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Histidine at the Active Site of *Neurospora* Tyrosinase†

E. Pfiffner and K. Lerch*

ABSTRACT: The involvement of histidyl residues as potential ligands to the binuclear active-site copper of *Neurospora* tyrosinase was explored by dye-sensitized photooxidation. The enzymatic activity of the holoenzyme was shown to be unaffected by exposure to light in the presence of methylene blue; however, irradiation of the apoenzyme under the same conditions led to a progressive loss of its ability to be reactivated with Cu^{2+}. This photoinactivation was paralleled by a decrease in the histidine content whereas the number of histidyl residues in the holoenzyme remained constant. Copper measurements of photooxidized, reconsituted apoenzyme demonstrated the loss of binding of one copper atom per mole of enzyme as a consequence of photosensitized oxidation of three out of nine histidine residues. Their sequence positions were determined by a comparison of the relative yields of the histidine containing peptides of photooxidized holo- and apotyrosinases. The data obtained show the preferential modification of histidyl residues 188, 193, and 289 and suggest that they consitute metal ligands to one of the two active-site copper atoms. Substitution of copper by cobalt was found to afford complete protection of the histidyl residues from being modified by dye-sensitized photooxidation.

Tyrosinase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing monooxygenase which occurs widespread in microorganisms, plants, and animals (Lerch, 1981). The enzyme catalyzes the ortho hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones, leading to melanin pigments and other polyphenolic compounds (Mason, 1965). The enzymes from the common mushroom *Agaricus bispora* and *Neurospora crassa* have been shown to contain a copper pair at the active site which upon reduction of Cu^{2+} to Cu^{1+} binds oxygen reversibly (Schoot Uiterkamp & Mason, 1973; Makino et al., 1974; Deinum et al., 1976; Jolley et al., 1974; Lerch, 1976). In a recent resonance Raman study of *Neurospora* oxytyrosinase, the coordinated oxygen was shown to be bound as peroxide (Eickman et al., 1978), as was previously reported for the oxygen transporting hemocyanins (Freedman et al., 1976). In contrast to the rapidly increasing information on the involvement of the metal in the binding and activation of oxygen in these copper proteins (Eickman et al., 1979; Brown et al., 1980; Larrabee & Spiro, 1980; Himmelwright et al., 1980), the knowledge of the part played by the protein moiety especially with regard to the metal ligands and the active-site residues is still limited. Although a number of investigations implicate the involvement of histidyl residues as metal ligands of the active-site copper (Salvato et al., 1974; Tallandini et al., 1975; Engelborghs & Lontie, 1973; Gutteridge et al., 1977), progress has been hampered by the complexity and molecular heterogeneity of these molecules. In this report, the residues of the active-site region of *Neurospora* tyrosinase were identified by chemical modification employing dye-sensitized photooxidation. In conjunction with the recently determined primary structure of *Neurospora* tyrosinase (Lerch, 1978), the data suggest the involvement of histidyl residues 188, 193, and 289 in the binding of one of the two copper atoms of this monooxygenase.

Materials and Methods

Neurospora crassa wild-type strain (FGSC 320) producing the thermolabile form of tyrosinase was grown according to Horowitz et al. (1970). The enzyme from cycloheximide-derepressed cultures was isolated as described previously (Lerch, 1976). The specific activity of the purified enzyme was 1200 ± 100 units/mg as measured according the Fling et al. (1963). Protein concentration was determined by using the extinction coefficient $A_{280}^{1\%,1cm} = 22$ (Lerch, 1976). Amino acid analyses were performed after hydrolysis of peptide samples in 6 N HCl in sealed evacuated tubes for 22 h at 110 °C on a Durrum (Model D-500) amino acid analyzer. Tryptophan was determined by acid hydrolysis with 4.0 M methanesulfonic acid + 0.2% tryptamine (Inglis et al., 1976). Dye-sensitized photooxidation of holo- and apotyrosinases was carried out according to Forman et al. (1973). The reaction mixtures were exposed to a 250-W lamp of an ordinary slide projector at a distance of 30 cm. At given times, samples were withdrawn for activity measurements and amino acid analysis.

Apotyrosinase was prepared by treating the holoenzyme (100 μM with potassium cyanide (100 mM) for 2 h at 4 °C in 0.1 M sodium phosphate, pH 8.5, and subsequent gel filtration on Sephadex G-25 in 50 mM sodium phosphate, pH 7.5. This procedure resulted in apotyrosinase samples still containing 0.1–0.2 g-atom of copper per mol of enzyme, a value found to be independent on the residual enzymatic activity (0.1–1% of the native enzyme). Apotyrosinase was reconstituted by incubating the apoenzyme with a 20-fold molar excess of $CuSO_4$ for 20 h at 4 °C. The specific activity of the reconstituted enzyme was between 80% and 90% of the native enzyme. Prior to enzymatic digestion, holo-and apotyrosinases were freed from methylene blue by gel filtration on Sephadex G-25 in 25 mM ammonium acetate and lyophilized. The copper content of holo- and apotyrosinases was determined by atomic absorption spectrometry (Instrumen-

† From the Biochemisches Institut der Universität Zürich, CH-8028 Zürich, Switzerland. *Received March 12, 1981.* This work was supported by Swiss National Science Foundation Grants 3.018.76 and 3.420.78.

