Primary Structure of Tyrosinase from *Streptomyces glaucescens*†

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ABSTRACT: The complete amino acid sequence of *Streptomyces glaucescens* tyrosinase is reported. The molecule consists of 273 amino acids and has a M_r of 30 900 including two copper atoms. The primary structure was determined by a combination of amino acid and DNA sequence analysis. Peptide sequence information was derived from the cyanogen bromide, tryptic, and thermolytic fragments of apotyrosinase by automated Edman degradation and aminopeptidase M and carboxypeptidase C digestions. The nucleotide sequence of the tyrosinase gene cloned into the *Pvu*II site of pBR322 was determined. The enzyme contains no apparent leader peptide despite the fact that it is secreted into the culture medium. As observed for a number of different *Streptomyces* genes, the tyrosinase gene shows a strong preference (97%) for codons ending in G or C. A comparison of the amino acid sequence of *Streptomyces glaucescens* tyrosinase with that of *Neurospora crassa* tyrosinase reveals an overall sequence homology of only 24.2%. However, the sequence homology is much higher in those regions thought to be involved in metal binding of the binuclear active site copper of this monooxygenase.

Tyrosinase is a copper-containing monooxygenase catalyzing the ortho hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Mason, 1965). The enzyme occurs widely in nature and is responsible for the formation of melanin pigments (Lerch, 1981). The enzymes from *Agaricus bisporus* (Strothkamp et al., 1976) and *Neurospora crassa* (Lerch, 1983) have been investigated most extensively from both a structural and functional point of view.

Among the group of *Streptomycetes*, many species are characterized by the production of dark-colored melanin pigments that are synthesized by phenol oxidases (Küster, 1976). *Streptomyces* tyrosinases have been isolated from *Streptomyces nigrificans* (Nambudiri et al., 1972) and *Streptomyces glaucescens* (Lerch & Ettlinger, 1972). The molecular properties of *S. glaucescens* tyrosinase were shown to be similar to those of tyrosinases from eucaryotic organisms (Lerch & Ettlinger, 1972).

S. glaucescens tyrosinase was found to occur both as a cell-associated form and as an extracellular form in liquid cultures (Baumann & Kocher, 1976). More recently, the two forms were shown to be indistinguishable in terms of their molecular properties and the amino acid sequence of their NH_2 termini (Crameri et al., 1982).

To arrive at a better understanding of the mechanism of secretion and the active site structure of *S. glaucescens* tyrosinase, we set out to determine its primary structure. In this paper, the complete amino acid sequence is reported and compared to that of *N. crassa* tyrosinase.

Experimental Procedures

Assay and Isolation of S. glaucescens Tyrosinase. Tyrosinase was isolated from liquid cultures of *S. glaucescens* as described previously (Lerch & Ettlinger, 1972). Enzyme activity was determined with L-DOPA[1] as substrate (Fling et al., 1963).

Cyanogen Bromide Cleavage. Apotyrosinase was prepared from the native enzyme by incubation in 50% formic acid and 10 mM EDTA (1 h at room temperature) and subsequent gel filtration on Sephadex G-25 in 7% formic acid. The metal-free protein was cleaved with cyanogen bromide in 70% formic (40-fold excess cyanogen bromide to methionine) for 20 h at room temperature.

Enzymic Methods. Tryptic digestion of apotyrosinase was performed in 0.1 M ammonium bicarbonate, pH 8.5, with 2% by weight trypsin for 1 h at 37 °C. A second portion of trypsin was added after 1 h and the mixture further incubated for 3 h at 37 °C. Hydrolysis with thermolysin was performed in 0.1 M ammonium bicarbonate, pH 8.0, at 37 °C for 24 h, with 5% by weight enzyme.

Gel Chromatography. Cyanogen bromide fragments were separated on a Sephadex G-50 superfine column (2.5 × 140 cm) in 7% formic acid. Peptides were identified by their absorbance at 280 nm.

Ion-Exchange Chromatography. Tryptic and thermolytic peptides of apotyrosinase were fractionated on Beckman M-72 ion-exchange resin (0.9 × 20 cm) equilibrated in 0.05 M pyridine/acetate, pH 2.5, at 55 °C. The column was developed with a four-chamber gradient containing 200 mL each of 0.05 M pyridine/acetate, pH 2.5, 0.2 M pyridine/acetate, pH 3.1, 0.5 M pyridine/acetate, pH 3.75, and 2.0 M pyridine/acetate, pH 5.0. The separation of peptides was monitored with a Technicon Auto-Analyzer (Hill & Delaney, 1967). Pooled fractions were desalted on a Sephadex G-25 column (0.9 × 140 cm) in 50% acetic acid.

