Correction. In the article "Amino acid sequence of tyrosinase from Neurospora crassa" by Konrad Lerch, which appeared in the August 1978 issue of Proc. Natl. Acad. Sci. USA (75, 3635–3639), the author requests that the following correction be noted. In the sequence, residue 94 is cysteine and residue 96 is histidine (instead of vice versa). Appropriate changes should be made in lines 10 and 11 of the Abstract, in Fig. 2, and in lines 19 and 37 of the left-hand column on p. 3638.

Correction. In the article "Methylation of Herpesvirus saimiri DNA in lymphoid tumor cell lines" by Ronald C. Desrosiers, Carel Mulder, and Bernhard Fleckenstein, which appeared in the August 1979 issue of Proc. Natl. Acad. Sci. USA (76, 3839–3843), two errors occurred in the Proceedings editorial office. In the right column on p. 3839, the sentence beginning on line 24 should read "The dinucleotide C-G is . . . ." In the footnote on p. 3840, the third name should be C. Mulder.
Amino acid sequence of tyrosinase from *Neurospora crassa*

(phenoloxidase/copper monooxygenase/protein primary structure)

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Communicated by Norman H. Horowitz, May 12, 1978

ABSTRACT  The amino-acid sequence of tyrosinase from *Neurospora crassa* (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is reported. This copper-containing oxidase consists of a single polypeptide chain of 407 amino acids. The primary structure was determined by automated and manual sequence analysis on fragments produced by cleavage with cyanogen bromide and on peptides obtained by digestion with trypsin, peptic, thermolysin, or chymotrypsin. The amino terminus of the protein is acetylated and the single cysteiny1 residue 96 is covalently linked via a thioether bridge to histidyl residue 94. The formation and the possible role of this unusual structure in *Neurospora* tyrosinase is discussed. Dyesensitized photooxidation of apotyrosinase and active-site-directed inactivation of the native enzyme indicate the possible involvement of histidyl residues 188, 192, 289, and 305 or 306 as ligands to the active-site copper as well as in the catalytic mechanism of this monooxygenase.

Tyrosinase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing mixed-function oxidase catalyzing the o-hydroxylation of monophenols and the oxidation of o-diphenols to o-quinones (1). The enzyme is found in different microorganisms, plants, and animals, and its prime function is directed towards formation of pigments such as melanins and other polyphenolic compounds (2, 3). Highly purified enzyme preparations have been obtained from a large variety of sources (3), but information on the structure of this oxidase is scant due to its molecular heterogeneity. The enzyme from the mushroom *Agaricus bispora* has been reported to contain a contiguous copper pair at the active site on the basis of electron paramagnetic resonance measurements of the NO complex (4), magnetic susceptibility studies (5), and the stoichiometric reaction of one H2O2 per two copper ions (6). A recent study on the quaternary structure of this tyrosinase revealed the presence of two types of polypeptide chains with molecular weights of 45,000 and 13,400 (7). The distribution of the copper between these two subunits is not known.

Different wild-type strains of the ascomycete *Neurospora crassa* have been reported to produce allelic forms of a tyrosinase with a molecular weight of 33,000 and containing only one mole of copper per mole of enzyme (8, 9). In a reinvestigation it was shown, however, that this tyrosinase contains one contiguous copper pair per functional unit of 44,000 daltons (10, 11). As a further step towards a better understanding of the structure and function of this copper monooxygenase, a study of its amino acid sequence was undertaken. Tyrosinase from *Neurospora crassa* was found especially suitable for the reasons that it can be obtained in sizable amounts, that it has allelic forms differing in thermostability and electrophoretic mobility (12), and that it has been reported to crystallize (8).

In this communication the complete amino acid sequence of the thermolabile form of *Neurospora* tyrosinase is presented. In addition, the involvement of histidyl residues as ligands to the active-site copper is examined by chemical modification studies.