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Table I: Photooxidation of Native Tyrosinase at pH Values between 6.0 and 7.5[a]

pH	activity (%) after 60-min irradiation		mol of histidine/mol of enzyme		copper content (%)	
	without methylene blue	with methylene blue	without methylene blue	with methylene blue	without methylene blue	with methylene blue
6.0	94	95	9.1	9.0	0.27	0.27
6.5	93	94	9.1	8.7	0.27	0.27
7.0	95	93	8.9	8.6	0.27	0.25
7.5	92	93	9.2	8.9	0.26	0.26

[a] Holotyrosinase was photooxidized for 1 h as described under Materials and Methods. Amino acid analysis data are normalized on the basis of 20 arginine, 33 alanine, and 17 lysine residues per molecule of enzyme (Lerch, 1978). Activity of 100% is defined as the value measured before irradiation. A copper content of 0.27% corresponds to two Cu atoms per molecule.

tation Laboratory, Model IL 157). Apotyrosinase which was photooxidized and reconstituted for subsequent copper measurements was treated with a 200-fold molar excess of EDTA[1] for 2 h at 4 °C. The reconstituted samples were then freed from the Cu^{2+}–EDTA complex and methylene blue by gel filtration on Sephadex G-25 in 50 mM sodium phosphate, pH 7.5. Preparation of the cobalt-substituted enzyme was carried out as described previously (Rüegg & Lerch, 1981).

Enzymatic digestions of the polypeptide chain of tyrosinase were performed by established procedures. The resulting peptides were fractionated on a column (0.9 × 20 cm) of Beckman M-72 resin at 55 °C by using pyridine/acetate buffers (Baumann et al., 1975). The peptides were further purified by high-voltage electrophoresis on paper at pH 1.9 or by gel filtration on a Sephadex G-25 fine column (0.9 × 140 cm) equilibrated with 50% acetic acid. Reagents of best grade available were purchased from Fluka AG (Buchs, Switzerland) and Merck AG (Darmstadt, Germany). Trypsin and pepsin were obtained from Worthington and thermolysin from Serva (Munich, Germany). All solutions were prepared with deionized, destilled water and freed from copper by Chelex-100. Acid-washed glassware was utilized in all experiments involving apotyrosinase and copper measurements.

Results

Dye-Sensitized Photooxidation of Holo- and Apotyrosinases. Holotyrosinase was exposed to light in the presence of 0.002% methylene blue and 50 mM sodium phosphate at different pH values and 10 °C. The reaction course was followed by activity measurements and amino acid and copper analyses. After photooxidation at pH values between 6.0 and 7.5, the enzymatic activity of the holoenzyme remained practically unchanged. Also, the histidine and copper contents stayed essentially constant (Table I), demonstrating that the holoenzyme was unaffected by dye-sensitized photooxidation. The quantitative measurement of the amino acids tyrosine, methionine, and tryptophan, which are also susceptible to this reaction, showed that the number of tyrosines remained unchanged whereas one methionine and two tryptophan residues were modified after 1 h of photooxidation. Contrary to the native enzyme, apotyrosinase underwent a progressive loss of reconstitutable enzymatic activity upon dye-sensitized photooxidation. The rate of loss of enzyme activity at different pH values is depicted in Figure 1A. Enzymatic activity of 100% is defined as the value measured in the absence of methylene blue and irradiation following reconstitution of the apoenzyme with Cu^{2+}. From a semilogarithmic plot of these data, it is evident that photoinactivation of apotyrosinase obeys first-order kinetics. The calculated apparent first-order rate constants and their dependency on pH are plotted in Figure 2A. A pK_a value of 7.2, typical for the photochemical oxidation of histidine (Ray, 1967;