Amino Acid Analysis. Samples were hydrolyzed for 22–72 h at 110 °C in vacuum-sealed glass tubes and analyzed with a Durrum D-500 amino acid analyzer. Values given in the

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[1] Abbreviations: L-DOPA, 3,4-dihydroxy-L-phenylalanine; CB, cyanogen bromide peptides; CPC, carboxypeptidase C; APM, aminopeptidase M; PTH, phenylthiohydantoin; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; R, regeneration of PTH derivative; HPLC, high-pressure liquid chromatography; Quadrol, *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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Table I: Amino Acid Composition, Yields, and Molecular Weights of Cyanogen Bromide Fragments from *Streptomyces glaucescens* Tyrosinase[a]

amino acid	CB1	CB2	CB3	CB4	CB5	CB6	sum, CB1–6	total protein
Lys	2.7 (3)		0.8 (1)		1.0 (1)		4.5 (5)	4.8
His	3.1 (3)		3.0 (3)	3.1 (3)	2.0 (2)		11.2 (11)	10.8
Arg	14.2 (14)	6.0 (6)	3.1 (3)	3.8 (4)			27.1 (27)	27.6
Asx	11.1 (11)	4.6 (4)	7.0 (7)	6.0 (6)	5.8 (6)	0.4	34.9 (34)	34.0
Thr	8.9 (9)	1.9 (2)	1.3 (1)	2.0 (2)	3.7 (4)	1.0 (1)	18.8 (19)	18.6
Ser	7.9 (8)	2.7 (3)	2.2 (2)	2.0 (2)	1.2 (1)	0.4	16.4 (16)	15.7
Glx	7.8 (8)	2.8 (3)	2.5 (2)	1.6 (1)			14.7 (14)	15.1
Pro	4.2 (4)	3.2 (3)	5.0 (5)	1.2 (1)	1.7 (2)		15.3 (15)	15.4
Gly	8.7 (8)	5.0 (5)	3.6 (3)	5.4 (5)	0.4	1.3 (1)	23.8 (22)	23.1
Ala	13.1 (13)	6.2 (6)	4.2 (4)	3.1 (3)	2.2 (2)	1.0 (1)	29.8 (29)	29.7
Val[b]	7.3 (7)	5.2 (5)	4.3 (4)	4.0 (4)			20.8 (20)	19.8
Met								4.8
Hse	0.8 (1)	0.9 (1)	0.9 (1)	0.8 (1)		0.8 (1)	4.3 (5)	
Ile[b]	1.8 (2)	0.9 (1)					2.7 (3)	2.9
Leu	11.0 (11)	3.8 (4)	4.0 (4)	2.0 (2)	2.1 (2)		22.9 (23)	23.1
Tyr	2.9 (3)	1.1 (1)	2.0 (2)	0.9 (1)	0.9 (1)		7.8 (8)	7.5
Phe	5.8 (6)	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)		9.9 (10)	9.8
Trp	nd[d] (4)	nd (1)	nd (3)	nd (3)	nd (1)		(12)	11.5[c]
total residues	115	46	46	39	23	4	273	
yield (%)	50	69	48	62	71	50		
M_r	13072	4867	5301	4452	2644	361	30697	
NH_2 terminus	Thr	Asp	Ser	Ala	Lys	Ala		

[a] Residues per molecule. Integral values in parenthesis obtained from DNA sequence analysis. [b] Maximum values obtained after 72 h of hydrolysis. [c] Determined spectrophotometrically by the method of Edelhoch (1967). [d] nd, not determined.

tables are corrected for the partial loss of serine and threonine during the hydrolytic procedure or for incomplete liberation of valine and isoleucine after 22 h of hydrolysis.

HPLC. Peptides were further purified by HPLC on a Lichrosorb RP-18 column (0.46 × 25 cm). The buffer system used was a mixture of buffer A, 0.01 M sodium perchlorate in 0.1% phosphoric acid, pH 2.1, and buffer B, the same as buffer A except with 60% (v/v) acetonitrile. Chromatography was carried out with a linear gradient of buffer B (1%/min) at room temperature with a flow rate of 1.0 mL/min. Absorbance was recorded at 220 nm.

Sequence Analysis. Automated Edman degradation was performed on a Beckman sequenator, Model 890-B (updated), with Quadrol Beckman program 060275, using the Edman & Begg (1967) procedure as modified by Hermodson et al. (1972). The peptide (50–500 nmol) was dissolved in 0.5 mL of 50% acetic acid containing 4 mg of Polybrene. Solvent was removed by following Beckman application program 031872 before the sequencing program was commenced. Thiazolinone derivatives were converted to phenylthiohydantoins by treatment with 1 M HCl at 80 °C for 10 min. The PTH derivatives were identified qualitatively by thin-layer chromatography on Kieselgel sheets (Hermodson et al., 1972) and quantitatively by GLC (Hermodson et al., 1972) or amino acid analysis after back-conversion of the PTH derivatives with 6 M HCl containing 0.1% $SnCl_2$ (Mendez & Lai, 1975). Repetitive yields varied between 85 and 94%, depending on the peptides sequenced.

Hydrolysis of Peptides with APM and CPC. Peptides (20 nmol) were hydrolyzed for 2–30 min with APM or CPC (100:1 peptide to enzyme molar ratio) at 37 °C in 100 μL of 0.2 M *N*-ethylmorpholine/acetate, pH 8.5, or 0.1 M pyridine/acetate, pH 5.5, respectively. Portions (20 μL) of the hydrolysate were lyophilized and subjected directly to amino acid analysis.