MATERIALS AND METHODS

*Neurospora crassa* wild-type strain (Fungal Genetic Stock Center no. 320, Arcata, CA) producing the thermolabile form of tyrosinase was grown according to Horowitz et al. (13). The enzyme from cytochrome-derpressed cultures was isolated as described previously (10). The protein was cleaved with cyanogen bromide in 75% trifluoroacetic acid or in 70% (vol/vol) formic acid. The resulting fragments were separated by gel filtration on Sephadex G-100 in 7% formic acid. The smaller fragments were further purified by repeated gel chromatography on Sephadex G-50 superfine. The purity and approximate size of the fragments were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by gel filtration in 6 M guanidine hydrochloride (10). Enzymatic digestions of the whole polypeptide chain and the cyanogen bromide fragments were performed by established procedures. Specific cleavage at arginyl residues was accomplished after maleylation of the ϵ-amino groups of lysyl residues (14). The resulting peptides were fractionated on a column (0.9 × 20 cm) of Beckman M-72 resin at 55° by using pyridine/acetate buffers. The peptides were further purified by high-voltage electrophoresis on paper at pH 1.9 or 6.5 or by gel filtration on a Sephadex G-25 fine column (0.9 × 140 cm) equilibrated with 50% (wt/vol) acetic acid. Depending on the size and composition of the purified peptides, sequence analyses were carried out by either automated or manual Edman degradation techniques. Automated sequence analysis was performed on a Beckman sequencer model 890 B (updated) using the Edman and Begg procedure (15) as modified by Hermanson et al. (16). The phenylthiohydantoin derivatives were identified by gas chromatography (16, 17), by thin-layer chromatography (15, 17), and by amino acid analysis after reconversion to the free amino acids by hydrolysis with H1 (18) or with 5.7 M HCl containing 0.1% SnCl2 (19). Amino acid analyses were performed on a Durrum (model D-500) amino acid analyzer. 35S-Labeled tyrosinase was isolated from a culture grown on a [35S]sulfate-supplemented medium. Dyesensitized photooxidation of holo- and apotyrosinase was carried out according to Forman et al. (20). Apotyrosinase was prepared by treating the holoenzyme (100 μM) with KCN (100 mM) and subsequent gel filtration on Sephadex G-25 in 50 mM sodium phosphate, pH 7.5. Apotyrosinase was reconstituted by incubating the apoenzyme with a 20-fold excess of CuSO4 for 20 hr at 4°. The specific activity of the reconstituted enzyme varied between 80 and 90% of that of the native enzyme. Inactivation of tyrosinase (0.175 μM) using catechol (1 mM) as a substrate was carried out in the presence of excess ascorbate (10 mM) at 25° in oxygen-saturated 10 mM sodium phosphate, pH 6.0.

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RESULTS AND DISCUSSION

The strategy of the amino acid sequence determination of *Neurospora* tyrosinase is outlined schematically in Fig. 1. Cleavage of the protein with cyanogen bromide yielded four major fragments and one minor overlap peptide due to incomplete cleavage of a methionyl-threonyl bond. Automatic sequence analysis of the cyanogen bromide fragments CB1, CB2, and CB4 allowed an unambiguous sequence determination of the first 25 to 30 residues, whereas CB3 turned out to be blocked by an acetyl group, previously identified by mass spectrometry (21). The amino-terminal sequence of the overlap fragment CB2-4 was found to be identical with the one of CB4 and because fragment CB2 lacks homoserine the four primary cyanogen bromide fragments were aligned in the order CB5-CB1-CB4-CB2 (Fig. 1). Subdigestion of the cyanogen bromide fragments CB3, CB1, and CB2 with trypsin allowed the isolation in pure form of all expected tryptic peptides with the exception of the amino-terminal fragment of CB1, which showed heterogeneity due to aggregation. Automated and manual Edman degradation of the intact tryptic peptides furnished more than 50% of the sequence information as depicted by the stippled areas in Fig. 1.

Cleavage of the maleylated protein with trypsin yielded besides a number of already known tryptic peptides four new fragments TM1, TM2, TM3, and TM4 (Fig. 1). Automated sequence analysis of these fragments permitted the alignment of eight small tryptic peptides and confirmed the position of the cyanogen bromide fragments CB1 and CB3.

The cleavage points of pepsin-treated tyrosinase are shown in the bottom bar of Fig. 1. Automated and manual sequence analysis of the peptic peptides provided the majority of the overlaps of the tryptic fragments. Subdigestion of the four primary cyanogen bromide fragments with thermolysin and of CB1 with α-chymotrypsin (not shown in Fig. 1) provided the remaining overlaps permitted to align unequivocally all the tryptic and peptic fragments.

