Short Communications

Biochemical Studies of the Peroxidase-Mediated Oxidation of Tyrosine to Melanin: Demonstration of the Hydroxylation of Tyrosine by Plant and Human Peroxidases

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The ability of peroxidases to mediate the oxidation of dopa (3,4-dihydroxyphenylalanine) and other polyphenols to melanin is known (Maehly & Chance, 1954; Polis & Schmukler, 1953). However, it is not generally believed that peroxidases can mediate the oxidation of tyrosine to melanin. For this reason mammalian peroxidases have not been considered to have roles in the major pathways involving tyrosine hydroxylation, namely melanin synthesis and catecholamine synthesis.

Previous histochemical studies in our laboratory were the first to demonstrate the peroxidase-dependent oxidation of tyrosine (in the presence of dopa as cofactor) to melanin in mast cells, eosinophils, melanocytes and neurons (Okun, Edelstein, Or, Hamada & Donnellan, 1970*a,b*; Okun *et al.* 1970*c*; Okun, Edelstein, Or, Donnellan & Lever, 1971*a*; Okun *et al.* 1971*b*); electrophoretic studies in our laboratory (Okun *et al.* 1971*b*) suggested that peroxidase, acting alone, could mediate the synthesis of melanin from tyrosine, since enzymic conversion of tyrosine into melanin was observed in peroxidase bands derived from the tissues studied.

Mason, Onoprienko & Buhler (1957) described the conversion of tyrosine into dopa by horseradish peroxidase, with dihydroxyfumarate as factor, but did not indicate whether further steps in the tyrosine-to-melanin sequence ensued. Elliott (1932) described the peroxidatic conversion of tyrosine into pigment by crude preparations of lactoperoxidase, but did not identify the pigment or the pathway of its formation.

No reports were found in the literature on the quantitative determination of peroxidase-mediated dopa formation. In addition, no reports were found in the literature indicating the ability of purified preparations of mammalian peroxidase to mediate the formation of dopa or melanin from tyrosine. Agner (1941) indicated that myeloperoxidase could

not oxidize tyrosine; however, he did not use dopa or other cofactor.

Because of our histochemical and electrophoretic studies, biochemical investigations of the ability of purified preparations of plant and mammalian peroxidases to oxidize tyrosine to melanin were carried out, with either dopa or dihydroxyfumarate as cofactor. As described below, these investigations confirmed the ability of these enzymes to mediate the synthesis of melanin from tyrosine, and they demonstrated that dopa and dopachrome are intermediates in this pathway.

Human myeloperoxidase was given by Dr Julius Schultz, Director of the Papanicolaou Cancer Research Institute, Miami, Fla., U.S.A. Horseradish peroxidase (type II) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. L-Tyrosine, DL-dopa, sodium ascorbate and dihydroxyfumaric acid were obtained commercially.

The dopa formed from the hydroxylation of tyrosine by peroxidase was measured by the quantitative methods described by Raper (1926) and Evans & Raper (1937*a,b*) to demonstrate dopa formed by the enzymic hydroxylation of tyrosine by plant tyrosinase. In this method the lead salt of dopa that is precipitated at alkaline pH is removed by centrifugation and dopa is recovered by the action of hydrogen sulphide on this compound.

Our experiments indicated that the peroxidase-mediated synthesis of melanin from tyrosine is much more rapid than that mediated by mushroom tyrosinase, with resultant greater difficulty in identifying dopa as an intermediate. The following measures were taken to increase the yield of dopa in the peroxidase-mediated reaction: (a) lowering of the concentration of enzyme; (b) lowering of the concentration of cofactor; (c) lowering of the concentration of hydrogen peroxide; (d) shortening of the reaction time; (e) use of a reducing agent (ascorbate).

Fig. 1. Spectral curve of dopa derived from the action of myeloperoxidase on tyrosine in the presence of dihydroxyfumarate. The absorption peak at 281 nm represents newly formed dopa.

