# PHOTOCHEMICAL ACTIVATION OF RANA PIPIENS TYROSINASE

Ross B. Mikkelsen[1], David H. Tang and Edward L. Triplett

Biochemistry and Molecular Biology Section
Department of Biological Sciences
University of California at Santa Barbara
Santa Barbara, California 93106

Received February 27, 1975

Summary: Purified tyrosinase from Rana pipiens is activated by light. An action spectrum for the process indicates that there are two absorption bands responsible for the activation (290nm and 334nm). The kinetics of the photochemical process show an initial activation followed by inhibition. Molecular oxygen is required. The ability of the protein to be photoactivated and the absorbancy of the protein at 334nm can be extracted with 50% acetone/water.

The action of light on skin resulting in increased melanin synthesis and dispersion has been a subject of considerable study (for a review see Ref. 1). Early work suggested that sulfhydryl compounds such as reduced glutathione are inhibitors of tyrosinase, an enzyme which catalyzes melanin synthesis. Furthermore the intracellular concentration of these inhibitors could be at least partially regulated by a photochemical mechanism (2,3). For example, Halprin and Okawara (3) present evidence for one target of UV irradiation in the skin being glutathione reductase.

Other inhibitors of tyrosinase activity that do not require a free sulfhydryl for activity have been found in extracts from several melanomas. Monochromotic light at 280nm and light in the 340 to 380nm range destroys their inhibitory power(4).

Tyrosinase in R. pipiens can be isolated in an inactive form that can be activated proteolytically (5,6,7). In our studies with this enzyme, another time dependent activation process became apparent (8,13). Further investigation revealed the process to be photochemical. We report here on these initial studies. To our knowledge, this is the first report of a photochemical activation of an extensively purified tyrosinase.

---

1. Present Address: Division of Radiobiology, New England Medical Center, 171 Harrison Ave., Boston, Mass. 02111.

Methods: Tyrosinase is purified according to the complete procedure in Ref. 8. The purified protein is homogeneous by the criteria of electrophoresis in 8M urea at low and at high pH, and in sodium dodecyl sulfate (8). Enzyme assays and source and grade of reagents have been stated previously (5,8).

For light activation, we use a quartz grating monochromoter with a Philips 125W high pressure mercury arc. Incident light intensities are measured with the ferrioxalate actinometer (9,10). The fraction of incident light absorbed is approximated from the absorbance of the irradiated solution at the illumination wavelength (10). A value proportional to the quantum yield is determined by dividing the enhancement in enzyme activity by the total quanta absorbed during illumination.

Typically, 2.0 ml of enzyme contained in a 1.0 cm light path quartz cuvette is irradiated and small aliquots (10-50 µl) are removed for analysis after different times of illumination. The experiments are performed under photographic red safelights. The temperature (20±1°) is constant under illumination for at least one hour. The entire contents of the cuvette are irradiated.

In those experiments where anaerobiosis is desired, we purge the reaction mixtures with nitrogen (11). The nitrogen is deoxygenated by passage through alkaline pyrogallol (12).

We extract tyrosinase by suspending the lyophilized protein in 50% acetone/water in a glass centrifuge tube at room temperature. The protein is precipitated by centrifugation, the solvent is decanted, and the procedure is repeated six times. The residual solvent is removed from the protein with a stream of nitrogen followed by evacuation under high vacuum in a dessicator for at least four hours. Incomplete removal of the organic solvent results in the failure of the protein to dissolve in an aqueous buffer even at extremes of pH or in 8M urea.

Results: The D1 variant of R. pipiens tyrosinase is used in the below experiments (8). Similar results are found with the D2 form. For discussion of quantitative effects of photochemical activation, we define 100% activity as the maximal activity achieved with trypsin activation under optimal assay conditions.

