Photoinactivation of *Agaricus bisporus* Tyrosinase: Modification of the Binuclear Copper Site†

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ABSTRACT: Irradiation of *Agaricus bisporus* tyrosinase in the presence of citrate at pH 5.6 with 300–420-nm light results in a loss of both catecholase activity and cresolase activity. The light-sensitive species appears to be an enzyme–citrate complex, most likely involving coordination of citrate to the active site copper. One copper ion from each binuclear active site can be removed from the inactivated enzyme, resulting in the formation of a met apo derivative. The electron spin resonance spectrum of met apo tyrosinase resembles that of met apo hemocyanin and half-met *Neurospora* tyrosinase. It is consistent with a distorted square-planar geometry around the copper and with either nitrogen or nitrogen and oxygen ligands. Amino acid analysis indicates that four histidines on the heavy subunit are destroyed during the inactivation process. Some or all of these histidines may serve as ligands to the copper ion which becomes labile after inactivation. Photoinactivation results in decarboxylation of citrate and does not require the presence of oxygen. The reaction may involve generation of a free radical from the citrate which then attacks nearby histidine residues.

Tyrosinase from the common mushroom, *Agaricus bisporus*, has a molecular weight of 1.2×10^5 and contains four copper atoms per molecule (Bouchilloux et al., 1963). It catalyzes the ortho hydroxylation of phenols and the oxidation of the resulting catechol to an *o*-quinone (Mason, 1965; Solomon, 1980). Each active site contains a pair of antiferromagnetically coupled cupric ions in the resting enzyme (Makino et al., 1974; Schoot Uiterkamp & Mason, 1973). Mushroom tyrosinase is composed of two subunits having molecular weights of 4.3×10^4 (H) and 1.3×10^4 (L) and has a quaternary structure of H_2L_2 (Strothkamp et al., 1976). Tyrosinase from *Neurospora crassa*, which also contains a binuclear copper active site, is a single polypeptide chain of M_r 4.6×10^4 (Lerch, 1982; Deinum et al., 1976).

Treatment of tyrosinase with H_2O_2 in the presence of oxygen results in the appearance of an oxygenated form of the protein, oxytyrosinase (Jolley et al., 1972, 1974). Oxytyrosinase and oxyhemocyanin, the oxygen transport protein found in some molluscs and arthropods, are similar in many respects, which suggests an evolutionary relationship (Jolley et al., 1974; Eickman et al., 1978). Recent spectroscopic work on hemocyanin has involved both the native protein and derivatives where the binuclear copper site was altered in some way. These experiments provide information about the mode of oxygen binding (Freedman et al., 1976; Thamann et al., 1977) and the identity of the ligands and geometry around the copper ions (Brown et al., 1980; Larrabee & Spiro, 1980; Co et al., 1981; Eickman et al., 1979). Both half met [Cu(II)Cu(I)] and met apo [Cu(II)] forms of the binuclear site were prepared and used in some of these studies. A half-met form of *Neurospora* tyrosinase was made and shown to be similar to the half-met form of hemocyanin (Himmelwright et al., 1980).

The present paper describes the photoinactivation of mushroom tyrosinase in the presence of citrate. In this process one copper ion of the binuclear site becomes labile, thus making it possible to prepare a met apo form of the enzyme. Photoinactivation is accompanied by the destruction of four histidine residues on the large subunit.

Materials and Methods

Tyrosinase was isolated from the common mushroom (*Agaricus bisporus*) as previously described (Nelson & Mason, 1970; Jolley et al., 1974). Protein concentrations were determined by the Lowry procedure (Lowry et al., 1951) with crystalline bovine serum albumin as the standard. Copper analyses were done by graphite furnace atomic absorption. The purified isozymes of tyrosinase were homogeneous on SDS[1]–polyacrylamide gel electrophoresis (Weber & Osborn, 1969). Enzymatic activity was measured with a Gilson Oxygraph and Clark oxygen electrode at 25.0 ± 0.2 °C by using a 4-*tert*-butylcatechol (catecholase activity) or *p*-cresol (cresolase activity) (Nelson & Mason, 1970).

Chelex-100 was obtained from Bio-Rad Laboratories. *p*-Cresol and 4-*tert*-butylcatechol were from Aldrich and were sublimed before use. Potassium ferrioxalate was prepared as previously described (Hatchard & Parker, 1956). [1,5-^{14}C]Citric acid was purchased from New England Nuclear. Tissue solubilizer, TS-1, for absorption of CO_2 was from Research Products International. Prepurified grade nitrogen gas, containing less than 5 ppm of oxygen, was used in the anaerobic experiments. All other reagents were of the highest purity available and were used without further treatment. All solutions were prepared with distilled, deionized water.

