due—at least partially—to the formation of such a complex. However, since the tyrosinase activity of this melanin–tyrosinase complex could not be increased by some of the procedures which increased the tyrosinase activity of melanosomes (2–5, 9–11), it would appear that this activation phenomenon would require a more complex structure such as the presence of a membrane surrounding these granules.

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