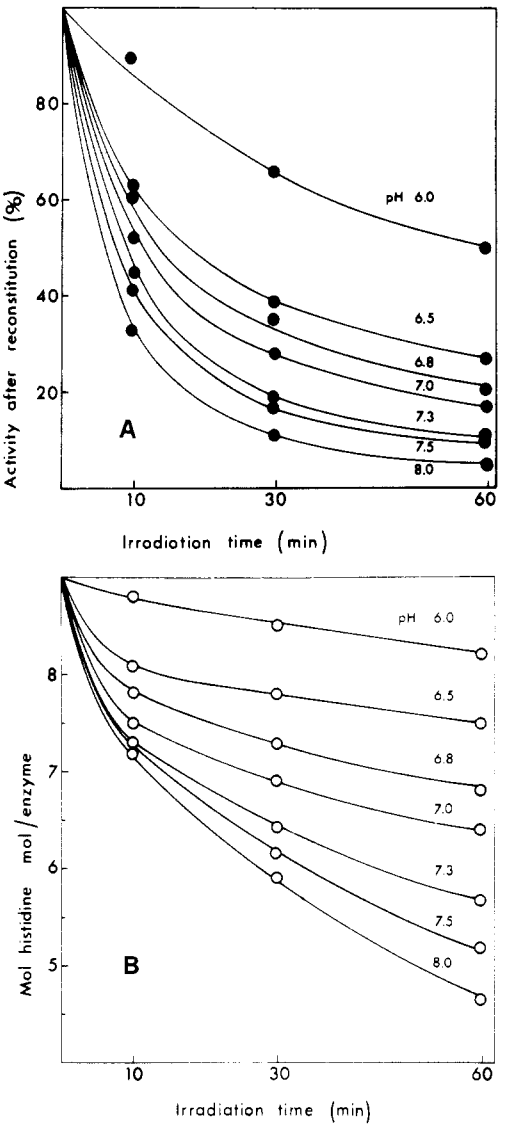

FIGURE 1: (A) Dye-sensitized photooxidation of apotyrosinase in the presence of methylene blue. Relation between the loss of ability of the apoenzyme to be reactivated by Cu^{2+} and time of irradiation at different pH values. Samples were withdrawn at given intervals and assayed for enzymatic activity after reconstitution with excess Cu^{2+}. (B) Histidine content of photooxidized apotyrosinase after 10-, 30-, and 60-min irradiation time. At different pH values, samples were removed at the indicated intervals and subjected to amino acid analyses. The calculation of histidyl residues is based on the same number of amino acids as indicated in Table I.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Ans, 1-anilino-8-naphthalenesulfonate; EPR, electron paramagnetic resonance.

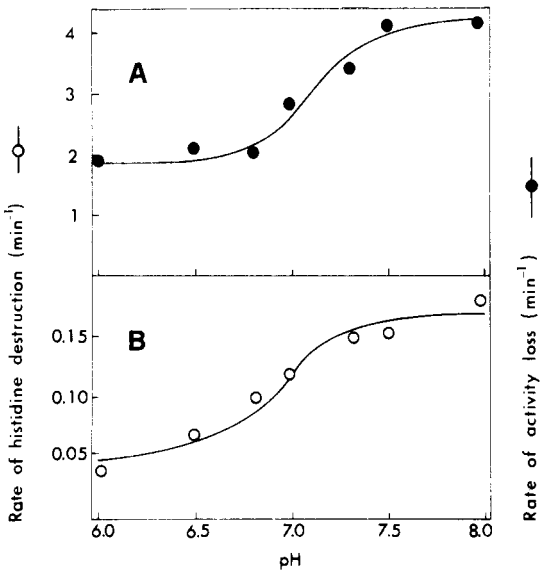

FIGURE 2: Effect of pH on the rate of photooxidation of apotyrosinase. (A) pH profile of the rate of loss of reconstitutable enzymatic activity. (B) pH profile of the rate of destruction of histidyl residues. Experimental conditions as outlined in Figure 1.

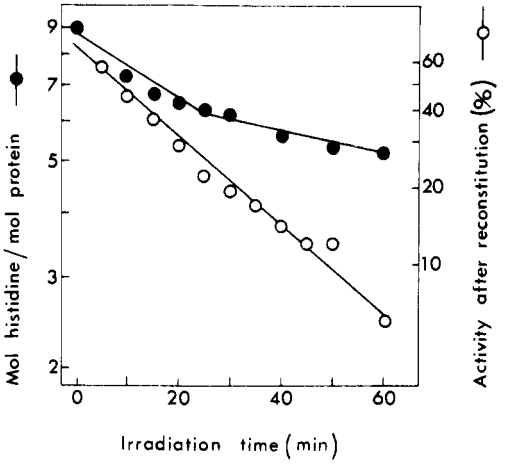

FIGURE 3: Photosensitized oxidation of apotyrosinase at pH 7.5. Semilogarithmic relation between loss of recoverable enzymatic activity after reconstitution with Cu^{2+} (O) between oxidation of histidyl residues (●) and irradiation time. Experimental conditions are outlined under Material and Methods.

Foote, 1968), is observed. Amino acid analyses of the photooxidized apoenzyme demonstrated that the photoinactivation is also associated with the destruction of histidine residues, the histidine content being dependent on the pH at which irradiation occurred (Figure 1B). As expected, the pH dependency of the rate constants of histidine destruction (Figure 2B) was found to be very similar to the one shown in Figure 2A, with an inflection point of around pH 7.0. Since the photoinactivation and the concomitant destruction of histidyl residues of apotyrosinase is optimal at pH 7.5, the reaction course was followed in more detail at this pH value. The loss of ability of the apoenzyme to be reactivated by Cu^{2+} follows first-order kinetics, the rate constant being 2.7 min^{-1} (Figure 3). In contrast, the oxidation of histidine residues appears to be biphasic in nature. The fast phase manifested by the disappearance of approximately three histidines after 25-min irradiation time is characterized by a rate constant of 0.35 min^{-1}. The rate constant of the slow phase was found to be 1 order of magnitude smaller. During the initial fast phase, a close correlation between the loss of histidine and the loss of reconstitutable enzymatic activity is observed. From a plot