ESR spectra were obtained with a Varian E-line X-band spectrometer at 77 K. Mn(II), naturally present as an impurity in strontium oxide, was used to calibrate the magnetic field (Bolton et al., 1972). ESR parameters were calculated as previously described (Malmstrom & Vanngard, 1960; Peisach & Blumberg, 1974). Paramagnetic copper was quantitated by double integration of the spectrum and comparison with a copper–EDTA solution. Amino acid analyses were carried out on a Beckman 120-C analyzer. Tryptophan was determined after hydrolysis with *p*-toluenesulfonic acid (Liu, 1972). Separation of tyrosinase subunits by SDS electrophoresis was carried out as previously described (Strothkamp et al., 1976).

Irradiation Experiments. Most of the experiments were performed with a 100-W high-pressure mercury vapor lamp (GE H100A4/T) and a Corning 5970 filter which transmitted

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[1] Abbreviations: SDS, sodium dodecyl sulfate; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetate.

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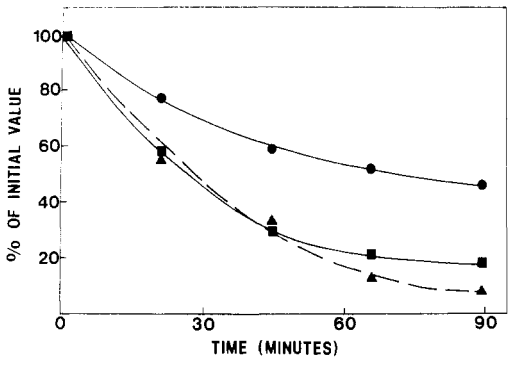

FIGURE 1: Loss of catecholase activity (■), cresolase activity (▲), and copper (●) from α-tyrosinase during irradiation with 300–420-nm light in 100 mM phosphate and 35 mM citrate, pH 5.6, at 11 °C. Aliquots were removed from the solution at various times and assayed for activity and copper as described under Materials and Methods.

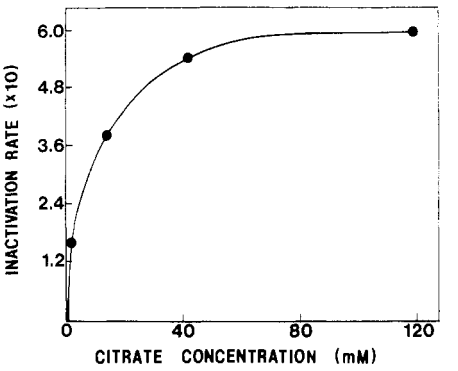

FIGURE 2: Dependence of the initial rate of photoinactivation of γ-tyrosinase on thhe citrate concentration in 100 mM phosphate, pH 5.6, at 11 °C. Each inactivation rate was obtained from a plot similar to Figure 1 by using 4-*tert*-butylcatechol as substrate.

wavelengths of 300–420 nm. For some experiments, narrow band filters or a combination of filters was used to isolate a narrow region of the mercury spectrum. One series of experiments employed a Quanta Ray Nd YAG laser emitting at 355 nm. The intensities of the various light sources were determined by actinometry with potassium ferrioxalate (Hatchard & Parker, 1956). The temperature of the enzyme solution during irradiation was maintained at 11 °C. Aliquots of the solution were removed at various times for determination of activity and copper content. Chelex-100 was used to remove adventitious copper from the solution prior to the start of the experiment and also after irradiation to remove the loosely bound copper resulting from photochemical damage to the protein. Chelex-100 did not remove copper from native tyrosinase. Control samples were treated identically except that they were maintained in complete darkness.

Anaerobic irradiation experiments were performed by using Thunberg cuvettes. The solution in the cuvette was subjected to eight cycles of evacuation and flushing with nitrogen. Deoxygenation was monitored by measuring the decrease in absorbance of the "intrinsic oxytyrosinase" (Jolley et al., 1974) at 345 nm in the difference spectrum of identical solutions of anaerobic vs. air-saturated tyrosinase.

