Primary Structure of Tyrosinase from *Neurospora crassa*

I. PURIFICATION AND AMINO ACID SEQUENCE OF THE CYANOGEN BROMIDE FRAGMENTS*

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Konrad Lerch‡, Claudia Longoni, and Erika Jordi

From the Biochemisches Institut der Universität Zurich, Zurichbergstrasse 4, CH-8028 Zurich, Switzerland

Cyanogen bromide (CB) cleavage of *Neurospora* tyrosinase resulted in four major fragments, CB1 (222 residues), CB2 (82 residues), CB3 (68 residues), and CB4 (35 residues), and one minor overlap peptide CB2-4 (117 residues) due to incomplete cleavage of a methionyl-threonyl bond. The sum of the amino acid residues of the four major fragments matches the total number of amino acid residues of the native protein. The amino acid sequences of the cyanogen bromide fragments CB2, CB3, and CB4 were determined by a combination of automated and manual sequence analysis on peptides derived by chemical and enzymatic cleavage of the intact and the maleylated derivatives. The peptides were the products of cleavage by mild acid hydrolysis, trypsin, pepsin, chymotrypsin, thermolysin, and *Staphylococcus aureus* protease V8. The cyanogen bromide fragment CB1 was found to contain two unusual amino acids whose chemical structure will be presented in the following paper.

Tyrosinase (monophenol, dihydroxyphenyl alanine:oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing monooxygenase catalyzing both the oxygen-insertion of monophenols and the oxidation of o-diphenols to o-quinones (1).

\[
\text{monophenol} + O_2 \rightarrow \text{o-quinone} + H_2O
\]

\[
2 \text{o-diphenol} + O_2 \rightarrow 2 \text{o-quinone} + 2 H_2O
\]

The enzyme occurs in different microorganisms, plants and animals and is mainly involved in the biosynthesis of melanins and other polyphenolic compounds (2-4). Tyrosinases from the common mushroom *Agaricus bisporus* and *Neurospora crassa* contain an antiferromagnetically spin-coupled copper pair (5-8) which in the cuprous state binds molecular oxygen (33,000 \(y_2\)). The oxygen in *Neurospora* oxytyrosinase is bound to the protein as peroxide (11), as was demonstrated earlier for oxyhemocyanin (12). In contrast to the wealth of information available on the mode of copper binding at the active site (8, 13) of tyrosinase, the knowledge of the covalent structure of the polypeptide chains is still very limited due to the occurrence of molecular heterogeneity (2, 4).

Tyrosinase from the ascomycete *N. crassa* has been reported to be a monomeric protein with a molecular weight of 33,000 (14, 15). More recently, this enzyme was found to consist of a single polypeptide chain of \(M_1\) of about 44,000 (10). To arrive at a better understanding of the structure and function of this copper-containing monooxygenase, we have carried out the determination of the complete amino acid sequence of *Neurospora* tyrosinase. In the first paper, the isolation and the sequence analysis of the cyanogen bromide fragments CB1 to CB4 are described.

The accompanying paper reports the complete primary structure of *Neurospora* tyrosinase (16). A preliminary report of some of this work has been published (17).

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Purification of Cyanogen Bromide Peptides—*Neurospora* tyrosinase is a single-chain protein with a molecular weight of 46,000 (10, 17). In order to determine its amino acid sequence, the protein was cleaved with cyanogen bromide in 75% trifluoroacetic acid. As predicted from the presence of three methionine residues, four major fragments (CB1, CB2, CB3, and CB4) were obtained. The peptides were isolated by gel filtration on Sephadex G-100 (Fig. 1). The fragments CB1 and CB4 were found to be sufficiently pure for sequence analysis. The mixture of CB2 and CB3 was resolved by repeated gel chromatography on Sephadex G-50 (superfine) in 7% formic acid (Fig. 3). Alternatively, the mixture was maleylated and subsequently separated by DEAE-cellulose chromatography (Fig. 5). Cyanogen bromide cleavage of the enzyme with 70% formic acid yielded, in addition to the four fragments described above, a fifth fragment which was isolated in good yield and found to consist of 127 residues.

1. The abbreviations used are: CB, cyanogen bromide; PTH, phenylthiohydantoin; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; DMBA, N,N'-dimethylbenzylamine; TLC, thin layer chromatography; GLC, gas liquid chromatography; R, regeneration of PTH derivative; HPLC, high pressure liquid chromatography; HVE, high voltage electrophoresis. For other abbreviations see "Nomenclature" in the miniprint section.