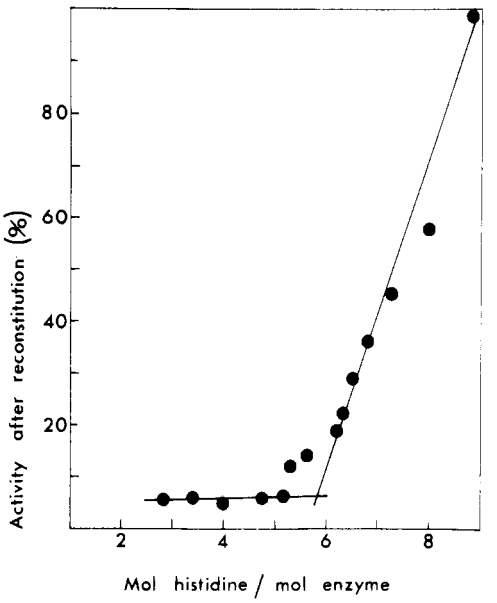

FIGURE 4: Relation between activity of photooxidized apotyrosinase after reconstitution with Cu^{2+} and corresponding loss of histidyl residues after dye-sensitized photooxidation at pH 7.5. Experimental values as obtained in Figure 3.

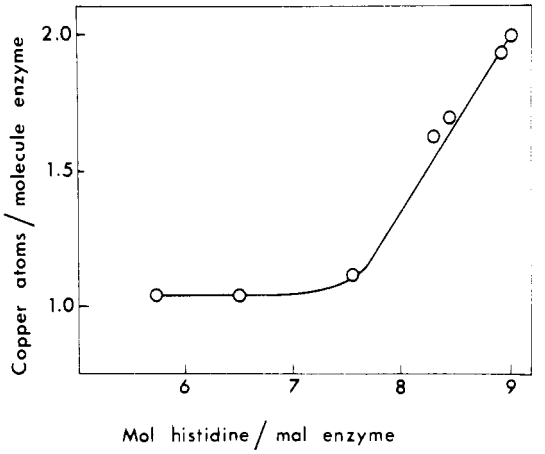

FIGURE 5: Relation between histidine content and ability of copper binding of photooxidized apotyrosinase. Samples were withdrawn at intervals and treated with 20-fold excess of Cu^{2+}. Copper measurements were performed after incubation with EDTA and removal of excess reagents by gel filtration as mentioned under Materials and Methods.

of the enzymatic activity vs. the histidine content (Figure 4), it is apparent that the destruction of these histidines is strongly correlated with the loss of reconstitutability of enzymatic activity. Thus, upon photooxidation of three histidines, more than 85% of the tyrosinase activity is irreversibly lost. Complete amino acid analyses of the photooxidized apoenzyme samples demonstrated that the other residues known to be susceptible to photooxidation, one methionyl and two tryptophyl residues were also modified after 60 min. The same losses were found for the photooxidized holoenzyme, incidently (see above).

Copper Binding of Photooxidizing Apotyrosinase. The copper binding capacity of the photooxidized apoenzyme was assessed after reconstitution with Cu^{2+}, addition of EDTA, and subsequent removal of low molecular weight components by gel filtration. Figure 5 illustrates the decrease of copper binding as a function of the number of histidyl residues destroyed. Initially the copper content decreases linearly and then gradually levels off to reach approximately 1 g-atom of

FIGURE 6: Amino acid sequence of *Neurospora* tyrosinase (Lerch, 1978). One letter amino acid abbreviations [see IUB–IUPAC (1968)]. Cysteinyl residue 94 is linked to histidyl residue 96 via a thioether bridge. Asterisks indicate the histidyl residues lost after dye-sensitized photooxidation of the apoenzyme.

copper per mol of enzyme. The fact that in general slightly more than 1 g-atom of copper is bound possibly reflects uncomplete removal of copper from the holoenzyme (see Materials and Methods).

Photooxidation of Cobalt-Substituted Tyrosinase. From metal substitution experiments, it is known that apotyrosinase binds cobalt with the same stoichiometry as found for copper (Rüegg & Lerch, 1981), yet being enzymatically inactive. In an attempt to study a possible protective effect of this metal ion on the histidyl residues destroyed during photooxidation of apotyrosinase, cobalt-substituted enzyme was treated as described for the native enzyme. Amino acid and metal analyses of the photooxidized cobalt tyrosinase showed the number of histidyl residues and the cobalt content to be constant during an irradiation period of 30 min. Incubation of a sample of photooxidized apotyrosinase with cobalt resulted in binding of but one Co atom per molecule analogous to its reduced ability to bind Cu^{2+} (see above).