Experiments using [^{14}C]citrate were also carried out in Thunberg cuvettes. An enzyme solution in buffer containing [^{14}C]citrate was placed in the cuvette, and 0.50 mL of tissue solubilizer (TS-1) was placed in the side well to trap CO_2. The cuvette was then sealed and irradiated in the usual manner. In those experiments where the presence of CO_2 was being determined, 100 μL of 5 N H_2SO_4 was injected into the enzyme solution through a rubber septum. The Thunberg cuvette was shaken gently for 30 min at room temperature to allow for complete absorption of the CO_2 by the tissue solubilizer. The amount of radioactivity in the tissue solubilizer was then determined by liquid scintillation counting.

Experiments were also designed to detect the incorporation of radioactivity from the citrate into the protein. The enzyme solution was irradiated in the presence of [^{14}C]citrate in the usual manner. At the end of the irradiation, ^{14}C not bound to the protein was removed by exhaustive dialysis against buffer containing nonradioactive citrate until the radioactivity in the dialysate was reduced to the background level. The protein solution was then assayed for bound ^{14}C by scintillation counting.

Results

Figure 1 shows the time course of the photoinactivation process in 100 mM phosphate and 35 mM citrate, pH 5.60.

Table I: Decarboxylation of [1,5-^{14}C]Citrate in the Presence and Absence of β-Tyrosinase

buffer[a]	pH	nmol of enzyme	nmol of CO_2 formed	condition[b]
phosphate-citrate	5.6	3.32	0.0147	nonirradiated
phosphate-citrate	5.6	3.32	7.70	irradiated
phosphate-citrate	5.6		0.949	irradiated
phosphate-citrate + 0.6 mM $CuSO_4$	5.6		0.360	irradiated
phosphate-citrate	8.0		0.664	irradiated
phosphate-citrate	8.0	3.32	0.0244	nonirradiated
phosphate-citrate	8.0	3.32	0.306	irradiated

[a] 100 mM phosphate and 25 mM citrate. [b] Nonirradiated samples were kept refrigerated in the dark. Irradiation was carried out at 11 °C using 300–420-nm light.

Catecholase and cresolase activities were lost at about the same rate. A loss of >80% of the initial activity was accompanied by a 50% loss of copper upon Chelex-100 treatment. There was no loss of either type of activity or of copper when the solution was kept in the dark. Irradiated samples were stored for days in the refrigerator without further loss of activity or copper. Addition of copper to inactivated samples did not restore any of the activity. Logarithmic plots of either percent activity or copper remaining vs. time were linear, indicating apparent first-order kinetics. The first-order rate constants for loss of catecholase activity [$(2.8 \pm 0.2) \times 10^{-2}$ min^{-1}] and cresolase activity [$(2.3 \pm 0.3) \times 10^{-2}$ min^{-1}] were the same within ±1 standard deviation.

Photoinactivation was dependent both on the presence of citrate and on the pH of the buffer. Inactivation and copper loss did not occur in phosphate–citrate buffer at pH 8.0 or in phosphate buffer alone at pH 5.6 or 8.0. Other carboxylic acids also produced photoinactivation at pH 5.6 with the following order of effectiveness: citric > succinic > acetic > malonic. Irradiation of the enzyme in the presence of oxalic acid also resulted in a loss of activity. In contrast to all the other acids tested, however, oxalic acid also caused a loss of activity in the dark, although the rate of activity loss was greater when the sample was irradiated. All three isozymes of tyrosinase showed similar behavior when irradiated in the presence of citrate. The rates of photoinactivation were $\alpha \gtrsim \beta > \gamma$.

Figure 2 shows the dependence of the initial rate of inactivation on the citrate concentration. The inactivation rate became constant above about 80 mM citrate. This suggests the formation of an enzyme–citrate complex which is the photolabile species. Table I shows the production of CO_2 from buffer containing [1,5-^{14}C]citrate under various conditions,

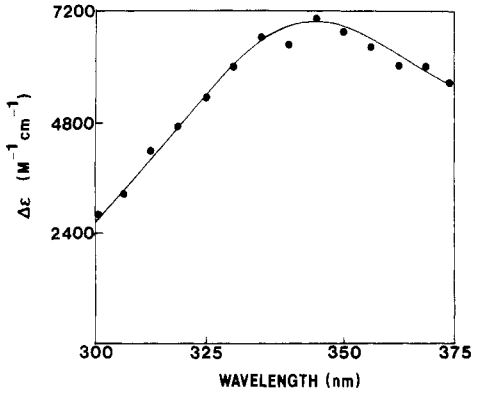

FIGURE 3: A difference spectrum of nonirradiated minus irradiated β-tyrosinase in 100 mM phosphate and 25 mM citrate, pH 5.6. The irradiated sample had lost 70% of its initial catecholase activity. The Δε values are calculated per mole of enzyme by using a molecular weight of 1.2×10^5.