The complete amino acid sequence of the 407 residues of *Neurospora* tyrosinase is shown in Fig. 2. The amino-acid composition calculated from the sequence is 24 Asp, 20 Asn, 22 Thr, 42 Ser, 17 Glu, 17 Gln, 31 Pro, 25 Gly, 33 Ala, 1 Cys, 23 Val, 3 Met, 13 Ile, 33 Leu, 20 Tyr, 24 Phe, 12 Trp, 10 His, 17 Lys, and 20 Arg. This composition is in good agreement with the one reported by Fling et al. (8) if corrected for the larger molecular weight of 46,000. However, in contrast to previous
Ac-Ser-Thr-Asp-Ile-Lys-Phe-Ala-Ile-Thr-Gly-Val-Pro-Thr-Thr-Pro-Ser-Ser-Asn-Gly-Ala-Val-Pro-Leu-Arg-Arg-Glu-Leu-Arg-Asp-Leu


Gln-Val-Ala-Gly-Ile-His-Gly-Met-Pro-Phe-Lys-Pro-Trp-Ala-Gly-Val-Pro-Ser-Asp-Thr-Asp-Trp-Ser-Gln-Pro-Gly-Ser-Ser-Gly-Phe

Gly-Gly-Tyr-His-Thr-Cys-Ser-Ser-Ile-Leu-Phe-Ile-Thr-Trp-His-Arg-Pro-Tyr-Leu-Ala-Leu-Tyr-Glu-Gln-Ala-Leu-Tyr-Ala-Ser-Val

Gln-Ala-Val-Ala-Gln-Lys-Phe-Pro-Val-Glu-Gly-Gly-Leu-Arg-Ala-Lys-Tyr-Ala-Ala-Ala-Lys-Asp-Phe-Arg-Ala-Pro-Tyr-Phe-Asp

Trp-Ala-Ser-Gln-Pro-Pro-Lys-Gly-Thr-Leu-Ala-Phe-Pro-Glu-Ser-Leu-Ser-Ser-Arg-Thr-Ile-Gln-Val-Val-Asp-Val-Asp-Gly-Lys-Thr

Lys-Ser-Ile-Asn-Asn-Pro-Leu-His-Arg-Phe-Thr-Phe-His-Pro-Val-Asn-Pro-Ser-Pro-Gly-Asn-Phe-Ser-Ala-Ala-Asp-Val-Asp-Arg-Tyr-Pro

Ser-Thr-Val-Arg-Tyr-Pro-Asn-Arg-Leu-Thr-Gly-Ala-Ser-Arg-Asp-Glu-Arg-Ile-Ala-Pro-Ile-Leu-Ala-Asp-Glu-Leu-Ala-Ser-Arg

Asn-Asn-Val-Ser-Leu-Leu-Leu-Ser-Ser-Leu-Thr-Lys-Asp-Phe-Asp-Ala-Phe-Ser-Tyr-Asn-Arg-Trp-Asp-Pro-Asn-Thr-Asn-Pro-Gly-Asp-Phe

Gly-Ser-Leu-Glu-Asp-Val-Asn-Glu-Ile-Asp-Arg-Thr-Gly-Gly-Gly-His-Met-Ser-Ser-Leu-Glu-Val-Ser-Ala-Phe-Asp-Pro

Leu-Phe-Trp-Leu-His-Val-Asn-Ala-Ser-Arg-Leu-Trp-Ser-Ile-Trp-Gln-Asp-Leu-Pro-Asn-Arg-Asp-Met-Thr-Pro-Arg-Pro-Ala

Pro-Tyr-Thr-Phe-Val-Ala-Gln-Glu-Gly-Glu-Asp-Gln-Ser-Lys-Ser-Thr-Pro-Leu-Glu-Pro-Phe-Trp-Asp-Lys-Ser-Ala-Ala-Asp-Phe

Trp-Thr-Ser-Glu-Gln-Val-Lys-Asp-Ala-Asn-Arg-Thr-Leu-Ser-Glu-Gly-Tyr-Ala-Tyr-Pro-Glu-Thr-Gln-Lys-Trp-Lys-Tyr-Ser-Ser-Val-Lys-Glu-Tyr