Location of the Photooxidized Histidyl Residues in the Primary Structure. The striking difference between holo- and apotyrosinases in the susceptibility of the histidyl residues toward photosensitized oxidation prompted us to investigate the specificity of this reaction by comparing the yields of the histidine-containing peptides of the two forms. The assignments of these peptides and the location of the sequence of the affected residues were greatly facilitated owing to the knowledge of the complete primary structure of *Neurospora* tyrosinase (Lerch, 1978; Figure 6). Samples of holo- and apotyrosinases which had been in vivo labeled with [2,5-$^3H_2$]histidine were photooxidized for 20 min in the presence of methylene blue and digested with pepsin. From a comparison of the ion-exchange elution profiles of the peptide mixtures (Figure 7) the main differences are to be found in position III. The fractions indicated by a bar were pooled and further analyzed by gel filtration on Sephadex G-25. The radioactivity of the eluate from the holoenzyme sample emerged quantitatively as a single peak representing a pure peptide P_3 (residues 274–293) with three histidine residues (residues 277, 281, and 289). In contrast, the yield of this fragment in the apoenzyme sample (Figure 7B) was found to be drastically reduced. These results suggest that at least one

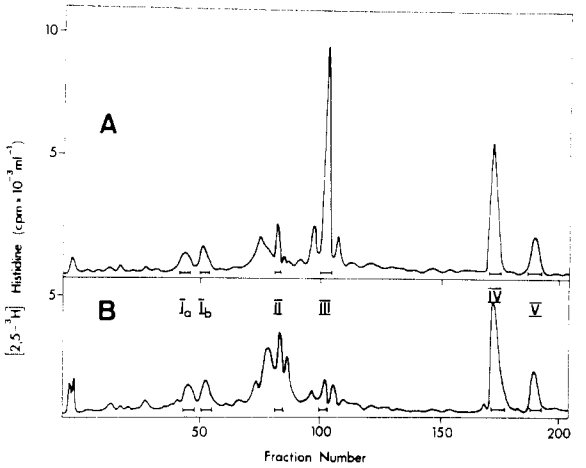

FIGURE 7: Peptide elution profile from a cationic exchange column of a peptic digest of in vivo labeled [2,5-$^3H_2$]histidine holotyrosinase (A) and apotyrosinase (B) after dye-sensitized photooxidation. Fractions marked by horizontal lines were pooled and further purified as indicated under Materials and Methods.

of the three histidyl residues normally present in this peptide was modified by photooxidation. Gel filtration of the fractions eluting in positions Ia and Ib demonstrated the presence of the same petide P_1 (residues 60–81) in both peaks. The peptides in positions Ia and Ib differed, however, in the oxidation state of the single methionine residue. In agreement with its elution behavior, the peptide in fragment Ia contained methionine sulfoxide as compared to unmodified methionine in fragment Ib. From a comparison of the yields of these peptides in photooxidized holo- and apotyrosinases, it can be concluded that histidyl residue 66 is not susceptible to photooxidation in the apoenzyme. Peptide P_2 (residues 91–96) containing histidyl residue 96 eluted in position II with comparable yields in photooxidized holo- and apotyrosinases. This histidyl residue which is linked in an unusual way via a thioether to cysteinyl residue 94 is therefore considered to be also nonsusceptible toward photoinactivation. High-voltage electrophoresis at pH 6.5 of the fractions eluting in position IV revealed a mixture of two histidine-containing peptides in comparable yields. Peptide P_{4a} with a single histidyl residue (residue 105) and peptide P_{4b} containing two histidyl residues (residues 305–306) were unambiguously assigned in the sequence spanning residues 102–111 and 303–309, respectively. The fragment P_5 eluting in position V was identified as an overlap peptide of P_{4b} extended by three amino acid residues (residues 310–312). These data suggest that histidyl residues 105, 305, and 306 are resistant toward photooxidation.

The destiny of the remaining five histidyl residues with respect to their susceptibility toward photooxidation was explored by cleavage of the photooxidized apoenzyme and the native protein with thermolysin and trypsin. The elution profile of the thermolytic digest of photooxidized apotyrosinase superimposed on the one of the native enzyme is shown in Figure 8. The two histidine-containing peptides Th 1 (residues 273–279) and Th 4 (residues 280–283) were found to be present in comparable yields in both samples. Histidyl residues 277 of peptide Th 1 and 281 of peptide Th 4 are therefore considered to be resistant to photochemical modification. As already shown, these two residues are present together with histidyl residue 289 in the peptic fragment eluting in position III (see Figure 7), which has been demonstrated to be drastically reduced in the apoenzyme sample. These data thus imply that histidyl residue 289 of the peptic peptide (residues 247–293) has been destroyed during photosensitized oxidation of the apoenzyme. The yield of peptide Th 2 (residues 65-69)

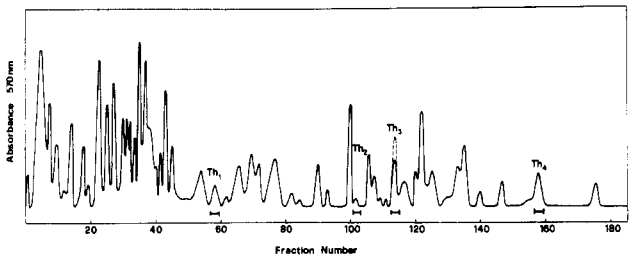

FIGURE 8: Peptide elution profile from a cationic exchange column of a thermolytic digest of photooxidized apotyrosinase (—) and native enzyme (---). Fractions marked by a horizontal bar were pooled and processed under Materials and Methods.