Source of Plasmid Containing the Tyrosinase Gene. The *Kpn*I fragment of plasmid pMEA 4 containing the *S. glaucescens* gene (Hintermann et al., 1985) was cloned into the *Pvu*II site of pBR322 with *Kpn*I linkers (P-L Biochemicals). This yielded two plasmids that contain the tyrosinase gene in opposite directions (pMEA 6 and pMEA 7). Both plasmids showed no expression of the *S. glaucescens* gene in *Escherichia coli* ED 8767 (Murray et al., 1977). However, the gene was shown to be still active after retransformation of a melanin-negative mutant of *S. glaucescens*. In this work plasmid pMEA 7 was used exclusively. Plasmid DNA was isolated as described by Gunsalus et al. (1979). Digestions with restriction endonucleases were performed according to the suppliers' instructions (Boehringer, Amersham) and the fragments recovered by low-melting agarose gels (Weislander, 1979).

Nucleotide Sequence Determination and Analysis. Nucleotide sequences were determined by the method of Maxam & Gilbert (1977, 1980). The polyacrylamide/urea gel system described by Sanger & Coulson (1978) was used. Nucleotide sequences were analyzed by a computer program (Queen & Korn, 1980).

Results

Amino Acid Sequence Analysis. Purification of Cyanogen Bromide Peptides. S. glaucescens tyrosinase is a single-chain protein with a previously estimated molecular weight of 29 500 (Lerch & Ettlinger, 1972). In order to determine its amino acid sequence, the protein was cleaved with cyanogen bromide. As predicted from the presence of five methionine residues, six major fragments (CB1–CB6) were obtained. The peptides were isolated by gel filtration on Sephadex G-50 (Figure 5 of the supplementary material; see paragraph at end of paper regarding supplementary material).

Characterization of Cyanogen Bromide Fragments. Amino acid compositions, molecular weights, NH_2 termini, and yields of the six major fragments are listed in Table I. The amino acid compositions obtained are in good agreement with those determined by sequence analysis (shown in parentheses). The sum of the six cyanogen bromide peptides also corresponds closely to the composition of native *S. glaucescens* tyrosinase (last column).

Amino-Terminal Sequences of Cyanogen Bromide Fragments CB1, CB2, and CB4. Automatic sequence analysis of the fragment CB1 allowed the unambiguous sequence determination of the first 28 residues (Figure 1, Table V of the supplementary material). This sequence is identical with the amino-terminal sequence reported earlier for intact *S. glaucescens* tyrosinase (Crameri et al., 1982), establishing CB1 as the amino-terminal cyanogen bromide fragment. The ad-

