Phytochemistry, 1977, Vol. 16, pp 517–519. Pergamon Press Printed in England

PHOTOCHEMICAL OXIDATION OF TYROSINASE

STEVEN GUTTERIDGE, GEORGE DICKSON and DONALD ROBB
Department of Biochemistry, University of Strathclyde, The Todd Centre, 31 Taylor Street, Glasgow G4 0NR, Scotland

(*Received* 27 *September* 1976)

Key Word Index—*Neurospora crassa*; *Agaricus bispora*; mushroom; tyrosinase; photo-oxidation.

Abstract—Dye sensitized photo-oxidation inactivates tyrosinases isolated from *Neurospora* and *Agaricus*. The rate of inactivation is enhanced by cyanide and is dependent on pH.

INTRODUCTION

Tyrosinase (E.C. 1.14.18.1), which occurs extensively throughout the plant kingdom [1], is a copper containing protein which catalyses both the hydroxylation of phenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. Thus it is implicated in the early stages of melanin formation. While recent work has emphasized the role played by the copper prosthetic group [2, 3], little progress has been made in establishing the part played by the protein, particularly in connection with active site residues and residues forming the ligands of the copper. Therefore we have begun attempts to identify such residues by the technique of covalent modification with group specific reagents. Previous studies on the irreversible inhibition of tyrosinase are chiefly concerned with the use of chelating agents to remove the copper, but a few methods causing covalent modification have been described. The best known example of irreversible inhibition is the phenomenon of reaction–inactivation in which tyrosinase is inactivated as it oxidizes *o*-diphenols to *o*-quinones [4]. Since this process is accompanied by the incorporation of substrate into the enzyme, it has been proposed that reaction–inactivation is due to the reaction of some nucleophilic group in the vicinity of the active site with the *o*-quinone molecules formed at that site, in a manner analogous to the Michael addition of nucleophiles to α,β-unsaturated carbonyl systems [5]. Copper is lost from the enzyme in the course of reaction-inactivation [6] suggesting that the ligands of the copper may be targets for the reaction with *o*-quinones. However, tyrosinase activity appears to be resistant to general electrophilic and acylating agents such as iodine, succinic anhydride [7], imido-esters [8] and diethyl pyrocarbonate [9]. Phenylhydrazine has been shown to be a relatively specific inactivator [10] but its mechanism of action is not clear [11]. This report shows that tyrosinases from two fungi, *Neurospora crassa* and *Agaricus bispora*, are sensitive to dye sensitized photochemical oxidation [12], and suggests that destruction of histidine is responsible for inactivation.

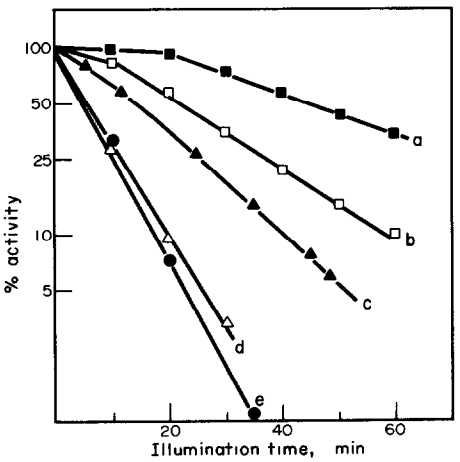

Fig. 1. Photo-oxidation of mushroom tyrosinase, in the absence (a) and presence (b–e) of 1 mM cyanide. The studies were conducted at various pH values, (a) and (b) 6.5, (c) 7.0, (d) 7.5 and (e) 8.0, using rose bengal as sensitizer.

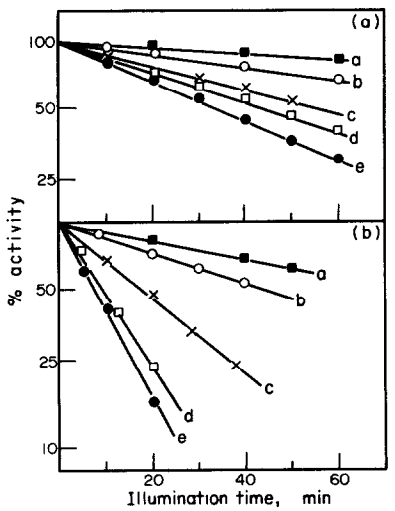

Fig. 2. Photo-oxidation of *Neurospora* tyrosinase mediated by rose bengal, (a) in the absence and (b) in the presence of 1 mM cyanide. pH values used were (a) 6.0, (b) 6.5, (c) 6.8, (d) 7.0 and (e) 8.0.