2. Portions of this paper (including "Experimental Procedures" and part of "Results and Discussion," Figs. 1-9, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-2727, cite authors, and include a check for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

3. Figs. 10-32 and Tables II—XXXVI are deposited in the *J. Biol. Chem.* depository for scientific data. They are available as JBC Document Number 81M-2727B, in the form of 2 microfiches or 92 pages. Orders for supplementary material should specify the title, authors and reference to this paper and the JBC Document number, the form desired (microfiche or full size photocopy) and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814 and must be accompanied by remittance to the order of the Journal in the amount of $2.50 per microfiche ($5.00 per set) or $36.80 per complete set of full size photocopies. The complete document is included in the microfilm edition of the Journal that is available from Waverly Press.
Amino Acid Sequence of Neurospora Tyrosinase

Scribed, an overlap peptide of CB2 and CB4 which arises from incomplete cleavage of a methionyl-threonyl bond in this solvent (Fig. 2). This fragment was obtained in pure form by rechromatography on Sephadex G-50 (superfine) in 7% formic acid (Fig. 4).

Characterization of Cyanogen Bromide Peptides—Amino acid compositions, molecular weights, NH2 termini, and yields of the cyanogen bromide fragments are listed in Table I. The amino acid compositions obtained from amino acid analysis are in good agreement with those determined by sequence analysis (shown in parentheses). The sum of the four major fragments CB1 to CB4 corresponds closely to the composition of native Neurospora tyrosinase (last column). The amino acid composition of the minor overlap peptide CB2-4 is in good agreement with the sum of the compositions of CB2 and CB4. Thus, all amino acids of Neurospora tyrosinase are contained within the four cyanogen bromide fragments CB1 to CB4.

Amino Acid Sequence of Fragment CB1—The elucidation of the amino acid sequence of the cyanogen bromide fragment CB1 relies to a great extent on the tryptic peptides of the unmodified fragment and of the maleylated derivative (Fig. 6A). With the exception of two aggregated peptides designated as Tc (core peptides) all tryptic peptides were isolated in good yields. These peptides yielded more than 80% of the total primary structure of CB1 including the sequence information of residues 1 to 35 which was obtained by automated sequence analysis of the unmodified fragment. For details, see Miniprint. For the alignment of the tryptic peptides of CB1, suitable overlap peptides were obtained by digestion of the maleylated derivative with Staphylococcus aureus protease and by cleavage of the unmodified fragment with dilute acetic acid (Fig. 6A). To complete the NH2-terminal sequence of CB1 and to unambiguously align the remaining tryptic peptides, the unmodified fragment was further cleaved with pepsin, chymotrypsin, and thermolysin (Fig. 6B). For details of the isolation and sequence analyses of these peptides, see Miniprint. CB1 is the largest of the four cyanogen bromide fragments of Neurospora tyrosinase, containing 222 amino acid residues. As a consequence of its size, a large number of peptides (>140) had to be isolated to arrive at the primary structure. Despite the efforts made in the course of this work in obtaining large fragments of CB1, the major portion of the amino acid sequence was eventually derived from small peptides generated by different proteases.

Amino Acid Sequence of Fragment CB2—The amino acid sequence of the cyanogen bromide fragment CB2 was determined by a combination of automated and manual sequence analysis of the intact fragment and of peptides derived from proteolytic cleavage of CB2 and its maleylated derivative with trypsin, thermolysin, and pepsin (Fig. 7). Complete documentation of the isolation and sequence analyses of these peptides is given in the Miniprint.

The cyanogen bromide fragment CB2 contains 82 of the 407 amino acid residues of Neurospora tyrosinase. It is devoid of histidine and homoserine; hence, it is placed at the COOH-terminal end of the intact protein.

Amino Acid Sequence of Fragment CB3—Like native Neurospora tyrosinase, the cyanogen bromide fragment CB3 was found to be resistant to Edman degradation. Chromatography of the tryptic digest of CB3 on the ion exchange resin M-72 gave the pentapeptide T3 (Fig. 8), which was blocked. The amino acid sequence of this peptide is N-acetyl-Ser-Thr-Asp-Ile-Lys, previously determined by computer-assisted mass spectrometry (32). The complete amino acid sequence of CB3 (Fig. 8) was determined by automatic and manual sequence analysis on peptides generated by cleavage with trypsin, dilute acetic acid, and thermolysin. For details, see Miniprint.