both with and without tyrosinase present. The CO_2 arises from decarboxylation of the citrate. Samples of phosphate–citrate buffer at pH 5.6 or 8.0 containing tyrosinase show insignificant formation of CO_2 when kept in the dark. The mole ratio of CO_2 formed to protein is <0.01. When irradiated, the pH 5.6 sample produces more than 2 mol of CO_2/mol of enzyme. Irradiation of buffer at pH 5.6 or 8.0 in the absence of the enzyme results in minimal CO_2 production in comparison to buffer plus enzyme. Comparable amounts of CO_2 are formed at pH 8.0 whether or not tyrosinase is present. Addition of a large quantity of Cu(II) to the buffer has no effect on CO_2 production. These results demonstrate that significant decarboxylation of citrate occurs only upon irradiation in the presence of tyrosinase and only at pH 5.6. Formation of CO_2, therefore, correlates with inactivation of the enzyme and is not induced by the presence of aqueous Cu(II). The results are consistent with formation of CO_2 from an enzyme–citrate complex which is present at pH 5.6 but not pH 8.0. There was no incorporation of ^{14}C into the enzyme under any of the experimental conditions.

Figure 3 is a difference spectrum showing the change in molar extinction coefficient of tyrosinase as a result of photoinactivation. The maximum in Δε at 345 nm results from a loss of "intrinsic oxytyrosinase" during the irradiation. The 345-nm absorption band of the intrinsic oxytyrosinase is not the light-absorbing chromophore responsible for inactivation, however, because photoinactivation occurs at the same rate under anaerobic conditions, where the absorption due to intrinsic oxytyrosinase has been eliminated. The anaerobic experiments also rule out the possibility that dioxygen, or some species derived from it, is involved in the inactivation process.

Investigation of the wavelength dependence of the photoinactivation process involved comparing the inactivation rate obtained by using all the mercury lines in the 300–420-nm region with the 366-nm line of the mercury lamp and the 355-nm line of the Nd YAG laser. Actinometry was used to measure the light intensity at the sample in each case and rate constants were normalized to a constant intensity. The results, shown in Table II, indicate that 355-nm light is the most effective of the wavelengths tested. Extending the range of wavelengths to include the visible lines of the mercury spectrum does not increase the inactivation rate. The light intensity in this case could not be accurately measured but was greater than when only the 300–420-nm region of the spectrum was used. Visible light, therefore, does not produce photoinactivation. The chromophore responsible for photoinactivation appears to have an absorption maximum in the 300–420-nm region, but it is not possible to observe this band directly in the ultraviolet spectrum of the enzyme in citrate buffer. This is most likely due to its low extinction coefficient as well as overlapping absorption from other chromophores.

Table II: Irradiation of Tyrosinase at Various Wavelengths[a]

wavelength[b] (nm)	light intensity (photons/min) ($\times 10^{-21}$)	rate constant for inactivation[c] (min^{-1})	rate constant per 1.0×10^{21} photons/min (min^{-1})
300–420	8.25	1.54×10^{-3}	1.87×10^{-4}
355	7.83	1.63×10^{-3}	2.08×10^{-4}
366	4.21	0.26×10^{-3}	0.62×10^{-4}
310–800		1.15×10^{-3}	

[a] The solution composition for all experiments was 100 mM phosphate and 25 mM citrate, pH 5.6, containing 0.040 μM β-tyrosinase. [b] Irradiation of solutions with light of different wavelengths and actinometry measurements were performed as described under Materials and Methods. [c] Each inactivation rate was determined from a graph of ln % catecholase activity vs. time.

Table III: Amino Acid Composition of Photoinactivated Tyrosinase[a]

amino acid	α-tyrosinase		β-tyrosinase	
	% of nonirradiated value	residues destroyed[b]	% of nonirradiated value	residues destroyed[b]
Lys	93		100	
His	65	4	75	4
Arg	99		100	
Asp	114		126	
Thr	102		103	
Ser	100		112	
Glu	93		107	
Gly	108		100	
Ala	91		97	
Val	103		100	
Met	85		100	
Ile	93		93	
Leu	91		96	
Tyr	61	6	100	
Phe	79	4	100	
Trp	65	3		

[a] Enzyme solutions were prepared and divided into two portions. Half was irradiated in the usual way, and the other half was kept in the dark in the refrigerator. Both samples were then subjected to amino acid analysis. Values for α-tyrosinase are averages of 24- and 70-h hydrolysates while those for β-tyrosinase are from a 24-h hydrolysate. Tryptophan was determined as described under Materials and Methods. [b] Per active site.