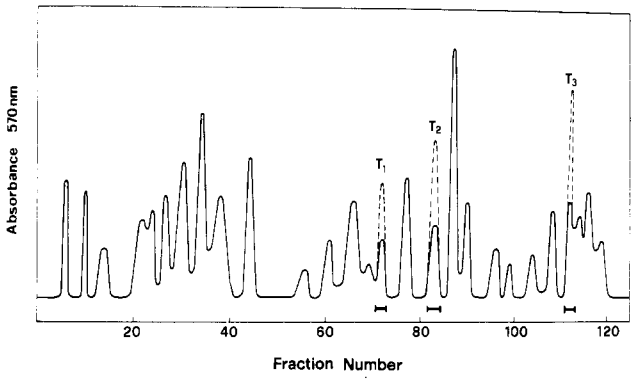

FIGURE 9: Chromatographic fractionation of a tryptic digest on a cation-exchange resin of photooxidized apotyrosinase (—) and native enzyme (---). Fractions indicated by a horizontal bar were pooled and further purified as outlined under Materials and Methods.

was shown to be essentially the same for the holo- and apoenzyme samples, confirming the resistance of histidyl residue 66 toward photooxidation (compare fractions Ia and Ib of Figure 7). Strong evidence for the photochemical oxidation of histidyl residue 193 was obtained from the lack of peptide Th 3 (residues 192–194) in the apoenzyme sample. This result is further supported by a sharply decreased yield of tryptic peptide T_2 (residues 190–208) of the apoenzyme shown in Figure 9. A striking difference in the elution profiles of the tryptic digests of photooxidized apotyrosinase and the native enzyme is in addition seen for peptide T_3 (residues 182–189), suggesting that histidyl residue 188 is destroyed during photosensitized oxidation of apotyrosinase. The decreased yield of peptide T_1 (residues 261–283) containing histidyl residues 277 and 281, already shown to be resistant to photooxidation in the apoenzyme, is assumed to arise from photochemical oxidation of tryptophyl residue 261. The data presented in this section are summarized in Table II, emphasizing the preferential modification of the three histidyl residues His-188, His-193, and His-289 (marked as italics in Table II and by asterisks in Figure 6) during dye-sensitized photooxidation of apotyrosinase.

Discussion

When native tyrosinase is subjected to dye-sensitized photo oxidation in the presence of methylene blue, no change in enzymatic activity is observed. In contrast under the same conditions, apotyrosinase gradually looses its ability to be reactivated with Cu^{2+} in parallel with the destruction of histidine residues. The rate of photoinactivation was found to be strongly pH dependent with an apparent pK_a of 7.0, characteristic for the oxidation of histidine residues (Ray, 1967; Foote, 1968). At pH 7.5, three histidine residues were destroyed in an initial fast reaction, with a concomitant decrease of activity of more than 90%. This destruction of histidine

Table II: Summary of Histidine-Containing Peptides Characterized after Peptic (P), Thermolytic (Th), and Tryptic (T) Digestion of Photooxidized Apo- and Holotyrosinases[a]

sequence position of histidine-containing peptide	yield of histidine-containing peptide in apotyrosinase[b]	sequence position of histidyl residues
P_1 (60–81)	100	66
P_2 (91–96)	95	96
P_3 (274–293)	8	277, 281, *289*[c]
P_{4a} (102–111)	84	105
P_{4b} (303–309)	85	305, 306
P_5 (303–312)	83	305, 306
Th_1 (273–279)	100	277
Th_2 (65–69)	90	66
Th_3 (280–283)	95	281
Th_4 (192–194)	5	*193*
T_1 (261–283)	60	277, 281[d]
T_2 (190–208)	8	*193*
T_3 (182–189)	6	*188*

[a] Peptic peptides derived from photooxidized in vivo labeled [2,5-$^3H_2$]histidine holo- and apotyrosinases. Thermolytic and tryptic peptides were obtained from photooxidized apotyrosinase and native enzyme. [b] Compared to the yield of photooxidized holotyrosinase (peptic peptides) and of the native enzyme (thermolytic and tryptic peptides) taken as 100%. [c] Sequence positions of histidyl residues destroyed during photochemical oxidation are given in italics. [d] Decreased yield is ascribed to photosensitized oxidation of tryptophan residue 261.