The cyanogen bromide fragment CB3 is composed of 68 residues and is devoid of tryptophan. It contains an Asn-Gly bond (residues 18 and 19) whose presence was confirmed by the isolation of two fragments after hydroxylamine cleavage (29) of peptide Th1 (Fig. 8). Finally, the amide assignments of the aspartic acid residues of CB3 were found to be in complete agreement with the known specificity of cleavage at Asp by dilute acetic acid (33).

Amino Acid Sequence of Fragment CB4—The primary structure of CB4 was determined by a combination of automated and manual sequence analysis of peptides derived from proteolytic cleavage of the unmodified fragment with thermolysin, pepsin, and chymotrypsin (Fig. 9). For details of isolation and sequence analyses of these peptides, see Miniprint.

The cyanogen bromide fragment CB4 consists of 35 amino acid residues (Fig. 9) lacking lysine and tyrosine. It shows a high content of hydrophobic amino acids (43%) including 3 out of a total of 12 tryptophans in Neurospora tyrosinase. This unusual amino acid composition could at least partially be responsible for the observed resistance of cleavage of the Arg 21-Leu 22 bond by trypsin.

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Amino Acid Sequence of Neurospora Tyrosinase

Materials

Amino acid sequence of Neurospora crassa tyrosinase (GenBank accession number: 692,453, 692,453) was predicted by the thermoplastic film, with a final model being constructed by computer graphics. The protein sequence was linearized using a computer program to minimize the number of amino acid replacements. The final model was refined using a molecular dynamics simulation to ensure accuracy.

Experimental Procedure

- Preparative chromatography: The enzyme was purified by chromatography on a Sephadex G-200 column (1.5 x 78 cm) equilibrated with 0.1 M sodium borate buffer, pH 8.0. The column was eluted with 0.1 M sodium borate buffer, pH 8.0, and the fractions were monitored for tyrosinase activity.

- Purification by ion exchange chromatography: The enzyme was purified by ion exchange chromatography on a DEAE-cellulose column (1.5 x 78 cm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0. The column was eluted with a linear gradient of 0.05 M to 0.5 M sodium citrate buffer, pH 6.0.

- Purification by affinity chromatography: The enzyme was purified by affinity chromatography on a specific tyrosinase ligand column (1.5 x 78 cm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0. The column was eluted with a linear gradient of 0.05 M to 0.5 M sodium citrate buffer, pH 6.0.

- Purification by gel filtration chromatography: The enzyme was purified by gel filtration chromatography on a Sephadex G-200 column (1.5 x 78 cm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0. The column was eluted with 0.05 M sodium citrate buffer, pH 6.0.

- Purification by solvent extraction: The enzyme was purified by solvent extraction using a specific solvent system. The solvent was evaporated to dryness and the enzyme was obtained as a white powder.

- Purification by crystallization: The enzyme was purified by crystallization from a solution of 0.05 M sodium citrate buffer, pH 6.0, and 0.2 M potassium phosphate buffer, pH 7.0. The crystals were obtained by slow evaporation of the solution at room temperature.

- Purification by dialysis: The enzyme was purified by dialysis against 0.05 M sodium citrate buffer, pH 6.0, and 0.2 M potassium phosphate buffer, pH 7.0. The enzyme was obtained as a white powder.

- Purification by electrophoresis: The enzyme was purified by electrophoresis on a 12% polyacrylamide gel. The enzyme was visualized by staining with Coomassie blue dye.

- Purification by reversed-phase HPLC: The enzyme was purified by reversed-phase HPLC on a C18 column (250 x 10 mm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0, and 0.2 M potassium phosphate buffer, pH 7.0. The column was eluted with a linear gradient of 0.05 M to 0.5 M sodium citrate buffer, pH 6.0.

- Purification by affinity chromatography on a specific ligand column: The enzyme was purified by affinity chromatography on a specific ligand column (250 x 10 mm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0. The column was eluted with a linear gradient of 0.05 M to 0.5 M sodium citrate buffer, pH 6.0.

- Purification by gel filtration chromatography on a Sephadex G-200 column: The enzyme was purified by gel filtration chromatography on a Sephadex G-200 column (250 x 10 mm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0. The column was eluted with 0.05 M sodium citrate buffer, pH 6.0.

- Purification by solvent extraction and crystallization: The enzyme was purified by solvent extraction and crystallization from a solution of 0.05 M sodium citrate buffer, pH 6.0, and 0.2 M potassium phosphate buffer, pH 7.0. The crystals were obtained by slow evaporation of the solution at room temperature.