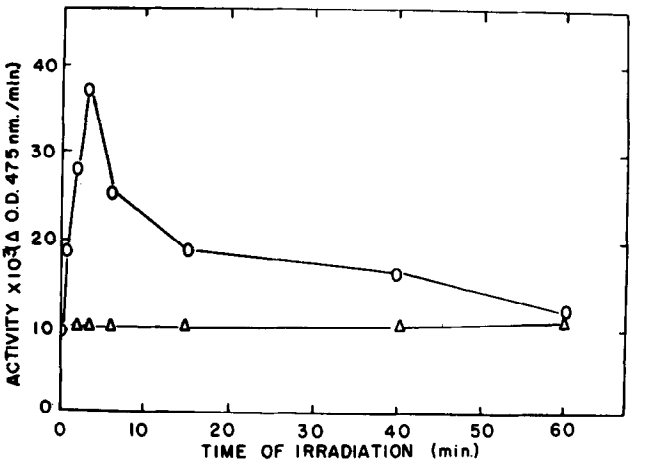


Figure 1. Light Activation Kinetics at λ = 334nm. Enzyme activity is measured spectrophotometrically at 475 nm, the absorption maximum of dopachrome. Protein concentration is 2.0 mg/ml. Incident light intensity is 15.7 x $10^{13}$ quanta/sec. Dopa oxidase activities with and without trypsin are 0.08 and 0.01 O.D. 475 nm/min. 0-0-0, with $0_2$ during illumination Δ-Δ-Δ, anaerobic during illumination.

The results are presented in terms of enhancement in dopa oxidase activity since we are unable to detect changes in the tyrosine oxidase activity of tyrosinase due to illumination.

A typical illumination time course for a wavelength that activates the enzyme (λ = 334nm) is seen in Figure 1. A time course of activation followed by inactivation is observed for all wavelengths that are affective. Enzyme inactivated by prolonged irradiation is still fully activatable with trypsin.

The requirement for oxygen in the light activation process is also demonstrated in Figure 1.

The maximum dopa oxidase activities from time courses such as Figure 1 for each wavelength are used to construct the action spectrum ( Figure 2). This action spectrum demonstrates: (1) the spectral energy distribution involved in activation is in the biologically important near UV wavelength region; (2) the absorption maximum around 330nm is not characteristic of amino acids normally found in proteins; (3) there are two absorption bands ( ∿290nm and ∿334nm) involved in the process.

With the enzyme preparation used in experiments for Figures 1 and 2, 50%

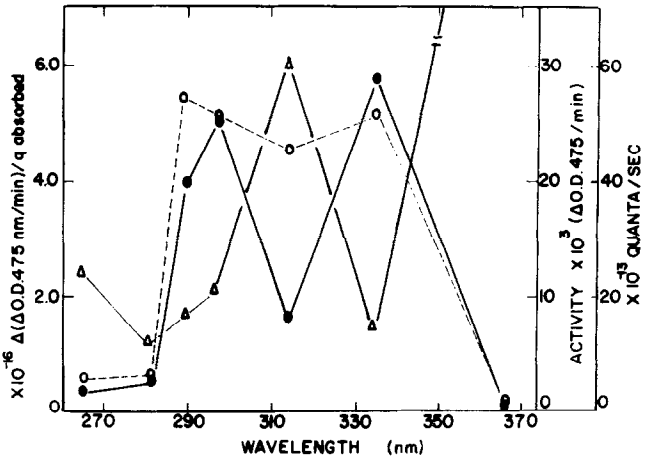


Figure 2. Action Spectrum of the Light Activation of Dopa Oxidase Activity. The conditions of illumination are identical to Figure 1. Δ-Δ-Δ, incident light intensity in quanta/sec. 0-0-0, O.D. 475 nm/min (dopa oxidase activity). ●-●-●, ( ΔO.D. 475nm/min)/quanta absorbed ( activation per quanta absorbed).

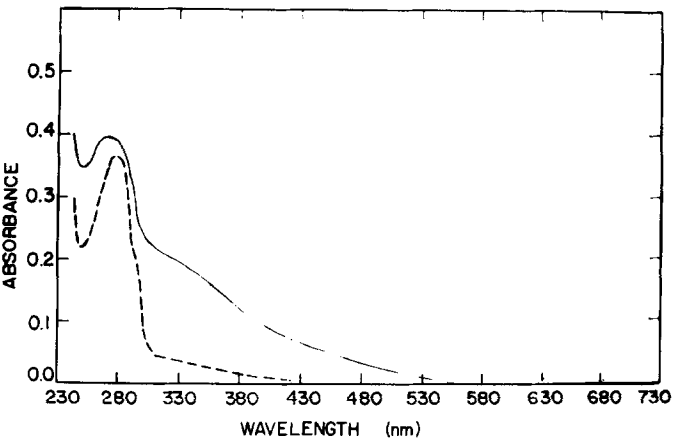


Figure 3. UV. and Visible Spectra of Tyrosinase. The spectra are recorded at room temperature in 0.02M sodium phosphate (pH 7.2). ———, native enzyme. - - -, enzyme extracted in 50% acetone. Protein concentration is 0.25 mg/ml.