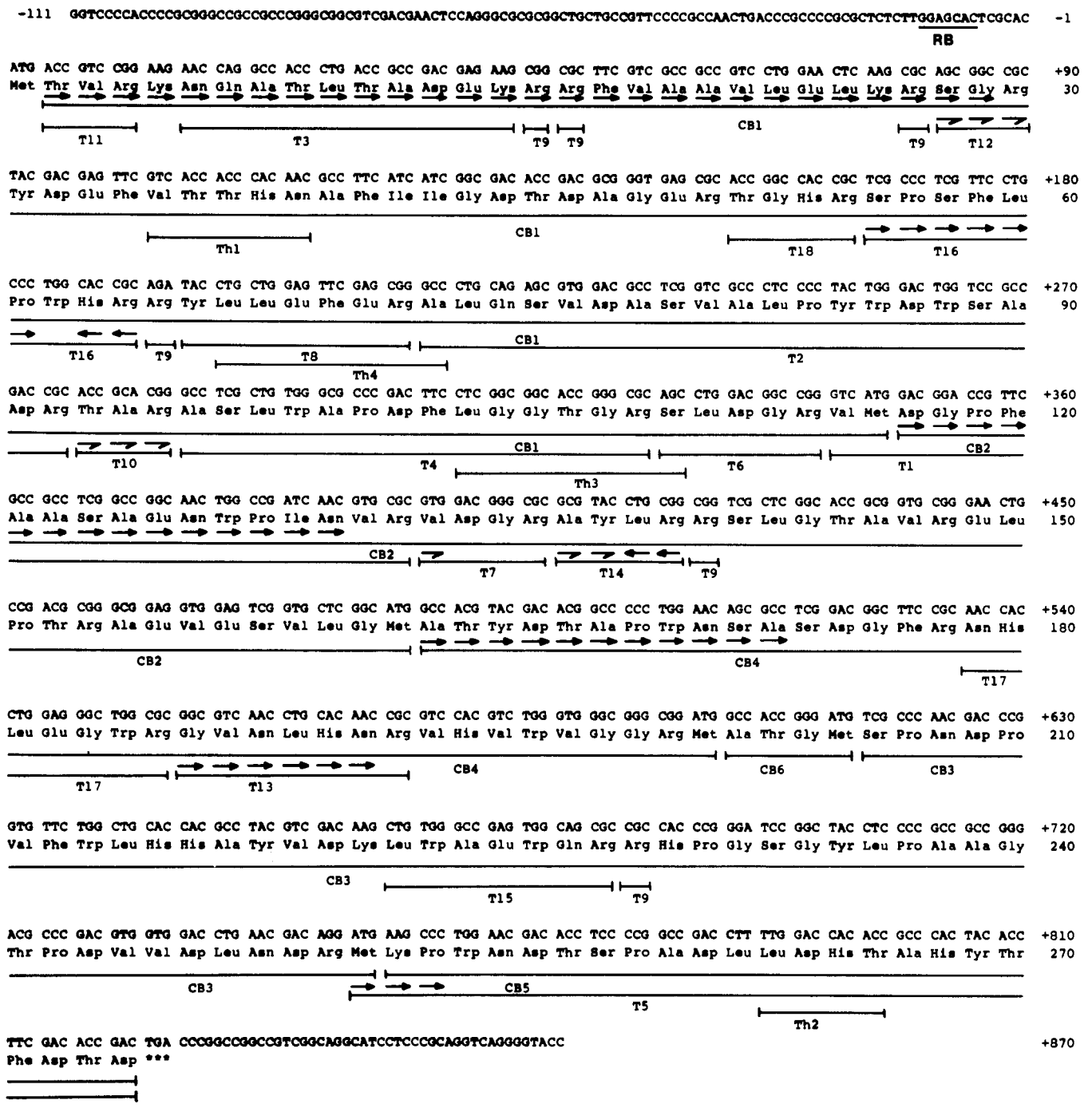

FIGURE 1: Primary structure of *S. glaucescens* tyrosinase. The nucleotide sequence of the tyrosinase gene was obtained from a DNA fragment cloned into *E. coli* plasmid pBR322. The deduced amino acid sequence is shown underneath, together with the peptides obtained after cleavage of the enzyme with cyanogen bromide (CB), trypsin (T), and thermolysin (Th). The following symbols were used to indicate the method of sequential degradation: →, automated Edman degradation; ⇀, aminopeptidase M; ←, carboxypeptidase C. RB denotes a possible ribosome binding site.

ditional cyanogen bromide fragments (CB2 and CB4) were also successfully subjected to automated Edman degradations (Figure 1, Tables VI and VII of the supplementary material).

Tryptic Peptides of S. glaucescens Tyrosinase. Apotyrosinase was cleaved with trypsin and the peptide mixture separated by ion-exchange chromatography (Figure 6 of the supplementary material). The peptides were further purified by gel filtration and HPLC (Table III of the supplementary material). Eighteen peptides were obtained in pure form and subjected to partial sequence analysis by automated Edman degradation and CPC and APM digestions (Tables VIII–XIV of the supplementary material). The results are summarized in Figure 1.

Thermolytic Peptides of S. glaucescens Tyrosinase. Four peptides were isolated from a thermolytic digest of the intact protein by ion-exchange chromatography and HPLC (Figure 7 of the supplementary material). The amino acid compositions are shown in Table IV (see supplementary material), and their positions in the amino acid sequence are shown in Figure 1.

Nucleotide Sequence. The *Kpn*I–*Sph*I fragment derived from pMEA 7 (Figure 2a) was the source of smaller DNA fragments used to determine the *S. glaucescens* tyrosinase nucleotide sequence as shown in Figure 2b. Due to the high G+C content (71.4%), compression problems on sequencing gels were occasionally observed. In those cases, the nucleotide

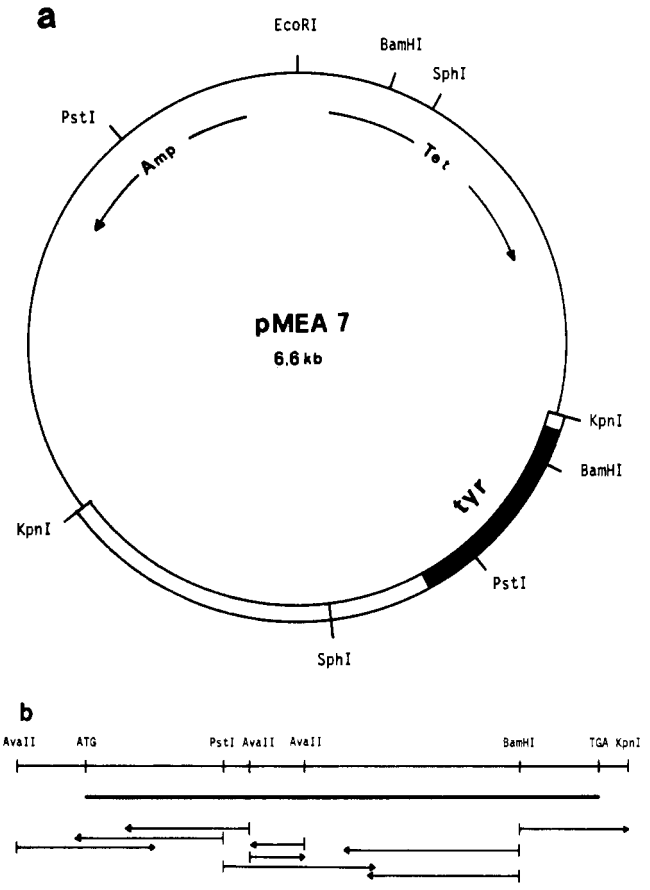

FIGURE 2: (a) Restriction map of pMEA 7. The *Kpn*I fragment containing the *S. glaucescens* tyrosinase gene was cloned into the *Pvu*II site of pBR322 with *Kpn*I linkers. tyr designates the tyrosinase gene. (b) Restriction endonuclease sites and nucleotide sequence determinations used to establish the primary structure of *S. glaucescens* tyrosinase. Arrows indicate the extent of each sequence determination.

