Identification of copper ligands in *Aspergillus oryzae* tyrosinase by site-directed mutagenesis

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Copper ligands of the recombinant tyrosinase from the fungus *Aspergillus oryzae* expressed in *Saccharomyces cerevisiae* or *Escherichia coli* were identified by site-directed mutagenesis. The recombinant protyrosinases expressed in *S. cerevisiae* were assayed for catalytic activities of mono-oxygenase and L-dopa oxidase at pH 5.5 after acid shock at pH 3.0. Replacements of His-63, His-84, His-93, His-290, His-294, His-332 or His-333 with asparagine resulted in mutant enzymes exhibiting no activities. The site-directed mutant Cys82Ala showed that Cys-82 was also an essential residue for the activity. We obtained homogeneous preparations of activated tyrosinases from mutated thioredoxin fusion gene products expressed in *E. coli* by acid shock. The copper contents of engineered mutants and wild-type enzyme expressed in *E. coli* were determined by atomic absorption spectrophotometry. The wild-type enzyme contained 2 g-atoms of copper/mol of the subunit. The His63Asn, His84Asn, His93Asn, His290Asn, His294Asn, His332Asn, His333Asn or Cys82Ala substitution decreased copper binding by approx. 50%, indicating that the mutants contain only approx. 1 g-atom of

copper/mol of the subunit. The five mutants His63Asn, His93Asn, His290Asn, His294Asn and Cys82Ala contain only one copper ion, which is fully detectable by EPR. From the correlation of $g_{\parallel}$ and $^{63}\text{Cu}A_{\parallel}$, we deduced that the nitrogen or sulphur donors in the copper ligands should be in a square or a distorted tetrahedral geometric environment. In further atomic absorption spectrophotometry experiments, no copper atom was observed in the seven double mutants His63Asn/His290Asn, His63Asn/His294Asn, His63Asn/His332Asn, His63Asn/His333Asn, Cys82Ala/His290Asn, His84Asn/His333Asn and His93Asn/His290Asn. We propose a new structure of active sites of tyrosinase from *A. oryzae*: the most likely binding sites of tyrosinase for Cu(A) are His-63, His-84 and His-93, with the remaining conserved Cys-82 providing the fourth ligand. Cu(B) liganded by four histidine residues, His-290, His-294, His-332 and His-333, is identified as new binding motif of Cu(B).

Key words: copper ligands, electron paramagnetic resonance (EPR), tyrosinase (*Aspergillus*).

INTRODUCTION

Copper in biological systems has been classified into three types according to its optical and magnetic properties [1]. Type I copper (blue copper) shows several charge-transfer bands, the most characteristic of which appears at approx. 600 nm. Type I copper functions as the electron mediator from the substrate to the trinuclear centre. Type II copper (non-blue copper) does not show any strong charge-transfer bands in the visible region and its $A_{\parallel}$ value in EPR spectra is normal. Type III copper is not detectable by EPR, because they are strongly antiferromagnetically coupled in pairs. Type II copper and III copper form a trinuclear centre to reduce dioxygen to two water molecules.

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1; and catechol oxidase, EC 1.10.3.1) is a type III copper protein and is an important enzyme participating in the process of melanin biosynthesis [2]. The enzyme catalyses both the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase; EC 1.14.18.1) and the oxidation of *o*-diphenols to *o*-quinones (catechol oxidase; EC 1.10.3.1). Tyrosinase is widespread in animals, insects, plants, fungi and bacteria. The biological role of the enzyme is thought to be participation not only in the biosynthesis of melanin but also in the biosyntheses

of lignins, bioflavonoids, tannin, adrenaline and other compounds; the starting materials for these compounds are phenols [2]. Albinism, which is due to a lack of melanin pigment, is one of the oldest known mutations in mice [3]. Tyrosinase activity is deficient in patients with classic type I oculocutaneous albinism; more than 50 distinct mutations have now been identified in the tyrosinase genes of such patients [3]. In higher plants, the enzyme protects the plant against insects and micro-organisms by catalysing the formation of an impervious scab of melanin against further attack [4]. Physiologically, the function of tyrosinase in fungi is not yet understood but it might be protective [4].

Two histidine residues, His-62 and His-189, were identified as copper ligands in *Streptomyces glaucescens* tyrosinase by Huber and Lerch [5]. The native enzyme contains two copper atoms at the active site that are not detectable by EPR. The two mutants, His62Asn and His189Asn, have lost their enzymic activity and their ability to bind oxygen, and contain only one copper ion that is fully detectable by EPR. The EPR parameters indicate that the remaining copper is in a tetragonally distorted ligand environment.

