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Deletion Mutational Mapping of the Catalytic Activities of Human Tyrosinase

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Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is an essential enzyme in the biosynthesis of melanin pigment, which has a protective effect against injurious ultraviolet radiation.[1] Tyrosinases are abundant in mammals, plants, fungi, and bacteria. Human tyrosinase was isolated from melanoma cells and the full-length human tyrosinase gene cloned.[2] Human tyrosinase is a membrane glycoprotein containing 529 amino acids, including an 18 amino acid N-terminal signal sequence.[3] The active sites of tyrosinases are well conserved among the different species.[4] The crystal structures of the bacterial tyrosinases from *Streptomyces castaneoglobisporus* and *Bacillus megaterum* have been determined and found to implicate the six histidine residues coordinating the two copper ions (CuA and CuB) in the active site.[5,6] From these studies, the structure and catalytic mechanism of bacterial tyrosinase have been extensively studied. However, the active sites and the substrate binding sites of mammalian tyrosinase remain largely unknown.

To gain further insight into the relationship between the structure and functions of human tyrosinase, in this study, we synthesized three deletion mutants of human tyrosinase as follows: (1) a mutant lacking the C-terminal 455-481 amino acids (hTyr-C); (2) a mutant lacking the N-terminal 1-92 amino acids (hTyr-N); (3) a mutant lacking the N-terminal 1-92 amino acids and the C-terminal 455-481 amino acids, (hTyr-C&N) (Fig. 1). The constructed three deletion mutants were expressed in *Escherichia coli* in a soluble form and the resultant encoded proteins were purified by immobilized metal affinity column chromatography.

The expression plasmid pET-hTyr-ecto containing the human tyrosinase ectodomain and C-terminal polyhistidine sequence tag has been previously constructed in this lab.[7,8] The genes of the deletion mutants were cloned by PCR and the amplified genes ligated with plasmid expression vector pET-16b(+), containing the N-terminal 6xHis tag for generating the recombinant plasmids, pET-hTyr-C, pET-hTyr-N and pET-hTyr-C&N. To maximize the yield of the soluble deletion mutants, *E. coli* BL21 Star (DE3) cells harboring pET-hTyr-C, pET-hTyr-N and pET-hTyr-C&N were grown to a measured, cellular, optical density of 0.2 at 600 nm and exposed to the inducer (0.4 mM IPTG) for 12 h. The total activities of the deletion mutants (approximately 300 units/1.0 L culture) were similar to that of the previous expression system of the human tyrosinase ectodomain.[8]

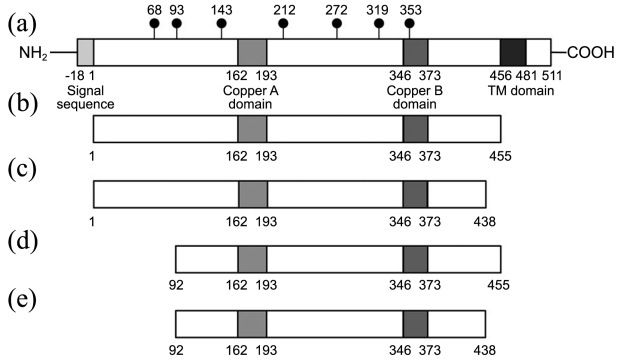

Figure 1. Schematic representation of the sequences of human tyrosinase and deletion mutants. (a) The mature human tyrosinase (529 amino acids long) includes an 18-amino acid long N-terminal signal peptide (SS) that targets the nascent polypeptide to the ER for folding, modification and sorting; seven *N*-glycosylation sites (G), two copper binding sites (CuA and CuB) and one transmembrane (TM) domain followed by a relatively short carboxyl tail that contains the essential signals for sorting and targeting to the melanosomes. (b) Human tyrosinase ectodomain (hTyr-TM: From 18 to 473 amino acid), (c) Human tyrosinase C-terminal deletion mutant (hTyr-C: From 18 to 456 amino acid), (d) Human tyrosinase N-terminal deletion mutant (hTyr-N: From 110 to 473 amino acid), (e) Human tyrosinase N- and C-terminal deletion mutant (hTyr-C&N: From 110 to 456 amino acid) (Ning and Daniel, 2006).

The recombinant human tyrosinase deletion mutants expressed from the *E. coli* BL21 Star (DE3) cells containing plasmid pET-hTyr-C, pET-hTyr-N and pET-hTyr-C&N were purified to electrophoretic homogeneity by immobilized metal affinity chromatography (result not shown). In the purification procedure, the yields of the purified human tyrosinase deletion mutants (approximately 40-60%) were similar to that of this lab's previous expression system of the human tyrosinase ectodomain.[8] The purified deletion mutants were then used to carry out subsequent characterizations.

