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5-S-CYSTEINYLDOPA AS A SUBSTRATE FOR TYROSINASE

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Abstract. Oxidation of 5-S-cysteinyldopa by mushroom tyrosinase was studied by measuring 5-S-cysteinyldopa consumption with HPLC. 5-S-Cysteinyldopa was found to be a substrate for tyrosinase, but our results suggests that a self-catalysed oxidation induced by enzymatically formed 5-S-cysteinyl dopaquinone also takes place. Dopa oxidation by tyrosinase was determined by measuring substrate consumption with HPLC. The oxidation of 5-S-cysteinyldopa was markedly accelerated in the presence of dopa. This could be explained by dopaquinone oxidation of 5-S-cysteinyldopa, which has a lower oxidation potential than dopa.

Key words: Dopa; 5-S-Cysteinyldopa; Tyrosinase; Melanin

It is well established that phaeomelanins, the pigment of red hair and feathers, are formed by a deviation of the eumelanin pathway involving the formation of cysteinyldopas by nucleophilic addition of cysteine to dopaquinone (6). When first described, cysteinyldopas were regarded merely as the building stones of phaeomelanin and were thought only to exist in melanocytes that formed such pigments. It is now known that cysteinyldopas are produced in all active melanocytes, and the oxidation products of cysteinyldopas apparently also co-polymerize with dopa oxidation products in many types of melanin. The metabolism of cysteinyldopa has therefore become of central interest in pigment biology (8).

Like tyrosine and dopa, the most important dopaquinone-cysteine adduct, 5-S-cysteinyldopa, is a substrate for tyrosinase (7). Whereas the kinetics of the oxidation of tyrosine and dopa by tyrosinases of various origins have been extensively studied (4), nothing is known about the enzymatic oxidation of 5-S-cysteinyldopa. We now describe the kinetics of oxidation of 5-S-cysteinyldopa by mushroom tyrosinase, and the acceleration of oxidation of 5-S-cysteinyldopa in the presence of dopa.

MATERIAL AND METHODS

The enzyme used was mushroom tyrosinase (4 000 U/mg, Sigma). L-Dopa was obtained from Merck. 5-S-Cysteinyldopa was prepared by a method previously described (1). 5-S-Cysteinyldopa oxidation with tyrosinase and 5-S-cysteinyldopa oxidation in the presence of dopa were performed for 3–5 min at 23°C with constant air bubbling, using 0.1 M phosphate buffer at pH 7.4. The incubations were interrupted by dilution with the mobile phase described below.

The analyses of 5-S-cysteinyldopa were performed with HPLC using a chromatographic system consisting of a Waters 6000-A high-pressure pump, a Reodyne model-7120 sample valve injector fitted with a 100 μl loop, a 200×4.6 mm stainless-steel column packed with Nucleosil C_{18} 5 μ, and a Vari-Chrom UV-detector working at 220 nm (2). All experiments were carried out by isocratic elution (flow rate 1.5 ml/min) using an aqueous solution containing 2.9 g phosphoric acid and 4 g methanesulphuric acid per litre. The pH was adjusted to 3.5 with 5 M NaOH.

Different amounts of L-dopa were incubated with different amounts of tyrosinase in 3 ml HEPES buffer at pH 7.4. The incubations were run at 23°C for 30 sec with constant air bubbling, and were then interrupted by addition of 7 ml absolute ethanol and 10 000× dilution of a sample with water. The diluted incubates were analysed on the HPLC system without previous purification, using an electrochemical detector with a graphite electrode working at 0.7 V vs. Ag/AgCl reference electrode.

Cyclic voltammetry was done with a CV-1A cyclic voltammetry instrument (Bioanalytical Systems Inc., N. La Fayette, In.) equipped with a carbon paste working electrode and an Ag/AgCl reference electrode. Solutions of dopa and 5-S-cysteinyldopa were prepared by dissolving 0.5 mg of the catecholic amino acid in 10 ml 0.1 M phosphate buffer at pH 7.4. All potentials are reported vs. Ag/AgCl reference electrode, and the experiments were run at 20°C.