Gln-Ala-Ala-Ile-Arg-Lys-Ser-Leu-Val-Thr-Ala-Leu-Tyr-Gly-Ser-Asn-Val-Phe

**Fig. 2.** Amino acid sequence of *Neurospora* tyrosinase. Histidyl residue 94 is covalently linked to the cysteiny1 residue 96 via a thioether bridge. Asterisks (*) denote tentative identifications of histidyl residue destroyed after dye-sensitized photooxidation of the apoenzyme and the pluses (+) indicate the histidyl residues lost after active-site directed inactivation of the native enzyme (see text).
indications (8, 22) that methionine is the sole sulfur-containing amino acid in Neurospora tyrosinase, the enzyme was found to contain also a single chemically modified cysteine residue. Digestion of in vivo labeled [35S]tyrosinase with pepsin resulted, in addition to the three methionine peptides, in a fourth radioactive peptide with the amino acid composition 0.95 Thr, 2.00 Gly, 0.91 Tyr, and an unknown ninhydrin-positive, radioactive species "X" eluting just after aspartic acid. The yield and the specific radioactivity of this peptide was identical with that of the three methionine peptides, thus strongly suggesting the presence of a modified sulfur-containing residue in this peptide.

The tryptic digestion of the peptic fragment yielded two peptides: the tripeptide Gly-Gly-Tyr (residues 91–94, Fig. 2) and a new radioactive fragment yielding only Thr and the unknown species X after acid hydrolysis. The structure of this neutral peptide, which showed a strong absorption at 255 nm (pH 2.0) was identified by mass spectrometry (L. Witte, K. Lerch, and H. Nau, unpublished data) as His-Thr-Cys with a thioether linkage between the cysteylnyl- and the histidyl residue. The presence of a thioether bond in this peptide was confirmed by its cleavage with Ag2SO4 (23) and the subsequent identification of cysteine as cysteic acid.

While the circumstances that lead to the formation of a histidylcysteine thioether bridge in Neurospora tyrosinase remain to be elucidated, it is most likely that this unusual crosslink is an inherent structural feature of this enzyme, generated in a post-translational event. In this context, it is of interest that Neurospora tyrosinase has been reported by Fox and Burnett (24) to be present in crude extracts in both an active and an inactive form (protyrosinase). From kinetic studies of the activation of protyrosinase, these authors postulated that this process may involve an intramolecular rearrangement catalyzed by an activating enzyme. In the light of the present findings, the activation of protyrosinase could be visualized to be brought about by an enzymatic formation of the thioether linkage between histidyl residue 94 and cysteylnyl residue 96. It is also conceivable that this unusual structure in Neurospora tyrosinase could be involved in the binding of the active-site copper or serve a functional role during the catalytic cycle of this monoxygenase.

A remarkable feature of Neurospora tyrosinase is its relatively high content of Pro, Gly, and Asn residues, which are known to occur preferentially in β-turn structures (25). A predictive analysis of the secondary structure carried out according to the rules of Chou and Fasman (26) indicates the presence of 45 β turns. α-Helical segments were predicted in residues 23–32, 51–56, 109–127, 138–146, 157–193, 202–208, 231–240, 243–256, 273–283, 292–298, 301–307, 334–339, 352–367, 378–383, and 391–396. β-Sheet regions were found to occur in residues 6–11, 38–45, 60–65, 92–96, 99–104, 159–162, 170–174, 312–317, 324–326, 370–376, and 396–400. On the basis of these calculations the secondary structure may be described as follows: 34% α helix, 15% β sheet, 31% β turn, and 20% coil structure. These values are close to the average of seven globular proteins of known x-ray structure (25), suggesting a globular conformation of this protein.

The involvement of histidyl residues as ligands in metalloproteins has been amply documented (27). Because imidazole groups are regarded as “valence-non-specific” copper ligands, histidyl residues have also been proposed to play an important role in the metal binding of the redox-active copper proteins (28). With the exception of superoxide dismutase (29) and plastocyanin (30), where the three-dimensional structures are known, the participation of histidyl residues as ligands in such proteins has been deduced mainly from electron paramagnetic and nuclear magnetic resonance data (31, 32). Two nitrogen groups have been implicated in the binding of the copper in mushroom tyrosinase on the basis of the electron paramagnetic resonance superhyperfine structure of the NO complex (4). The possible involvement of histidyl residues in the binding of the active site copper of Neurospora tyrosinase has also received support from dye-sensitized photoinactivation studies documented in Fig. 3 (data of E. Pfiffner and K. Lerch, to be published in more detail elsewhere). The activity of the holoenzyme is essentially unaffected by photooxidation in the presence of methylene blue, whereas the apoenzyme undergoes a progressive loss of its ability to be reactivated with Cu2+. The rate of disappearance of histidyl residues in the apoenzyme closely followed the rate of loss of the ability of being reactivated with Cu2+. In contrast, the number of histidyl residues in the holoenzyme remained unchanged during photoinactivation. These results taken together with the finding that the rate of photoinactivation depends strongly on pH (apparent pK 6.9) imply a participation of histidyl residues in the binding of the active-site copper. Complete amino acid analysis of the photoinactivated enzyme indicated no change in the tyrosine content, but one methionyl and one to two tryptophyl residues were destroyed by photooxidation after 60 min. However, chemical modification of the apoenzyme with N-chlorosuccinimide, a reagent known to specifically oxidize methionyl and tryptophyl residues (33), had no influence on its ability to be reactivated with Cu2+. The above-mentioned cysteinyl-histidine structure was also unaffected by photooxidation, as shown by amino acid analysis of the peptic peptide containing residues 91–96 (Fig. 2).