The substrate specificities of the human tyrosinase deletion mutants toward several compounds are shown in Table 2. The substrate specificities of the deletion mutants were

Abbreviations: L-DOPA: 3-(3,4-dihydroxylphenyl)-L-alanine; PAGE: polyacrylamide gel electrophoresis; SDS: sodium dodecyl sulfate; TM: transmembrane domain.

Table 1. Oligonucleotide primers used for the polymerase chain reaction

Name	Primer sequence	Restriction enzyme
pET16 hTyr (-C)	5'-ggaatcc**CATATG**cattttccgagagcctgtgtgtcttct-3' 5'-cgc**GGATCC**tgggtctgaatcttgtagatagcta-3'	*Nde* I *Bam*H I
pET16 hTyr (-N)	5'-ggaatcc**CATATG**aactgcacagagagacgact-3' 5'-cgc**GGATCC**cgttattagatccgactcgcttc-3'	Nde I *Bam*H I
pET16 hTyr (-C&N)	5'-ggaatcc**CATATG**aactgcacagagagacgact-3' 5'-cgc**GGATCC**tgggtctgaatcttgtagatagcta-3'	Nde I *Bam*H I

Table 2. Substrate specificity of the human tyrosinase deletion mutants towards monophenols, diphenols and triphenols

substrates	Wave length (nm)	Relative activity (%)		
		hTyr (-C)	hTyr (-N)	hTyr (-C&N)
Monohydroxyphenols				
ρ-Coumaric acid	410	14 ± 0.3	13 ± 0.3	6 ± 0.3
o-Coumaric acid	340	10 ± 0.7	0	0
Tyramine	410	27 ± 3	15 ± 2	10 ± 1
Dihydroxyphenols				
Caffeic acid	362	0	0	0
(±)-Catechin	410	233 ± 15	238 ± 28	230 ± 11
Catechin	480	135 ± 8	130 ± 4	120 ± 13
Catechol	324	94 ± 5	64 ± 3	50 ± 3
Chlorogenic acid	326	10 ± 0.3	0	0
L-DOPA	475	100	100	100
Epicatechin	470	59 ± 7	48 ± 4	64 ± 13
Trihydroxyphenols				
Phloroglucin	424	37 ± 3.3	28 ± 2.6	25 ± 1.4
Pyrogallol	334	332 ± 12	314 ± 26	290 ± 18

The enzyme activities were measured using 20.0 mM mono-, di-, or trihydroxyphenols as a substrate in 100 mM Tris-HCl, pH 7.5 at 37 °C; the relative activities were comparisons with that of L-dopa set at 100%. The values shown are means ± S.D., generally based on n ≥ 5.

moderately similar to that of the human tyrosinase ectodomain. The hTyr-C, hTyr-N and hTyr-C&N mutants showed high activity toward catechin, catechol, L-DOPA, and pyrogallol. The activities of the hTyr-C mutant towards tyramine and catechol were also approximately 2 times higher than those of the hTyr-N and hTyr-C&N mutants. These results demonstrated that the deletion of the N-terminal domain consisting of 1-92 amino acids in the human tyrosinase resulted in a significant structural change of the active site and might affect the conformation of the substrate binding site of the enzyme.

The kinetic parameters of the human tyrosinase deletion mutants were determined from the Lineweaver–Burk plot (Table 3). The deletion of the N-terminal domain consisting of 1-92 amino acids in the human tyrosinase scarcely affected the kinetic parameters. The K_m and k_{cat} values of the hTyr-N and hTyr-C&N mutants were moderately similar to those of the human tyrosinase ectodomain. On the other hand, the K_m values (1.06 μM and 0.21 mM) of the hTyr-C deletion mutant for L-tyrosine and L-dopa were 20-40% lower than those (1.32 μM and 0.34 mM) of the human tyrosinase ectodomain.[8] The data indicate that the N-terminal 1-92 amino acids in human tyrosinase have a higher affinity for substrates. From these results on the substrate specificity and the kinetic parameters, we suggest that the N-terminal domain in the human tyrosinase contributes to the binding of the substrates.