sequence was obtained from both strands. The complete nucleotide and amino acid sequences are shown in Figure 1. The termini and the reading frame of the tyrosinase gene are in complete agreement with the protein chemical data presented above.

Discussion

The primary structure of *S. glaucescens* tyrosinase has been determined by nucleotide and protein sequence analysis. The amino acid composition calculated from the nucleotide sequence information is in good agreement with the amino acid compositions of both the intact enzyme and the six cyanogen bromide fragments (Table I). In addition, eighteen tryptic and four thermolytic peptides isolated from the intact protein (Tables III and IV of the supplementary material) could be unambiguously positioned in the amino acid sequence (Figure 1). The three cyanogen bromide fragments CB1, CB2, and CB4 and eight tryptic peptides were also subjected to partial sequence analysis. The amino acid sequences obtained were found to be in complete agreement with those derived from the nucleotide sequence data.

S. glaucescens tyrosinase consists of 273 amino acids resulting in a molecular weight of 30 900 including two copper atoms. This value compares favorably with those obtained from SDS gel electrophoresis and sedimentation equilibrium analysis of the protein (Lerch & Ettlinger, 1972). The enzyme is devoid of cysteine and contains a rather high amount of arginine residues (9.9%). Moreover, 10 of the 27 arginine residues are found to occur juxtaposed to another arginine or a lysyl residue.

Table II: Codon Usage in the *Streptomyces glaucescens* Tyrosinase Gene

	T		C		A		G	
T	Phe 0	Ser 0	Tyr 0	Cys 0	T
	10	3	8	0	C
	Leu 0	0		0	A
	1	9		Trp 12	G
C	Leu 1	Pro 0	His 0	Arg 0	T
	6	9	11	15	C
	0	0	Gln 0	0	A
	15	6	3	10	G
A	Ile 0	Thr 0	Asn 0	Ser 0	T
	3	15	11	4	C
	0	0	Lys 0	Arg 1	A
	Met 5	4	5	1	G
G	Val 0	Ala 0	Asp 0	Gly 1	T
	10	23	23	14	C
	0	1	Glu 2	2	A
	10	5	9	5	G

	T	C	A	G	% G-C content
1st position	43	76	49	105	66.3
2nd position	61	75	72	65	51.3
3rd position	2	165	6	100	97.1

The codon usage for the *S. glaucescens* tyrosinase gene is shown in Table II. As was found for other *Streptomyces* genes (Thompson & Gray, 1983; Robbins et al., 1984), nearly all (97%) of the codons used end in G or C. The distribution of nucleotides in the other two positions is again very similar to those reported for other genes from streptomycetes. The overall G+C content of the tyrosinase gene is 71.4%. This value is close to the one found for total DNA of different *Streptomyces* species (Enquist & Bradley, 1971).

In a previous study (Crameri et al., 1982) the amino-terminal sequences of the intra- and extracellular tyrosinases from *S. glaucescens* were found to be indistinguishable. This fact led to the idea that no leader peptide is required for the secretion of tyrosinase. This conjecture is further supported by the nucleotide sequence 5′ upstream of the enzyme coding region (Figure 1). This nucleotide sequence shows no other ATG in the correct reading frame and, furthermore, contains a reasonable Shine–Dalgarno ribosome binding site (−13 to −7) close to the initial ATG. Alternatively, it is conceivable that *S. glaucescens* tyrosinase is secreted by an internal uncleaved leader sequence as proposed by Blobel (1980). However, no such sequence could be detected in *S. glaucescens* tyrosinase that would fulfill the criteria for leader structure according to von Heijne (1983).

Until now, only the amino acid sequence of tyrosinase from the ascomycete *Neurospora crassa* has been determined (Lerch, 1978, 1982). A comparison of this sequence with that of *S. glaucescens* tyrosinase shows that the bacterial enzyme is much smaller and has an overall sequence homology of only 24.2% (Figure 3). The sequence homology of the amino- and carboxy-terminal parts is particularly small (4.5 and 6.5%, respectively). They are followed by two regions that reveal a sequence homology of more than 50%. The middle parts (residues 80–187) again show a value of sequence homology clearly below average (14%). The occurrence in the two tyrosinase molecules of two stretches that are highly homologous suggests that they play an important role in the active site structure. In particular, they could provide the ligands of the binuclear copper site in tyrosinase. This conjecture is