In order to find out if specific histidyl residues were destroyed during photooxidation, the modified protein was digested with pepsin, thermolysin, and trypsin, and the resulting peptides were separated on a cationic exchange resin as outlined in Materials and Methods. From a comparison of the yields of the different histidine-containing peptides, it appears that histidyl residues 188, 193, and 289 (indicated by asterisks in Fig. 2) were destroyed during photoinactivation of the apotyrosinase.

In superoxide dismutase the involvement of histidyl residues in the binding of copper has been suggested on the basis of

**FIG. 3.** Dye-sensitized photooxidation of holo- and apotyrosinase. Reaction mixtures containing 60 μM methylene blue, 50 mM sodium phosphate buffer at pH 7.5, and 1.5 mg of holo- or apotyrosinase in 3.0 ml at 10° were exposed to a 250-W tungsten lamp at a distance of 30 cm. Samples were removed at different times for amino acid analysis and activity measurements. α, Mol histidine per mol holoenzyme; Δ, mol histidine per mol apoenzyme; ω, percent tyrosinase activity of holoenzyme; ω, percent tyrosinase activity of the apoenzyme after reconstitution with Cu2+. 
analogs photoinactivation experiments (20). These results have subsequently been confirmed by x-ray-diffraction analysis, which showed that the copper is indeed bound to four histidyl residues (29). It is therefore tempting to speculate that the three histidyl residues destroyed during photooxidation of apotyro- sinase also correspond to the copper-binding ligands; of course the possibility that an indirect conformational change in the enzyme accompanying the removal of the copper may have affected the reactivity of nonligand histidyl residues has to be considered too. However, circular dichroism measurements in the ultraviolet region failed to reveal significant differences between native and apotyrosinase, suggesting that the metal removal results in only small conformational changes, if any.

Tyrosinase has been reported to become irreversibly inactivated during the oxidation of catechol to o-quinone with a concurrent loss of the enzyme-bound copper (34, 35). This so-called “reaction inactivation” has been interpreted to originate from an active-site-directed attack of o-quinones on a nucleophilic group in the proximity of the active center (34). In an attempt to isolate an active-site peptide of Neurospora tyrosinase, the enzyme was inactivated using uniformly labeled [14C]phenol as the substrate. In contrast to a previous report (34), no incorporation of radioactivity into the enzyme could be observed (data of C. Dietler and K. Lerch). Amino acid analysis of the inactivated enzyme indicated, however, the loss of one histidyl residue per mole of enzyme. Subsequent peptide analysis of the modified protein indicated that one of the two adjacent histidyl residues 305 or 306 (shown by pluses in Fig. 2) was destroyed during this inactivation process. Because the number of catalytic events that occurred before the enzyme was irreversibly inactivated was found to be independent of the substrate concentration, the reaction inactivation of tyrosinase with catechol as the substrate may thus be regarded as a typical case of suicide inactivation (36). Similar results have been reported for a variety of flavin-dependent oxidases (37); however, in contrast to tyrosinase, the radioactively labeled inhibitors become covalently attached to these enzymes. While the exact nature of the suicide inactivation of tyrosinase is not known as yet, one may speculate that a reactive intermediate generated during the catalytic cycle (singlet oxygen, hydroxyl radical) might attack the histidyl residue that was shown to be lost during this reaction and that may be involved directly in catalysis and/or metal binding.

The author is indebted to C. Longoni, E. Boller, and D. Ammer for excellent technical assistance and to S. Fazur and E. Schwander for running the sequencer. I am grateful to Dr. K. Wilson for helpful suggestions and to Dr. J. Kaji for his continued interest and his helpful advice throughout this work. This project was supported by Schweizerischer Nationalfonds Grants 3.409-0.74 and 3.018.76.