is further accompanied by the binding capability of one of the two active-site copper atoms. Since the histidine content of the holoenzyme remained constant during photooxidation, it is suggested that the three histidine residues destroyed during the initial fast phase serve as ligands to one of the two copper atoms of *Neurospora* tyrosinase. This conjecture is further supported by the resistance of the cobalt-substituted enzyme toward dye-sensitized photooxidation. Similar studies had shown previously that chelation of Zn^{2+} and Co^{2+} protected histidine from photooxidation when bound to the protein active centers of superoxide dismutase (Forman et al., 1973) and alkaline phosphatase (Tait & Vallee, 1966). The second phase of dye-sensitized photooxidation of apotyrosinase is characterized by a rate of destruction of histidine residues 1 order of magnitude smaller than the one in the initial phase. It is possible that some of these residues represent metal ligands to the second copper atom; however, quantitative data were difficult to obtain because of the accompanying increase in protein denaturation. In contrast to these results, a recent investigation of the photooxidation of mushroom and *Neurospora* tyrosinase (Gutteridge et al., 1977) showed an inactivation of the holoenzyme and an acceleration of the photooxidation rate by the addition of cyanide. The different reaction conditions used in that study and the presence of cyanide leading to apotyrosinase may at least partially explain the apparent discrepancies.

In addition to the photochemical alterations of histidine residues, dye-sensitized photooxidation of proteins can result in the destruction of tyrosine, methionine, tryptophan, and cysteine residues (Ray, 1967). Upon irradiation of holo- and apotyrosinases, tyrosine residues were shown to be completely unaffected. These findings are in agreement with the results obtained from chemical modification studies with N-acetylimidazole and tetranitromethane, demonstrating a complete lack of reaction with both enzyme forms (E. Pfiffner and K. Lerch, unpublished results). These data suggest that the tyrosyl residues are buried in *Neurospora* tyrosinase, consistent with the observation that freshly isolated tyrosinase is com-

pletely devoid of autooxidized tyrosine residues. Such oxidation reactions leading to the formation of *o*-quinones have been observed to occur in the presence of tyrosinase in a number of peptides and proteins with exposed tyrosine residues (Sizer, 1953; Yasunobu et al., 1959; Cory & Frieden, 1967a,b). Upon photooxidation of holo- and apotyrosinases, a methionine and two tryptophan residues were found to be destroyed in both forms. Since the enzymatic activity and the copper content of the holoenzyme remained unchanged, these residues are thought to be unimportant for the copper binding of apotyrosinase. This conjecture is further supported by chemical modification of methionine and tryptophan with *N*-chlorosuccinimide (Schechter et al., 1975). This treatment had no influence on the extent of reconstitution of apotyrosinase with Cu^{2+} as compared to a control sample. The sole cysteinyl residue (residue 94) found in *Neurospora* tyrosinase which is linked in a rather unusual fashion via a thioether bridge to histidyl residue 96 is resistant toward photochemical oxidation. Hence, by exclusion, by the pH dependency of the photosensitized inactivation of apotyrosinase and the protein chemical data discussed above, it is most likely that the destruction of the histidyl residues is responsible for the partial abolition of copper binding capacity and of the ensuing loss of enzymatic activity.

A major objective of this work was to explore the reaction specificity of photooxidation with respect to each single histidine residue of *Neurospora* tyrosinase. A comparison of the yields of the histidine-containing peptic, thermolytic, and tryptic peptides of photooxidized apotyrosinase with those of the native and photooxidized holoenzyme revealed the preferential destruction of histidyl residues 188, 193, and 289. The destiny of the three photooxidized histidyl residues has not been elucidated as yet; indeed, a direct identification of the peptides involved is expected to be rather difficult as a consequence of a large number of reaction products generated during photochemical oxidation of the imidazole nucleus (Tomita et al. 1969). In a similar study aimed to identify histidyl residues of the much simpler enzyme pancreatic ribonuclease that were destroyed by photooxidation, the inherent difficulties of such an undertaking were emphasized by Kenkare & Richards (1966).

Ethoxyformic anhydride is known as a relatively specific reagent for the modification of histidyl residues at pH 6.0 (Melchior & Fahrney, 1970). As expected from the photooxidation data, the holoenzyme was unaffected by the treatment with this reagent. The exposure of the apoenzyme to ethoxyformic anhydride at concentrations up to 100 mM, however, had no influence at all on its ability to be reconstituted with Cu^{2+}. This surprising result suggests that the three histidyl residues destroyed upon photosensitized oxidation must be located in a rather peculiar microenvironment.

In a similar study on the photoxidation and ethoxyformylation of cytoplasmic aspartate aminotransferase by Polidoro et al. (1976), these authors have argued that the photooxidized histidyl residues which are also resistant toward modification with ethoxyformic anhydride might be located in a hydrophobic pocket. A strong argument in favor of a hydrophobic environment at the copper binding site of *Neurospora* tyrosinase comes from recent fluorescence measurements with Ans[1] (M. Beltramini and K. Lerch, unpublished results). In these studies, apotyrosinase was found to yield an Ans complex with Fluorescence properties very similar to the ones reported for hemocyanin (Ricchelli & Salvato, 1979). Since both copper proteins are capable of binding molecular oxygen reversibly with the same stoichiometry in a very similar fashion (Jolley et al., 1974; Lerch, 1976; Freedman et al., 1976; Eickman et al., 1978; Himmelwright et al., 1980), the hydrophobic character of the metal binding site of tyrosinase and hemocyanin could be of prime importance for the stability of their oxygen complexes as discussed earlier for oxymyoglobin and oxyhemoglobin (Wang, 1970; Perutz et al., 1968).