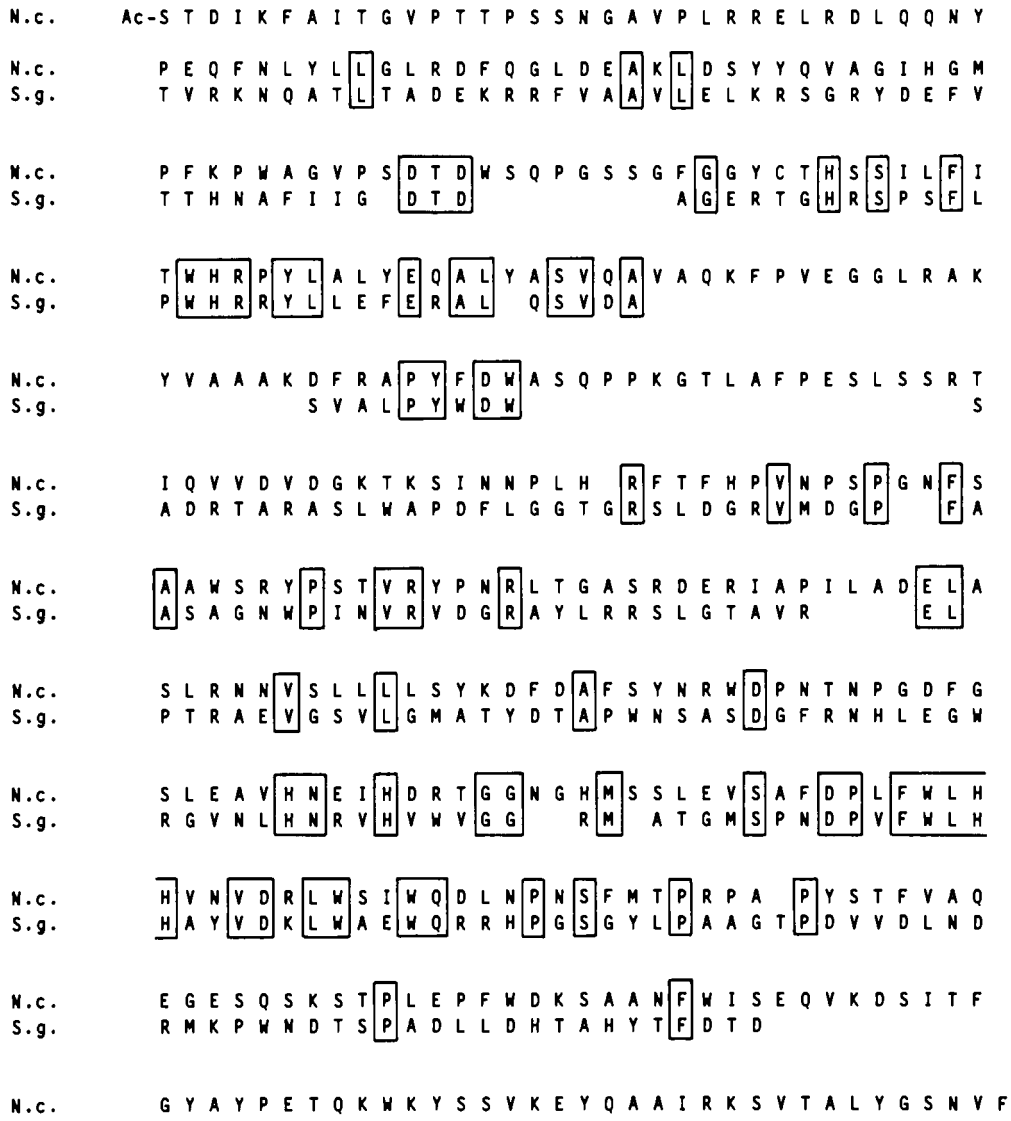

FIGURE 3: Amino acid sequence comparison of *S. glaucescens* (*S.g.*) and *N. crassa* (*N.c.*) tyrosinases (Lerch, 1982). Identical residues are indicated by boxes. The two amino acid sequences were aligned so as to obtain maximal sequence homology.