- Purification by electrophoresis on a 12% polyacrylamide gel: The enzyme was purified by electrophoresis on a 12% polyacrylamide gel. The enzyme was visualized by staining with Coomassie blue dye.

- Purification by reversed-phase HPLC on a C18 column: The enzyme was purified by reversed-phase HPLC on a C18 column (250 x 10 mm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0, and 0.2 M potassium phosphate buffer, pH 7.0. The column was eluted with a linear gradient of 0.05 M to 0.5 M sodium citrate buffer, pH 6.0.

- Purification by affinity chromatography on a specific ligand column: The enzyme was purified by affinity chromatography on a specific ligand column (250 x 10 mm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0. The column was eluted with a linear gradient of 0.05 M to 0.5 M sodium citrate buffer, pH 6.0.

- Purification by gel filtration chromatography on a Sephadex G-200 column: The enzyme was purified by gel filtration chromatography on a Sephadex G-200 column (250 x 10 mm) equilibrated with 0.05 M sodium citrate buffer, pH 6.0. The column was eluted with 0.05 M sodium citrate buffer, pH 6.0.

- Purification by solvent extraction and crystallization: The enzyme was purified by solvent extraction and crystallization from a solution of 0.05 M sodium citrate buffer, pH 6.0, and 0.2 M potassium phosphate buffer, pH 7.0. The crystals were obtained by slow evaporation of the solution at room temperature.
Amino Acid Sequence of Neurospora Tyrosinase

The amino acid sequence of the Neurospora tyrosinase was determined by automated Edman degradation. The sequence was deduced from the amino acid composition and the N-terminal amino acid sequence of the purified tyrosinase. The sequence of the tyrosinase was compared to the sequences of other tyrosinases from different sources. The results indicate that the Neurospora tyrosinase is closely related to the tyrosinases from other fungi and plants. The sequence was also compared to the sequences of other enzymes involved in the aromatic amino acid biosynthetic pathway.

Fig. 4. Amino acid sequence of the Neurospora tyrosinase. The sequence was deduced from the Edman degradation and the N-terminal amino acid sequence.

Fig. 5. Alignment of the amino acid sequences of the Neurospora tyrosinase with other tyrosinases.

Fig. 6. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 7. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other enzymes involved in the aromatic amino acid biosynthetic pathway.

Fig. 8. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 9. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 10. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 11. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

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Fig. 13. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 14. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 15. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 16. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 17. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 18. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 19. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.

Fig. 20. The amino acid sequence of the Neurospora tyrosinase was compared to the sequences of other tyrosinases from different sources.
Amino Acid Sequence of Neospora Tyrosinase

The figure shows a diagram of the amino acid sequence of Neospora tyrosinase and a schematic outline of the peptide used to elucidate the complete primary structure. The following symbols were used to indicate the method of sequencing and peptide degradation:

- Amino acid degradation
- Manual enzyme degradation
- Automated enzyme degradation
- Cleavage
- Cleavage
- Cleavage

Amino Acid Sequence of Peptide (CTS)

Trisaccharide peptides were isolated in good yield (Fig. 2) with the amino acid compositions given in Table I. The tryptic peptides 43 and 45 were partially sequenced (Table II). The peptide 42 was sequenced only when the portion of sequence was recovered for the peptide 43 and 54 (Table III). The trypsin peptide 43 was found to differ only by one amino acid from peptide 42 (Fig. 2). The portion of sequence was recovered for the peptide 43 and 54 (Fig. 2). The portion of sequence was recovered for the peptide 43 and 54 (Fig. 2).

To align the trypsin peptides, the tryptic peptides of fragment CTS were cleaved at Try with limited contact and direct analysis of the amino acids was done. The portion of sequence of the peptide 43 was recovered for the peptide 43 (Fig. 2), and the trypsin peptide 43 was sequenced by high-pressure liquid chromatography and mass spectrometry. The portion of sequence of the peptide 43 was recovered for the peptide 43 (Fig. 2), and the trypsin peptide 43 was sequenced by high-pressure liquid chromatography and mass spectrometry. The portion of sequence of the peptide 43 was recovered for the peptide 43 (Fig. 2), and the trypsin peptide 43 was sequenced by high-pressure liquid chromatography and mass spectrometry. The portion of sequence of the peptide 43 was recovered for the peptide 43 (Fig. 2), and the trypsin peptide 43 was sequenced by high-pressure liquid chromatography and mass spectrometry.

Theoretical section - Theoretical description of the trypsin peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS were isolated from a trypsin digest of the tryptic peptides CTS and CTS.
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