The biochemical properties of the recombinant human tyrosinase deletion mutants were investigated with the L-DOPA oxidation reaction. The optimum pH of the hTyr-N mutant was similar to that (pH 8.0) observed for the human tyrosinase ectodomain. On the other hand, the optimum pHs of the hTyr-C and hTyr-C&N mutants were 8.5 at 37 °C (Fig. 2(a)), showing less than 50% of its maximum activity below pH 6.5 and above 8.5, respectively. In order to understand the effects of temperature upon the enzyme activity, the reaction mixture was incubated at temperatures in the range from 20 to 80 °C. The optimum temperatures for the oxidation of L-dopa by the hTyr-C and hTyr-C&N mutants were 50 and 60 °C, respectively (Fig. 2(b)), which are higher than that of the human tyrosinase ectodomain (45 °C). On the other hand, the optimum temperature afforded by the hTyr-N mutant was lower than that of the human tyrosinase ectodomain. The thermal stabilities of the deletion mutants were moderately similar to that of the human tyrosinase ectodomain (Fig. 2(c)). From these results, we

Table 3. Kinetic parameters of human tyrosinase deletion mutants for L-tyrosine hydroxylation and DOPA oxidation reaction

Enzyme	L-Tyrosine			L-DOPA		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
hTyr(-C)	1.06 ± 0.05	36.6 ± 0.8	34.5	0.21 ± 0.02	20.4 ± 1.1	97.1
hTyr(-N)	1.43 ± 0.08	37.6 ± 1.3	26.3	0.35 ± 0.04	28.7 ± 1.8	82.0
hTyr(-C&N)	1.37 ± 0.14	40.4 ± 3.4	29.5	0.41 ± 0.06	26.2 ± 3.0	63.9

Values are Means ± S.D., generally based on n ≥ 5.

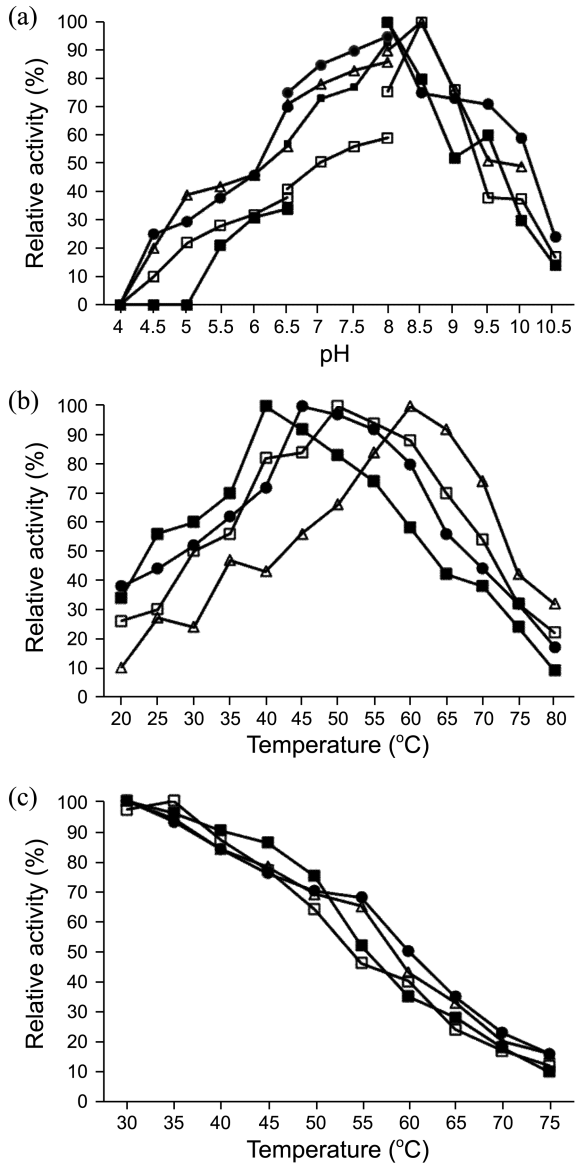

Figure 2. Effects of pH, temperature, and thermostability on the L-dopa oxidation activity of the human tyrosinase ectodomain (hTyr-TM, -○-), human tyrosinase C-terminal deletion mutant (hTyr-C, -●-), human tyrosinase N-terminal deletion mutant (hTyr-N, -■-), and human tyrosinase N- and C--terminal deletion mutant (hTyr-C&N, -▲-). (a) The effects of pH on the enzyme activity were determined by using the following buffers at the indicated pH: pH 4.0-6.5, 100 mM sodium acetate buffer; pH 6.5-7.5, 100 mM potassium phosphate buffer; pH 7.5-10.5, 100 mM Tris-HCl buffer. (b) The effects of temperature on the enzyme activity were determined in 100 mM Tris-HCl buffer (pH 7.5) at temperatures ranging from 20 to 80 °C. (c) Thermostability of the human tyrosinase deletion mutants.

suggest that the deletion of the C-terminal 455-481 amino acids in the human tyrosinase resulted in significant structural change of the active site and caused the optimum pH and temperature of the deletion mutant to be shifted.