In a typical experiment the reaction mixture consisted of 4.5 mg (25 μ mol) of L-tyrosine dissolved in 9.5 ml of 60 mM-sodium acetate-acetic acid buffer, pH 5.6, 0.25 mg (1.25 μ mol) of DL-dopa dissolved in 0.5 ml of the above buffer, 11.3 μ l (10 μ mol) of 3.0% hydrogen peroxide, 5.5 mg (25.4 μ mol) of sodium ascorbate and 0.2 mg of myeloperoxidase. The quantity of enzyme was increased to 2.0 mg in the case of horseradish peroxidase. In some experiments 9.7 mg (66 μ mol) of dihydroxyfumaric acid dissolved in 9.5 ml of 60 mM-acetate buffer, pH 5.6, was used as a cofactor instead of dopa. The reaction time was 1 h at 25°C.

In experiments with dopa as cofactor a control experiment was carried out under the conditions described above but with tyrosine omitted. Other controls included: (a) omission of enzyme; (b) use of boiled enzyme; (c) omission of cofactor; (d) omission of hydrogen peroxide.

The dopa formed by the catalysis of peroxidase was measured spectrophotometrically (Mason, 1948; Rowbottom, 1954). With dopa as cofactor newly synthesized dopa was indicated by the difference in absorption peaks at 281 nm between the curve of the test experiment and the curve of the control experiment. With dihydroxyfumarate

as cofactor the absorption peak at 281 nm (Fig. 1) was a measure of newly formed dopa. With dopa as cofactor the net gain in the absorption for dopa in the reactions mediated by myeloperoxidase and horseradish peroxidase was 0.21 and 0.20 E_{281} unit respectively. The absorption peak in the reaction mediated by myeloperoxidase, with dihydroxyfumarate as cofactor, was 0.44 E_{281} unit. The average yield of dopa on the basis of tyrosine was 3.0%. The low yield was due to the rapidity of the conversion of dopa into further reaction products rather than to a low efficiency of the hydroxylation of tyrosine. Spectrophotometric analysis for tyrosine at the end of the reaction indicated a consumption of 29.0%.

In the absence of reducing agent (ascorbate), dopachrome (peak absorption at 475 nm) was rapidly formed, as a result of the conversion of dopa into dopaquinone followed by cyclization of the dopaquinone. Ultimately the sequence led to the formation of insoluble melanin. The presence of ascorbate causes dopaquinone to shift back to dopa, but does not interfere with the hydroxylation of tyrosine (Evans & Raper, 1937b).

Qualitative determination of dopa formed in the reaction was carried out by performing the specific colour reaction described by Arnow (1937). In experiments with dihydroxyfumarate as cofactor a copper-red colour confirmed the presence of newly formed dopa. In experiments with dopa as cofactor the difference in intensity of this colour between the control situation and the test solution was visually evident, confirming the presence of newly formed dopa in the test solution. No reaction product was formed without enzyme or with boiled enzyme. Omission of hydrogen peroxide or cofactor resulted in virtually no formation of reaction product.

In the experiments with dopa as cofactor the possibility that the greater amount of dopa present in the test experiments was due solely to the retardation of the oxidation of dopa cofactor by competition of tyrosine for the active site on the enzyme was excluded by: (a) the increased rate of dopachrome formation in the test experiments; (b) the consumption of tyrosine. There was no evidence of oxidative polymerization of tyrosine. Confirmation of the ability of peroxidases to hydroxylate tyrosine was provided by the experiments with dihydroxyfumarate as cofactor.

Dihydroxyfumarate was also used as cofactor in histochemical experiments demonstrating peroxidase-dependent oxidation of tyrosine to melanin in granulocytes, mast cells, melanoma cells and neurons (Okun *et al.* 1971c).

Experiments in our laboratory (R. Patel, M. Okun, L. Edelstein & D. Epstein, unpublished work) have shown that the peroxidase-mediated oxidation of

tyrosine to melanin occurs very rapidly at neutral or slightly alkaline pH.

Biochemical confirmation of the ability of mammalian peroxidase to mediate the oxidation of tyrosine to melanin via dopa formation supports the proposal of Okun *et al.* (1970*a,b,c*, 1971*a,b*) that peroxidase may have a role in initiating melanin and catecholamine synthesis *in vivo*, and in some instances can alone mediate the synthesis of melanin and neuromelanin from tyrosine *in vivo*. The importance of peroxidase relative to that of tyrosinase (Duchon, Fitzpatrick & Seiji, 1968) and tyrosine hydroxylase (Weiner, 1970) in the synthesis of melanin and catecholamines respectively remains to be fully investigated.

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