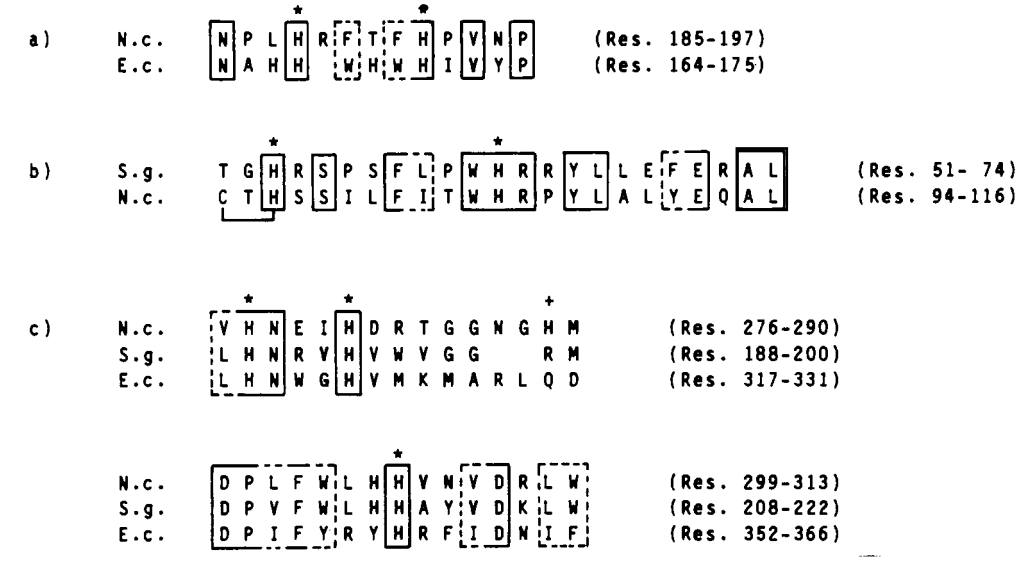

FIGURE 4: Amino acid sequence comparison of regions containing residues serving as potential ligands to the active site copper of tyrosinase and arthropod hemocyanins. (a) Amino acid sequence comparison of *Eurypelma californicum* (*E.c.*; Schneider et al., 1983) and *N. crassa* (*N.c.*) tyrosinases (Lerch, 1982). (b) Amino acid sequence comparison of *S. glaucescens* (*S.g.*) and *N. crassa* tyrosinases. (c) Amino acid sequence comparison of *S. glaucescens* tyrosinase, *N. crassa* tyrosinase, and *E. californicum* hemocyanin. Identical residues (—) and conservatively exchanged residues (---) are indicated by boxes. Asterisks denote histidyl residues proposed to be involved as ligands to the active site copper of tyrosinase and hemocyanin. The plus indicates a histidyl residue destroyed during photoinactivation of *N. crassa* tyrosinase (Pfiffner & Lerch, 1981). Cysteinyl residue 94 is covalently linked to histidyl residue 96 in *N. crassa* tyrosinase (Lerch, 1982).

supported by a structural comparison between tyrosinase and the oxygen-binding protein hemocyanin from mollusks and arthropods. Hemocyanins contain a binuclear copper complex that is remarkable similar to that in tyrosinase (Solomon, 1981; Lerch, 1981). Most prominent, the spectroscopic properties of the different functional states (oxy, deoxy, and met) of the two proteins are virtually indistinguishable. However, the accessibility of the binuclear copper site in tyrosinase is much higher than that in hemocyanin (Winkler et al., 1981). This difference is in agreement with the catalytic properties of tyrosinase, which is capable of binding organic substrate molecules in addition to molecular oxygen.

In a recent article (Gaykema et al., 1984), the three-dimensional structure of hemocyanin from the spiny lobster *Panulirus interruptus* has been reported. The two copper ions designated as Cu(A) and Cu(B) were found to be ligated each by three histidyl residues in a characteristic fashion. Amino acid sequence comparison of the region containing the ligands of Cu(B) shows that three histidyl residues of *S. glaucescens* and *N. crassa* tyrosinases are located exactly in the same positions (Figure 4c). In addition, the flanking sequences of the third histidyl residue of Cu(B) show a very high degree of homology in tyrosinase and arthropod hemocyanins. The involvement of the third histidyl residue as a copper ligand is also supported by protein modification experiments that were carried out with *N. crassa* tyrosinase. This histidyl residue was shown to be destroyed specifically during mechanism-based inactivation of tyrosinase with the concomitant loss of binding of one copper (Dietler & Lerch, 1982). In contrast to Cu(B), the three histidyl residues serving as ligands to Cu(A) in arthropod hemocyanins show only partial agreement of their positions with those in the sequence of *S. glaucescens* and *N. crassa* tyrosinase. Two histidyl residues of Cu(A) are found in a sequence that is similar in *N. crassa* tyrosinase and arthropod hemocyanins (Figure 4a). However, in *N. crassa* tyrosinase the two histidyl residues are separated by four in contrast to three amino acids in hemocyanin. In addition, the same histidyl residues (His-188 and -193, Figure 4a) have been implicated as copper ligands from photooxidation studies with *N. crassa* tyrosinase (Pfiffner & Lerch, 1980). The positions of the third histidyl residue of Cu(A) are totally different in tyrosinase and hemocyanin. In *P. interruptus* hemocyanin, this residue is separated by 26 amino acids (Gaykema et al., 1984); in *N. crassa* tyrosinase it was proposed to occur in the region of Cu(B) (Figure 4c, indicated by a +) on the basis of photoinactivation experiments (Pfiffner & Lerch, 1980). The two histidyl residues implicated as ligands to Cu(A) in *N. crassa* tyrosinase (His-188 and -193) are not found in *S. glaucescens* tyrosinase. However, the two tyrosinase molecules share a highly conserved sequence including two histidyl residues (Figure 4b) which are separated by eight amino acid residues. Although histidines 53 and -62 have not been identified as ligands to Cu(A) in *S. glaucescens* tyrosinase, it is attractive to speculate that they serve this function. In this context, it is interresting to note that His-96 of *N. crassa* tyrosinase (corresponding to His-53 in *S. glaucescens* tyrosinase) is covalently linked to Cys-94 (Figure 4b). This peculiar structure has been proposed earlier to be involved in the regulation of tyrosinase activity (Lerch, 1982). Thus it is conceivable that His-96 and -105 of *N. crassa* tyrosinase were originally ligands to Cu(A) but during evolution they were replaced by His-188 and -193 (see Figure 4a,b).