RESULTS AND COMMENTS

The disappearance of 5-S-cysteinyldopa in the presence of tyrosinase was proportional to the enzyme concentration. However, the velocity of 5-S-cysteinyldopa disappearance did not accord with an assumption of the simplest enzymatic kinetics. The reaction rate increased more than expected with higher concentrations of the substrate. A Lineweaver-Burk plot therefore did not give a straight line (Fig. 1). The curve could indicate that the substrate activates tyrosinase by also combining

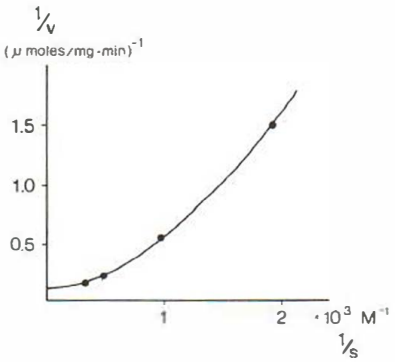

Fig. 1. Double-reciprocal plot of disappearance velocity and substrate concentration for 5-S-cysteinyldopa in the presence of tyrosinase.

with the enzyme molecule at sites other than at its active centre. Another explanation seems more probable, however. Prota et al. (7), who studied cysteinyldopa oxidation, assumed that 5-S-cysteinyldopaquinone undergoes an intramolecular cyclization reaction followed by elimination of water, producing as intermediate an *o*-quinoneimine derivative. This compound may be reduced to the corresponding dihydrobenzothiazine by another molecule of 5-S-cysteinyldopa which in turn is oxidized to the quinone. This type of self-catalysing reaction will inevitably constitute an obstacle to any exact definition of the enzyme kinetics. No true K_M or V_{max} can therefore be calculated from these experiments. In a typical experiment the maximum rate of 5-S-cysteinyldopa consumption was 10

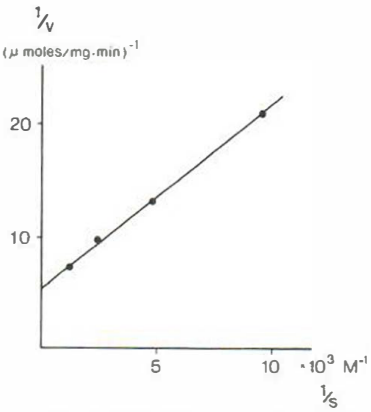

Fig. 2. Double-reciprocal plot of disappearance velocity and substrate concentration for dopa in the presence of tyrosinase.

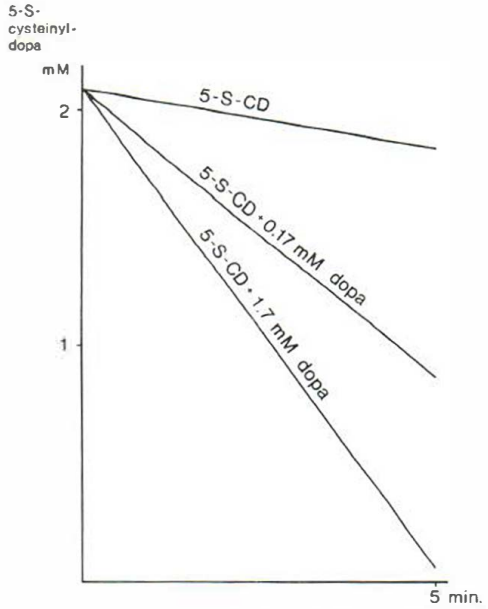

Fig. 3. Accelerated oxidation of 5-S-cysteinyldopa, incubated with tyrosinase, in the presence of dopa. 5-S-Cysteinyldopa 2.1 mM and tyrosinase 6.7 μg/ml in all experiments. Dopa decreased 8 and 10%.

μmol/mg and minute. The substrate concentration at half of this maximum rate was 2×10^{-3} M.

The double-reciprocal plot for the oxidation of dopa by tyrosinase gave K_M 3×10^{-4} and V_{max} 19 μmol/mg and minute (Fig. 2).