The involvement of histidyl residues as ligands to the active-site copper of tyrosinase has been invoked also on the basis of physicochemical evidence. An EPR[1] study of nitric oxide treated mushroom tyrosinase indicated the occurence of at least two nitrogen ligands per copper atom (Schoot Uiterkamp & Mason, 1973). In a recent resonance Raman study of *Neurospora* oxytyrosinase, the observed bands at low frequency were tentatively assigned to copper–imidazole vibrations in analogy to hemocyanin from different sources (Eickman et al., 1978). A very detailed view of the structure of the binuclear copper complex of oxyhemocyanin from *Busycon canaliculatum* has emerged recently by a combination of extended X-ray absorption fine structure analysis and resonance Raman spectroscopy (Larrabee & Spiro, 1980; Brown et al., 1980). From these data, a model of the oxygen binding site with each of the two copper ions containing three histidyl ligands and the metal ions being bridged by the bound peroxide has been proposed. With respect to one of the two copper ions bound to *Neurospora* tyrosinase, this model is supported by the present results of the photosensitized oxidation of three histidyl residues assigned to positions 188, 193, and 289 in the primary structure. Concerning the ligands of the second copper atom in *Neurospora* tyrosinase, the involvement of histidyl residues is not only plausible on the basis of the hemocyanin model but is also supported from active-site-directed modification of the native enzyme with catechol (Dietler & Lerch, 1981). The irreversible inactivation of the enzyme with catechol as a substrate was shown to be accompanied by a concurrent loss of one histidyl residue and one copper atom. Peptide analysis similar to that described here revealed a highly selective modification of histidyl residue 306. Although it has not been conclusively shown yet that the copper lost during reaction inactivation is identical with the one still capable of binding after dye-sensitized photooxidation of apotyrosinase, it is tempting to speculate that histidyl residues 188, 193, and 289 constitute the protein ligands of the first copper atom and histidyl residue 306 participates in the binding of the second copper atom of the binuclear complex of *Neurospora* tyrosinase.

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Purification and Comparative Study of Glyceraldehyde-3-phosphate Dehydrogenase from the Muscles of Young and Old Rats†

Ari Gafni

ABSTRACT: D-Glyceraldehyde-3-phosphate dehydrogenase (GPDH) was purified from the muscles of young and old rats. A marked difference was found between the (total) activities of these two enzyme preparations which originates in their different specific activities, while the concentrations of the enzyme in young and old tissues appear to be similar. Both "young" and "old" enzyme forms show four rapidly reacting sulfhydryl groups while six additional SH groups are revealed upon longer incubation with the sulfhydryl reagent. The UV absorption spectra and sedimentation coefficients of young and old GPDH molecules are also identical, and while the two enzyme forms partially dissociate into dimers in the presence of sodium chloride, the old enzyme appears to be more dissociable. The amino acid compositions of the GPDH molecules purified from young and old rats are remarkably alike and show a great similarity to the compositions of GPDH molecules from other mammals. Small differences in composition may, however, have escaped detection due to accuracy limitations of the determination and could be responsible for the differences in enzymatic activity. Alternatively, the activity differences may originate in postsynthetic conformational changes induced in the old enzyme during its longer "dwell time" in the tissue.

Aging phenomena at the molecular level have begun to attract attention in recent years when it was found that many enzymes lose part of their (specific) activity in old animals [for recent reviews on the subject, see Dreyfus et al. (1978) and Rothstein (1977)]. Only a few of the studies were devoted to aging of enzymes from mammalian tissues, some examples being mouse and rat liver aldolase (Gershon & Gershon, 1973; Weber et al., 1976), glucose-6-phosphatase (Grinna & Barber, 1975), rat liver superoxide dismutase (Reiss & Gershon, 1976), rat heart enolase (Rothstein et al., 1980), and rat muscle phosphoglycerate kinase (Sharma et al., 1980). Some enzymes, on the other hand, were found to retain their full activity in old animals (Gupta & Rothstein, 1976; Petell & Lebherz, 1979; Steinhagen-Thiessen & Hilz, 1976; Yagil, 1976), proving that altered enzymes are not necessarily concomitant of aging.

While the partial loss of enzymatic activity is common to all enzymes that are affected by aging, changes in other properties are less consistent. In some cases, a component with increased heat sensitivity was found (Reiss & Gershon, 1976; Bolla & Brot, 1975; Epstein & Gershon, 1972). Values of

† From the Department of Chemical Physics, The Weizmann Institute of Science, Rehovot 76100, Israel. *Received March 12, 1981.* This work was supported in part by a grant from the Israel Commission for Basic Research.

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