S. glaucescens tyrosinase is the smallest representative of this class known so far. In the course of evolution the enzyme tyrosinase has gradually increased in size (Lerch, 1981), reaching a value that is close to the average size of arthropod hemocyanins (Bannister, 1981). This change has occurred at both the amino- and carboxy-terminal ends (Figure 3). In the case of *P. interruptus* hemocyanin, the molecule was found to be divided into three distinct domains, with the second one representing the copper-binding region. It is therefore conceivable that the arthropod hemocyanins have evolved from a common ancestor of tyrosinase by extension of the copper-binding domain at the amino- and carboxy-terminal ends. As is evident from the X-ray work on *P. interruptus* hemocyanin, the extensions (domains 1 and 3) are shielding the binuclear copper complex in such a way as to prevent its interaction with organic substrate molecules, which is typical for tyrosinase. Hence, domains 1 and 3 are thought to be predominantly responsible in hemocyanin for only binding molecular oxygen in contrast to the monooxygenase properties of the related enzyme tyrosinase.

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SUPPLEMENTARY MATERIAL AVAILABLE

Tables III–XIV containing amino acid composition and amino acid sequence data and Figures 5–7 showing chromatograms of the separation of the cyanogen bromide, tryptic, and thermolytic peptides (12 pages). Ordering information is given on any current masthead page.

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Thermal Denaturation of Staphylococcal Nuclease†

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ABSTRACT: The fully reversible thermal denaturation of staphylococcal nuclease in the absence and presence of Ca^{2+} and/or thymidine 3′,5′-diphosphate (pdTp) from pH 4 to 8 has been studied by high-sensitivity differential scanning calorimetry. In the absence of ligands, the denaturation is accompanied by an enthalpy change of 4.25 cal g^{-1} and an increase in specific heat of 0.134 cal K^{-1} g^{-1}, both of which are usual values for small globular proteins. The temperature (t_m) of maximal excess specific heat is 53.4 °C. Each of the ligands, Ca^{2+} and pdTp, by itself has important effects on the unfolding of the protein which are enhanced when both ligands are present. Addition of saturating concentrations of these ligands raises the denaturational enthalpy to 5.74 cal g^{-1} in the case of Ca^{2+} and to 6.72 cal g^{-1} in the case of pdTp. The ligands raise the t_m by as much as 11 °C depending on ligand concentration. From the variation of the denaturational enthalpies with ligand concentrations, binding constants at 53 °C equal to 950 M^{-1} and 1.4×10^4 M^{-1} are estimated for Ca^{2+} and pdTp, respectively, and from the enthalpies at ligand saturation, binding enthalpies at 53 °C of −15.0 and −19.3 kcal mol^{-1}.

Staphylococcal nuclease A (deoxyribonucleate 3′-nucleotidohydrolase, EC 3.1.4.4) is a well-characterized globular protein containing 149 amino acid residues in a single peptide chain of molecular weight 16 807. It contains no sulfhydryl groups or disulfide bonds. Because of its relatively simple structure, ease of isolation and purification, and good thermal stability, it has been much studied (Anfinsen et al., 1971; Tucker et al., 1978, 1979a,b,c) with the aim of understanding the interrelations between the amino acid sequence and three-dimensional structure of the enzyme and its biological and physical properties. In this paper, we report the results of a high-sensitivity differential scanning calorimetric (DSC) study of the reversible thermal unfolding of staphylococcal nuclease (Nase). This study was undertaken primarily as the first step in a program of investigations of the effects of single amino acid replacements on the thermodynamics of unfolding of the protein.

The enzymic activity and many of the other properties of Nase are strongly affected by Ca^{2+}. Included in these properties is the binding of the strongest known inhibitor of the enzyme, thymidine 3′,5′-diphosphate (pdTp). We have therefore included in our study the thermal unfolding of Nase in the presence of either or both of these ligands.

Materials and Methods

All chemicals were of analytical reagent grade. pdTp (tetralithium salt, dihydrate) was obtained from Calbiochem-Boehring Corp. as A grade. Nase was isolated from homogenates of *Escherichia coli* cells that had been transformed with a recombinant plasmid containing the gene for the enzyme and inducers for production of the enzyme. This plasmid, obtained from Dr. Robert Fox of Yale University, was constructed by insertion of a *Sau*3A restriction fragment containing the structural gene for nuclease A in the unique *Bam*HI restriction site in the expression vector pAS1 (Rosenberg et al., 1983). This construction results in the synthesis of a modified nuclease in which the heptapeptide Met-Asp-Pro-Thr-Val-Tyr-Ser is appended to the amino-terminal alanine of nuclease A. By analogy with staphylococcal nuclease B (Davis et al., 1977), it is assumed that these extra residues

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