Amino acid analysis of native α- and β-tyrosinase were in excellent agreement with the reported values (Jolley et al., 1969). There was no change in the amino acid composition when the protein was placed in phosphate–citrate buffer at pH 5.6 or 8.0 and kept in the dark. Nor was there any change when the protein was irradiated with 300–420-nm light at pH 5.6 or 8.0 in the absence of citrate, conditions which do not result in a loss of activity. Changes in the amino acid composition were found when the enzyme was photoinactivated in phosphate–citrate at pH 5.6. Table III shows the amino acid composition of α- and β-tyrosinase after irradiation, expressed as a percentage of the native values. Four histidine residues are destroyed per active site in both the α and β isozymes. In addition, α-tyrosinase but not β-tyrosinase showed some loss of the aromatic amino acids. Activity loss and copper loss were the same for the two isozymes even though there was no loss of aromatic amino acids in β-tyrosinase. Thus, the loss of activity appears to correlate with the loss of histidine residues. The light and heavy subunits of

Table IV: ESR Parameters of Met Apo Tyrosinase and Related Proteins

protein	buffer (pH)	$g_\perp$ (or g_m)	$g_\parallel$	$A_\parallel$ (cm^{-1})
Agaricus bisporus tyrosinase (met apo)	phosphate–citrate (5.6)	2.06	2.25	0.0142
Neurospora tyrosinase (half-met NO_2^-)[a]	phosphate (6.3)	2.078	2.296	0.0131
Busycon hemocyanin (met apo)[b]	acetate (5.7)	2.083	2.314	0.0131
Busycon hemocyanin (half-met)[b]	acetate (5.7)	2.080	2.318	0.0141
bovine superoxide dismutase[c]	water	2.09	2.27	0.0145

[a] In this sample the copper has one or more nitrite ligands (Himmelwright et al., 1980). In the other samples water or buffer ions may be coordinated to the copper in addition to the protein ligands. [b] Himmelwright et al. (1978). [c] Fee & Gaber (1972).

photoinactivated β-tyrosinase were separated by gel electrophoresis and subjected to amino acid analysis. The observed loss of histidine residues was found to result from destruction of histidines on the heavy subunit and not the light.

Photoinactivated, copper-depleted tyrosinase gives an ESR signal characteristic of a mononuclear copper(II) complex. This signal is not present in the native enzyme. The failure of repeated treatment with Chelex-100 to diminish the signal indicates that the copper responsible for this spectrum is tightly bound to the protein. The signal represents one copper(II) ion for each active site destroyed during the irradiation and apparently results from the loss of one copper ion from each binuclear copper site which has suffered photochemical damage. This finding is consistent with the 50% loss of copper observed after inactivation of the enzyme. Table IV presents the ESR parameters of the observed spectrum along with those of related proteins and superoxide dismutase for comparison.

Discussion

The characteristics of the citrate-dependent photoinactivation of tyrosinase at pH 5.6 indicate an enzyme–citrate complex as the photolabile species. The leveling off of the inactivation rate at high citrate concentrations is characteristic of an equilibrium preceding the photochemical event. When the total citrate concentration is used, an apparent formation constant for the enzyme–citrate complex of 7.5×10^2 M^{-1} is calculated from a linear double-reciprocal plot of the data. There are two possible explanations for the pH dependence of the inactivation process. First, complex formation may not occur at pH 8.0, due to a chnage in the active site of the enzyme at the higher pH. Second, the necessary ionic form of citrate may not be present. At pH 8.0, citrate exists as the trianion, whereas at pH 5.6, 20% of the citrate is present as the dianion and 1% as the monoanion with the remainder as the trianion. If complex formation involved only the mono- or dianion of citrate, the formation constant would have to be calculated by using the concentration of the requied ionic form and not the total citrate concentration as was done. In this case, the true binding constant would be higher than the reported value. There are no data available on citrate complexes of tyrosinase to compare with the present results. The observation of saturation behavior with respect to the citrate concentration, however, is indicative of complex formation.

