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A Revised Concept of Mammalian Melanogenesis: The Possible Synergistic Functions of Aerobic Dopa Oxidase and Peroxidase. A Review

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THE PREVAILING HYPOTHESIS THAT MAMMALIAN MELANOGENESIS IS BASED ON THE ACTION OF A SINGLE AEROBIC OXIDASE, TYROSINASE

The enzymatic oxidation of tyrosine to melanin in mammalian cells is widely believed to be mediated solely by an aerobic, copper-dependent enzyme called tyrosinase (1). This enzyme is considered to be analogous to the tyrosinase of plants and insects, which has been shown to mediate the first two steps of the tyrosine to melanin sequence (2–4): hydroxylation of tyrosine to dopa and oxidation of dopa to dopa quinone.

This hypothesis superseded that of Bloch (5) which held that the key enzyme in melanogenesis was a specific dopa oxidase which was incapable of oxidizing tyrosine. Bloch's hypothesis prevailed until several decades ago when Lerner *et al.* (6) published their initial studies, which used manometric data obtained from crude extracts of mouse melanoma to provide evidence for the existence of a mammalian tyrosinase. These authors expressed the opinion that failure of Hogeboom and Adams (7) and Greenstein and Algire (8) to demonstrate activity toward tyrosine in their "dopa oxidase" melanoma fractions was because of their unawareness of the role of dopa as a required cofactor.

Other authors (9, 10), using similar methods, reported results corresponding to those of Lerner *et al.* (6). More recent studies with incompletely purified enzyme preparations from mammalian melanomas supporting the "tyrosinase" hypothesis include those of Pomerantz (11–13) and Miyazaki and Seiji (14).

Burnett (15) has described a highly purified "tyrosinase" preparation from mouse melanoma, but omitted data pertaining to the ability of this preparation to utilize tyrosine.

Fitzpatrick *et al.* described the histochemical demonstration of "tyrosinase" in normal and neoplastic melanocytes using unlabeled (16) and labeled (17) substrates. Both studies involved prolonged incubation of tissue in tyrosine without

added cofactor; in addition, a sulfhydryl copper-chelator, sodium diethyldithiocarbamate (DDC), was used in the incubation medium as a "specific" tyrosinase inhibitor.

EVIDENCE THAT PEROXIDASE CAN OXIDIZE TYROSINE, AS WELL AS DOPA, TO MELANIN

Although the ability of peroxidases to catalyze the oxidation of dopa to melanin is widely recognized (18, 19), the assumption that peroxidase cannot mediate the oxidation of tyrosine to melanin has been one reason the role of peroxidase in mammalian melanogenesis has been neglected.

Agner (20) and Polis and Shmukler (19) were unable to demonstrate oxididation of tyrosine by purified mammalian peroxidases. On the other hand Elliott (21) demonstrated oxidation of tyrosine to melanin by crude preparations of lactoperoxidase and Knox (22) demonstrated oxidation of tyrosine by plant and mammalian peroxidases.

A study by Mason *et al.* (23) suggested that peroxidase can mediate the first step in the tyrosine to melanin sequence: hydroxylation of tyrosine to dopa. In this study dihydroxyfumaric acid was used as cofactor.

HISTOCHEMICAL STUDIES IN OUR LABORATORY

Studies in our laboratory have shown that cells with peroxidase activity can oxidize tyrosine, as well as dopa, to melanin.

1. Mast cells, neutrophils, and eosinophils oxidized tyrosine to melanin in the presence of dopa or dihydroxyfumaric acid cofactor, in histochemical and autoradiographic–histochemical experiments EM-cytochemical experiments indicated that the subcellular distribution of this melanin corresponded to the known subcellular distribution of peroxidase (24–29).

2. Peroxidatic oxidation of dopa to melanin was also demonstrated in these cell types.

3. Peroxidase-dependent oxidation of tyrosine to melanin was determined by (a) correlation of the distribution of reaction product with that of standard histochemical techniques for peroxidase; (b) suppression of this melanin formation by catalase; (c) its resistance to *preincubation* with DDC. The use of DDC *in the incubation medium* was of no value in differentiating melanogenic activity of peroxidase from that of aerobic dopa oxidase ("tyrosinase"). Our results paralleled those of Randall (30) who demonstrated that thiols in the incubation medium suppress peroxidase activity. DDC and other thiols used in this way have nonselective inhibitory effects including substrate competition (30), antioxidant effect (30), and the effect of complexing with melanin intermediates (31–33).

4. Peroxidase activity and peroxidatic melanogenesis in mast cells (34) showed latency (requirement for structural damage to effect adequate substrate–enzyme interaction). For this reason only 1–3% of mast cells in cryostat sections showed positive peroxidase-dependent reactions in preparations of whole mast cells subjected to physical damage the proportion of positive cells approached 100%.