RESULTS

Both tyrosinases were slowly inactivated by photochemical oxidation in the presence of either rose bengal or pyridoxal phosphate. Inactivation required the presence of all components, was a first order process (Figs. 1 and 2), and was dependent on the pH, giving for example $t_{1/2}$ values of 200 min at pH 6.0 decreasing to 33 min at pH 8.0 for the *Neurospora* enzyme with rose bengal. Additionally, with the mushroom enzyme there was a distinct lag period which also showed a pH dependency, being more pronounced at low pH. Plotting the inactivation rate constants against pH produced similar titration curves for both enzymes, characterized by a pK_a of about 7.0. To confirm that the destroyed residues were essential for the activity of tyrosinase the process was studied in the presence of cyanide, an inhibitor which has a strong affinity for the oxygen binding site of both enzymes [13, 14]. Instead of affording protection, 1 mM cyanide caused about a fourfold enhancement in the rate of inactivation obtained with either photosensitizer, also decreasing the lag observed with the mushroom enzyme (Figs. 1 and 2). In spite of the enhancement, the pH dependency of photo-oxidation was very similar (Fig. 3), being characterized by a pK_a of 6.8 for the *Neurospora* enzyme and of 7.2 for the mushroom enzyme. Under these conditions cyanide behaved as a reversible inhibitor. Control studies showed that enzyme, cyanide and dye had to be irradiated together before the enhancement occurred and that cyanide had no effect on the rate of photo-oxidation of aldolase. Some protection was observed at cyanide concentrations of up to 100 μM and with the substrate analogue, L-phenylalanine. The latter is a competitive inhibitor with respect to 3-(3,4-dihydroxyphenyl)-DL-alanine, (DL-DOPA) [15] with a K_i of 1.2 mM at pH 8.0. At a phenylalanine concentration of 10 mM the rate constant for the inactivation of the mushroom enzyme was 20% lower than that obtained in its absence at this pH.

DISCUSSION

Since a pH dependent photo-oxidation is characteristic for histidine [12], these results suggest that histidine residues, with pK_a values of 6.8 and 7.2 for the *Neurospora* and mushroom enzymes respectively, are essential for the activity of tyrosinase. However methionine, tyrosine, cysteine and tryptophan are also susceptible to photo-oxidation and further studies will be necessary to establish whether histidine is uniquely responsible for the loss of activity.

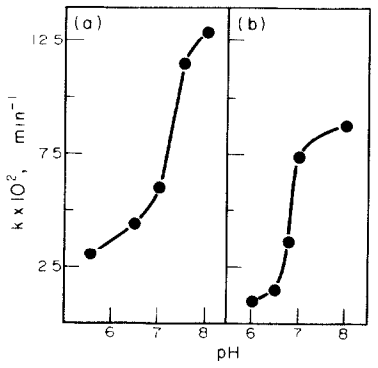

Fig. 3. Variation of the first order inactivation constant with pH, determined in the presence of 1 mM cyanide and rose bengal, for (a) mushroom and (b) *Neurospora* tyrosinases.

An explanation for the unexpected property of cyanide to accelerate the photo-oxidation of tyrosinase, is based on its ability to remove the copper from the enzyme during prolonged incubation at concentrations of 100 mM [16]. The immediate effect of adding this ligand to tyrosinase may be to expose at least one of the metal ion ligands for photo-oxidation. Without cyanide present, the metal ion bound to the residue may be acting protectively by preventing rapid oxidation occurring. It is interesting to find that a similar explanation was given for the enhancement of the photo-oxidation of carboxypeptidase by *o*-phenanthroline, a chelating agent used for the removal of Zn ions from the active site of this metalloenzyme [17]. An alternative explanation might be based on analogy with myoglobin. Cyanide also promotes the photo-oxidation of this protein, presumably because it converts the myoglobin to a low spin state [18]. However a similar circumstance is unlikely to apply to tyrosinase which is believed to be diamagnetic in the resting state [2, 3].

EXPERIMENTAL

Tyrosinase. Neurospora crassa tyrosinase was purified to homogeneity by the method reported previously [13]. Mushroom tyrosinase was prepared from commercial mushrooms by a modification of established methods [19, 20]: stalks and stipes were sliced thinly and freeze-dried. The enzyme was extracted from the dry powder with 25% acetone in water and purified by acetone and ammonium sulphate fractionations, followed by passage through DEAE-Sephadex A50 equilibrated with 0.3 M Na_2SO_4 in 10 mM imidazole buffer, pH 6.5. At this ionic concentration tyrosinase is not absorbed onto the ion exchanger but the associated brown pigments are. After dialysis against 0.03 M borate buffer pH 8.3, the preparation was freeze-dried and stored. Specific activity measurements and analysis by disc gel electrophoresis at pH 9.5 [21] show the enzyme to be *ca* 60% pure, the latter method indicating one major impurity. Staining the gels with the substrate, DL-DOPA showed only one enzyme band.

Photo-oxidation. Samples were incubated in a Perspex reaction vessel of 2 cm diameter which was mounted on a stirrer and surrounded by a constant flow water jacket maintained at 30°. A 500-W reflector lamp was fixed 15 cm from the cell and a 500 ml round flask full of H_2O was interposed between them. The flask acted as a crude lens and heat trap. Either rose bengal [12] or pyridoxal phosphate [22] was used as photosensitizer and the concentration of enzymes, rose bengal and pyridoxal phosphate employed were 0.5 mg/ml, 51 μM and 1 mM respectively in a volume of 2 ml. For studies at pHs 5.5 and 6–8, 25 mM citrate-50 mM phosphate, and 50 mM phosphate buffers were used respectively. With this arrangement the $t_{1/2}$ for the inactivation of aldolase with rose bengal at pH 7.6 was 1.6 min, comparable to published values [23]. The course of inactivation of tyrosinase was determined by assaying aliquots of the reaction mixture with DL-DOPA according to the spectrophotometric method described in [24] except a final volume of 2.5 ml was used.

Acknowledgements—We thank the Carnegie Trust for the award of a studentship to one of us (S.G.) and the Science Research Council for financial support.

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