---

of the total trypsinizable activity could be gained by illumination for 3 minutes at 334nm. From one enzyme preparation to another the amount of activity achieved with light ranged from 25% to 100% that obtained with trypsin. This variability might reflect physiological differences in animal populations used or in slight

alterations in enzyme purification procedures from one enzyme preparation to another, eg. amount of light exposure.

We are able to remove the absorbing material at 334nm and prevent photochemical activation while maintaining the inactive state of the enzyme. The procedure involves extraction with 50% acetone as described in Methods. For example, the specific activities before and after acetone extraction for one enzyme preparation are 68 and 70 units/mg with trypsin, and 8 and 16 units/mg with trypsin, respectively. The changes in the absorption spectrum after extraction are seen in Figure 3. There is an increase in the 280/260nm ratio and a decrease in absorbance at the higher wavelengths.

We have attempted extractions with solvent systems of lower polarity, eg. 100% acetone or 2:1 chloroform / methanol. With these solvents, extraction results in a partial activation with respect to trypsin (8,13).

When these 50% acetone extracts are analyzed for thiols or disulfides with Ellman's reagent and reduction with sodium borohydride (14), the results are negative for both of these functional groups.

Several attempts at reconstitution of the extract with the enzyme have proved unsuccessful with respect to inhibition of enzyme activity or recovery of photochemical activation ability.

Discussion: Our results can be summarized as follows. (1) The dopa oxidase but not tyrosine oxidase activity of tyrosinase can be activated to the same degree with either trypsin or light. (2) The photochemical activation requires oxygen and involves a chromophore with an absorption maximum around 334nm. (3) Extraction with 50% acetone prevents light but not trypsin activation.

The relationship of our findings with the earlier results of Chian and Wilgram (4) with different rodent melanomas is difficult to assess. The inhibitors from both organisms are photosensitive to near UV light and apparently do not contain free sulfhydryls. The mouse inhibitor appears to be more stable than that isolated from amphibian skin. These results suggest to us, however, that a photoactivation process might be common to tyrosinases from most organisms.

It is premature with the evidence at hand to propose a mechanism for the photochemical activation. However, we are currently investigating the possibility that intermediates in the oxidation pathway of dopa to melanin are the photosensitive inhibitors of tyrosinase.

References.

1. Pathak, M.A. (1971) in Biology of Normal and Abnormal Melanocytes (Kawamura,T., Fitzpatrick, T.B. and Seiji, M. eds.) pp. 149-159, University of Tokyo Press, Tokyo.
2. Rothman, S. (1946) Proc. Soc. Exp. Biol. Med. 62, 208-209.
3. Halprin, K. and Ohkawara, A. (1967) in Advances in Biology of Skin (Montagnu, W. and Hu, F., eds) vol. 8, pp. 241-268, Pergamon Ress, Oxford.
4. Chian, L.T.Y. and Wilgram, G.F. (1967) Science, 155, 198-200.
5. Miller, L.O., Newcombe, R. and Triplett, E.L. (1970) Comp. Biochem. Physiol., 32, 555-567.
6. Purvis, J.L. and Denstedt, O.F. (1957) Can. J. Biochem. Physiol. 35, 961-975.
7. McQuire, J.S. (1970) Biochem. Biophys. Res. Comm. 40,1084-1089.
8. Mikkelsen, R.B. and Triplett, E.L. (1975) J.Biol. Chem., 250, 638-643.
9. Parker, C.A. (1968) Photoluminescence of Solutions, pp. 208-214, Elsevier Publ. Co., Amsterdam.
10. Calvert. J.G. and Pitts, J.N. (1966) Photochemistry, pp. 795-798, John Wiley and Sons, Inc., New York.
11. Penzer, G.R. and Radda, G.K. (1971) Methods in Enzymology 18, 479-495.
12. Vogel, A. (1956) Textbook of Practical Organic Chemistry, p. 186, Longmans, London.
13. Mikkelsen, R.B. (1973) PhD Thesis, University of California at Santa Barbara.
14. Habeeb, A.F.S.A. (1972) Methods in Enzymology 25, 457-464.