BIOCHEMICAL STUDIES IN OUR LABORATORY

Purified plant and human peroxidases (35, 36) mediated the oxidation of tyrosine to dopachrome and insoluble melanin when dopa or dihydroxyfumarate was used as cofactor. Experiments using the radioassay method of Pomerantz (12, 13)

suggested that peroxidatic hydroxylation of tyrosine (37) is the first step in this sequence. Direct assay of peroxidatic hydroxylation of tyrosine to dopa is difficult, since the use of ascorbate to prevent further oxidation of dopa initiates a nonenzymatic, peroxide-dependent hydroxylating system (38, 39).

Oxidation of tyrosine to dopachrome by peroxidase was assayed by spectrophotometry and thin-layer chromatography with labeled substrate.

EVIDENCE THAT PEROXIDASE IS PRESENT AT SITES OF MELANOGENESIS

Histochemical studies in our laboratory. Peroxidase activity was demonstrated in normal and neoplastic melanocytes, using the benzidine method (24, 26, 27) and this was correlated with peroxidase-dependent oxidation of tyrosine or dopa to melanin (24–29). Peroxidase activity in melanocytes showed latency: positive normal melanocytes were more frequently observed in split epidermis than in cryostat sections positive melanoma cells were most frequently observed near edges or fissures in sections. Peroxidase activity in melanocytes was localized to melanosomes (24, 27).

Biochemical studies in our laboratory. Crude supernatant from mouse melanomas had intense peroxidase activity; acrylamide gel electrophoresis revealed two peroxidase bands.

100,000 *g* supernatant showed enzymatic oxidation of tyrosine to dopachrome, which was greatly increased by the addition of exogenous hydrogen peroxide to supplement the endogenous hydrogen peroxide present (40).

EVIDENCE THAT MAMMALIAN "TYROSINASE" IS A DOPA OXIDASE

Oxidation of tyrosine to melanin is easily demonstrated with preparations of plant tyrosinase. Our experiments have indicated, however, that the analogous mammalian enzyme is a dopa oxidase with no ability to oxidize tyrosine.

Histochemical studies in our laboratory. Histochemical (25, 27) and autoradiographic–histochemical (24–27) experiments demonstrated aerobic oxidation of dopa, but not tyrosine, in melanocytes. Tyrosine could not be oxidized aerobically even in the presence of dopa cofactor. Oxidation of tyrosine to melanin was always peroxide-dependent.

Biochemical studies in our laboratory (41). A partially purified "tyrosinase" preparation was obtained from Harding–Passey melanoma using the methods of Brown and Ward (9) and Burnett *et al.* (42).

Using spectrophotometric assay for dopachrome at 475 nm, this preparation readily oxidized dopa, but not tyrosine. When dopa was used as cofactor, the presence of tyrosine produced actual inhibition of dopachrome formation from dopa. Lack of tyrosine utilization by "tyrosinase" was confirmed by absence of tyrosine consumption as determined by the method of Raper (2) and Evans and Raper (3, 4).

Inability of this "tyrosinase" preparation to oxidize tyrosine was confirmed by radioassay of newly formed dopa, using alumina gel chromatography and ascorbate blockade of further dopa oxidation.

Critique of previous studies. The principal problem in histochemical and biochemical studies supporting the existence of mammalian "tyrosinase" was lack of a control for peroxidase.

An additional problem was the erroneous (see above) belief that thiol compounds in the incubation mixture acted as specific inhibitors of "tyrosinase."

EVIDENCE THAT PEROXIDASE AND AEROBIC DOPA OXIDASE ("TYROSINASE") MAY ACT SYNERGISTICALLY IN MAMMALIAN MELANOGENESIS (43, 44)

Oxidation of tyrosine to melanin in melanocytes was completely suppressed by catalase, and was therefore completely peroxide-dependent. However, in melanoma cells containing both aerobic dopa oxidase (in the GERL area and melanosomes) and peroxidase (in the melanosomes) preincubation with thiols resulted in a selective loss of reaction product in the GERL area, suggesting that aerobic dopa oxidase utilizes dopa synthesized by peroxidase.

There are many potential sources of hydrogen peroxide including peroxide generated by various oxidases and peroxide generated by autooxidation of various compounds, including intermediates in melanogenesis (2). Knox (22) has shown that peroxidase can utilize enzyme-generated peroxide to produce significant oxidation of tyrosine.

SUMMARY

Our studies indicate that mammalian melanogenesis may be based on the synergistic action of an aerobic dopa oxidase and a peroxidase. This evidence may be summarized as follows:

a. Heme-protein peroxidases can mediate the oxidation of tyrosine to melanin and the first step in this synthesis is the hydroxylation of tyrosine to dopa.

b. Peroxidase activity is present in normal and neoplastic melanocytes and is localized in melanosomes.

c. Mammalian "tyrosinase" appears to be a dopa oxidase with no ability to oxidize tyrosine, even in the presence of dopa cofactor. "Tyrosinase" activity described in previous studies may have been due to the presence of peroxidase.

d. Dopa oxidase in melanocytes appears to utilize dopa synthesized by peroxidase.

e. Oxidation of tyrosine to melanin by peroxidase is peroxide-dependent. There are many sources of hydrogen peroxide: (1) peroxide generated by various oxiddases (2) peroxide generated by autooxidation of intermediates in melanogenesis.

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