The 3-dimensional structure of human tyrosinase remains unresolved, although its amino acid sequence has been known since 1987.[2] Furthermore, human tyrosinase has not been shown to bind copper directly. However, a comparison study of the amino acid sequences of tyrosinases and hemocyanins suggested that two regions of apparent homology, termed CuA and CuB (Fig. 1), may be involved in the binding of the copper atoms which are necessary for its catalytic activity.[12] Mutational analysis of tyrosinase also suggested that the CuA and CuB sites are both required for its copper binding and catalytic activity.[13] In this study, we showed that the three deletion mutants exhibited tyrosinase activities towards the hydroxylation and oxidation reactions (Table 2). The hTyr-C deletion mutant showed different substrate specificity and had different affinities towards L-tyrosine and L-dopa (Table 2 and 3). These results suggest that the N-terminal 1-92 amino acids are important for the binding of the substrates. The hTyr-C and hTyr-C&N mutants also showed different optimum pH and temperature values from the human tyrosinase ectodomain and the hTyr-N deletion mutants (Fig. 2). These results suggest that the C-terminal 455-481 amino acids are important for maintaining the proper stable conformation of the enzyme. Moreover, the human tyrosinase deletion domain consisting of only 92-455 amino acids is indeed enzymatically active. This study offers information on the approximate region of the enzyme responsible for the enzyme-substrate interactions and catalytic properties of human tyrosinase and will be of great value in designing new inhibitors that may prove useful in cosmetics and chemotherapy and new enzymes having different substrate specificities.

Experimental Section

Synthesis of Oligonucleotides and Construction of the Expression Plasmid. The human tyrosinase ectodomain was obtained by the expression of the recombinant plasmid pET-hTyr-ecto in *E. coli* as described in a previous paper.[8] The oligonucleotide primers used for the polymerase chain reaction (PCR) are shown in Table 1. Primer-1 and primer-2 served as the 5'-sense (forward) and 3'-antisense (reverse) sequences of the deletion mutants in the human tyrosinase ectodomain, with the addition of the *Nde* I and *Bam*H I restriction sites, respectively. The PCR product was cloned into an expression vector pET-16b(+) using the *Nde* I and *Bam*H I sites. The resulting vectors were designated pET-hTyr-C, pET-hTyr-N, and pET-hTyr-C&N and used to transform the *E. coli* strain BL21 Star (DE3). The colony containing the appropriate insert was identified by DNA sequencing.[9]

Expression and Purification of the Deletion Mutants. The *E. coli* strain BL21 Star (DE3) harboring each of the constructed plasmids was grown in LB broth containing ampicillin (50.0 μg/mL) and 1.0 mM $CuSO_4$ at 37 °C and induced at OD_{600} = 0.2 with 0.4 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) for 12 h. The induced cells were harvested by centrifugation at 8,000 g for 10 min at 4 °C and resuspended in 20.0 mM Tris-HCl buffer (pH 7.9) containing 5.0 mM EDTA, 1.0 mM $CuSO_4$, and 100 μM phenylmethanesulfonyl fluoride. The resuspended cells were subjected to sonication using an ultrasonic processor (Sonics &

Materials Inc., Newtown, CT, USA) until lysed. After the aggregated proteins were removed by centrifugation at 10,000 g for 10 min, the supernatant was collected and stored at 4 ^{o}C.

The supernatant containing the deletion mutants bearing the His-tag was subjected to affinity column chromatography with Ni-conjugated agarose. The column was washed with 20.0 mM Tris-HCl buffer (pH 7.9) containing 12.0 mM imidazole. The deletion mutants were then eluted with 20.0 mM Tris-HCl buffer (pH 7.9) containing 100 mM imidazole. The pool of active fractions was concentrated by ultrafiltration and dialyzed against 20.0 mM potassium phosphate buffer (pH 6.8). The dialyzed purified deletion mutants were used for the next experiment.

Enzyme Assay and Kinetic Study. The assay of the tyrosine hydroxylase activity was performed as described by Tripathi *et al*.[10] The dopa oxidase activity of tyrosinase was determined at 37 °C by spectrophotometrically monitoring, at 475 nm, the appearance of the dopachrome product of the reaction as described by Hearing.[11] The specific activity was defined as the number of units of enzyme activity per mg of protein. To determine the substrate specificity, various mono-, di-, and tri-hydroxyphenol compounds were used as substrates. The rate of the reaction for the different substrates was measured at the maximum absorption of the reaction product between 300 and 500 nm. The relative activity was determined from the amount of enzyme that caused a change in absorbance of 0.001/min and was expressed with respect to L-dopa. The kinetic parameters for L-tyrosine and L-dopa were determined by the Lineweaver-Burk plot method. The parameters (with standard deviation) were determined by five separate experiments. The protein concentration was determined by the BCA assay reagent (Pierce Chemical Co., Rockford IL, USA) using bovine serum albumin as the standard protein.

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