The formation of CO_2 in significant quantity occurs only under those conditions where the enzyme undergoes photoinactivation. Decarboxylation of citrate, therefore, requires the presence of tyrosinase. This is further evidence for the involvement of an enzyme–citrate complex in the reaction. By analogy with the known photochemistry of ferric citrate complexes (Arzhankov & Poznyak, 1974), the light-absorbing species may be a citrate complex of the active site copper(II). In ferric citrate, absorption of a photon results in reduction of the iron to the ferrous state with the formation of CO_2 and a radical from the citrate. The analogous reaction involving citrate coordinated to the copper of tyrosinase would explain the formation of CO_2 in the photoinactivation process. In addition, the free radical resulting from decarboxylation of the citrate can then react further with amino acid residues in the active site resulting in destruction of histidines, as was observed. Some or all of the four histidines which are modified in this process may be the ligands to the one copper ion that can be removed from the active site after irradiation. Histidine destruction occurs on the heavy subunit of mushroom tyrosinase, which suggests it is the location of the active site. *Neurospora* tyrosinase also appears to have a number of histidine residues which serve as ligands (Pfiffner & Lerch, 1981). This conclusion results from dye-sensitized photooxidation of the apoenzyme, which leads to destruction of several histidine residues and a parallel loss of reconstitutability to the holoenzyme by the addition of copper. The present experiments, conducted on the native form of *Agaricus* tyrosinase, demonstrate the importance of histidine residues in copper binding to this enzyme. Thus, two different techniques, employed on tyrosinase from two different sources, indicate the involvement of histidine as a copper ligand. The heavy subunit of *Agaricus* tyrosinase, which contains the active site, may be homologous to the polypeptide chain of the *Neurospora* enzyme.

Loss of the characteristic absorption of intrinsic oxytyrosinase during photoinactivation (Figure 3) indicates that this minor component present in tyrosinase isolated from mushrooms is also modified during the inactivation process. Tyrosinase from both *Agaricus* and *Neurospora* is composed of intrinsic oxytyrosinase and the "resting" bicupric form of the protein. The relative contributions of each to the observed catalytic activity of the enzyme are unknown. Since intrinsic oxytyrosinase represents only a small fraction, on a weight basis, of the purified mushroom enzyme, it is possible that photoinactivation might have affected only the resting enzyme. The data of Figure 3 indicate, however, that this is not the case. Citrate can apparently bind to both the bicupric and oxy forms of tyrosinase and produce photoinactivation.

The finding that almost total loss of catalytic activity is accompanied by a 50% loss of copper when the inactivated enzyme is treated with Chelex-100 suggests that one copper ion is lost per binuclear active site. The ESR results, which show the appearance of a mononuclear copper signal corresponding to one copper ion per active site destroyed in the photoinactivated, copper-depleted enzyme, confirm this conclusion. The two copper ions of the binuclear site may differ slightly in their properties or in their accessibility to reagents such as citrate. This would account for the loss of only one copper ion of the pair. Photoinactivation, followed by removal of labile copper, results in the formation of a met apo form of the enzyme, analogous to the met apo form of the closely related protein hemocyanin. In addition to the half-met forms of the *Neurospora* enzyme (Himmelwright et al., 1980), the met apo form generated in the present work is another derivative of tyrosinase having a mononuclear copper ESR spectrum. A comparison of the ESR properties of these paramagnetic derivatives of tyrosinase and hemocyanin (Table IV) reveals only small differences, some of which may result

from differences in the composition and pH of the buffer used. The ESR spectrum of met apo tyrosinase provides further support for the proposed similarity of copper coordination in hemocyanin and tyrosinase and a common evolutionary origin of the two proteins.

The $A_{\parallel}$ and $g_{\parallel}$ values of met apo tyrosinase fall in the general range expected for a copper complex with four nitrogen or a mixture of oxygen and nitrogen ligands (Peisach & Blumberg, 1974). In bovine erythrocyte superoxide dismutase, for example, the copper is coordinated to four imidazole nitrogen atoms of histidine residues in a distorted square-planar geometry. There is also an axial water molecule bound to the copper (Richardson et al., 1975; Gaber et al., 1972; Boden et al., 1979). Met apo tyrosinase has $A_{\parallel}$ and $g_{\parallel}$ values very close to those of superoxide dismutase, which suggests the possibility of a similar coordination environment for the copper in the two proteins.

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Registry No. Monophenol monooxygenase, 9002-10-2; copper, 7440-50-8; citric acid, 77-92-9; L-histidine, 71-00-1.

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