Our method for measuring dopa consumption has not previously been applied for the assay of tyrosinase activity. The values it gives should be considered in the light of present knowledge of the Raper-Mason scheme of melanogenesis (3). The oxidation of dopa to dopaquinone can clearly be underestimated owing to dopa regeneration via the reduction of dopaquinone, for instance by dopachrome. The technique for measurement of dopa in tyrosinase assays is a useful innovation in studies of this type, because none of the techniques previously used directly measures this early step in melanogenesis. Our method gives the same K_M as the method involving measurement of dopachrome formation, but with the dopachrome method V_{max} is only about half that obtained with ours. A similar difference in measuring tyrosinase activity was found when comparing dopachrome formation with an isotope method measuring the formation of tritium-labelled water from tritium-labelled dopa (5).

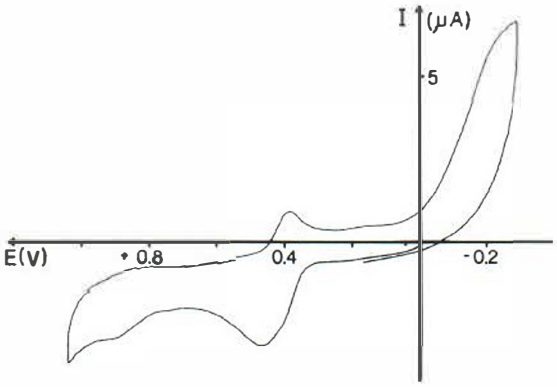

Fig. 4. Cyclic voltammetry of 5-S-cysteinyldopa; 0.5 mg in 10 ml 0.1 M phosphate buffer at pH 7.4. Scan rate 100 mV/s.

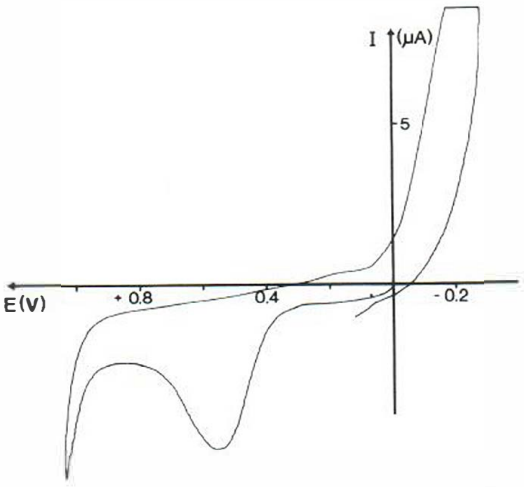

Fig. 5. Cyclic voltammetry of dopa; 0.5 mg in 10 ml 0.1 M phosphate buffer at pH 7.4. Scan rate 100 mV/s.

When dopa was added to the incubate of 5-S-cysteinyldopa and tyrosinase the rate of disappearance of 5-S-cysteinyldopa was strikingly increased (Fig. 3). It should also be noted that dopa consumption was small in these experiments.

We tried to explain the above-mentioned finding by studying the oxidation potentials of 5-S-cyteinyldopa and dopa. The cyclic voltammetry of dopa and 5-S-cysteinyldopa is shown in Figs. 4 and 5. On the first anodic sweep the catechols are oxidized to the corresponding *o*-quinones with peak potentials (E_{pa}) of 0.55 V for dopa and 0.47 V for 5-S-cysteinyldopa. The difference between the oxidation potentials for dopa and 5-S-cysteinyldopa shows that 5-S-cysteinyldopa is more readily oxidized than dopa itself. 5-S-cysteinyldopa can be oxidized by dopaquinone in a pure chemical step, and dopa is then regenerated, giving a better tyrosinase substrate than does 5-S-cysteinyldopa (Fig. 6).

The findings now reported on the influence of tyrosinase and dopa oxidation on the oxidation of 5-S-cysteinyldopa indicate that cysteinyldopas play an important role in the redox-systems of the melanocyte.

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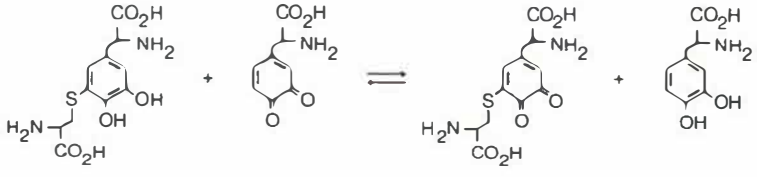

Fig. 6. Redox-system of dopa—5-S-cysteinyldopaquinone.

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