Our previous paper [6] described the unique process of activation of intracellular protyrosinase from *Aspergillus oryzae* by acid shock at pH 3.0. It was shown that the protyrosinase is a tetrameric enzyme with a molecular mass of 280 kDa; the four

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identical subunits each have a molecular mass of 67 kDa. N-terminal sequence analysis indicated that the N-terminus of protyrosinase was blocked. The 1671-bp nucleotide sequence of the protyrosinase gene, *melO*, from *A. oryzae* was determined [7]. The predicted amino acid sequence of the *melO* gene products has 17% and 15% identity with those from *Neurospora crassa* [8–11] and *Homo sapiens* [12] respectively. The protyrosinase from *A. oryzae* was assumed to contain 2 g-atoms of copper/mol of the subunits with a molecular mass of 67 kDa [7]. A coupled pair of three histidine residues [His-63, His-84 and His-93 for Cu(A); His-290, His-294 and His-333 for Cu(B)] of the proenzyme from *A. oryzae* was assumed [7] to correspond to the Cu(II) ligand in the similar tyrosinases from *Streptomyces antibioticus* [13], mouse [14,15] and *H. sapiens* [12].

Here we describe experiments showing the expressed of the *melO* gene [7] from *A. oryzae* RIB128 with the thioredoxin gene (*trxA*) fusion system [pET-32a(+)] system by means of an expression vector, pETY, in *Escherichia coli*. In the pET-32a(+) system, the *melO* gene was fused to the *trxA* gene mediating a spacer DNA containing corresponding protease restriction sites, a histidine tag and an S-tag for the purification and processing of products, as described previously [16]. The activated tyrosinase as a fusion protein had a molecular mass of 330 kDa and was composed of four identical subunits, each with a molecular mass of 83 kDa. The N-terminal amino acid sequence of activated recombinant tyrosinase was SDKIIHLTDD (single-letter amino acid codes), which was identical with that of thioredoxin. We found that the activated recombinant tyrosinase had monooxygenase activity at pH 5.5.

In the present study, site-directed mutagenesis of the *melO* was used to access the functional role of catalytic residues of the tyrosinase from *A. oryzae*. We determined seven essential histidine residues, His-63, His-84, His-93, His-290, His-294, His-332 and His-333, and a single essential cysteine residue, Cys-82, for the catalytic activity and copper ligands of the tyrosinase from *A. oryzae*. From the results it is possible to propose a new copper coordination and geometry in type III copper proteins for the copper sites in tyrosinase from *A. oryzae*.

EXPERIMENTAL

Materials

The protyrosinase gene (accession number D37929, DNA Data Bank of Japan, Mishima-shi) *melO*, described previously, was used [7]. T4 DNA ligase and the restriction endonucleases *NotI*, *NcoI* and *BamHI* were purchased from TaKaRa Shuzo (Kyoto, Japan). L-Tyrosine and L-dopa were purchased from Sigma (Madrid, Spain).

Strains, plasmids and media

A. oryzae RIB 128 (designated ATCC 22788) [17] was used as a source of cDNA of tyrosinase gene (*melO*) [7]. *E. coli* DH5 α , MV1184, BMH71-18*mutS* and BL21(DE3) [F⁻ *ompT* *hsdSB* (*rb*⁻*mb*⁻) *gal*(λ C1857 *ind1* *Sam7* *nin5* *lacUV5-T7* *gene1*) *dcm*(DE3)] were used for DNA manipulation. *S. cerevisiae* YPH 250 (*MATa*, *ura3*, *trp1*, *his3* and *leu2*) was used as a host for expression of *melO* encoding protyrosinase from *A. oryzae*. Plasmid pBluescript II SK⁺ was obtained from Toyobo (Osaka, Japan). M13mp18 was used for the preparation of single-stranded DNA. The soluble protein production vector pET-32-a(+) was purchased from Novagen (Madison, WI, U.S.A.). *E. coli* cells were grown in Luria–Bertani broth (1% (w/v) Bacto-tryptone/0.5% yeast extract/0.5% NaCl), 2 $\times$ YT [1.6% (w/v)

tryptone/1% (w/v) yeast extract/0.5% NaCl (pH 7.6)], and YT broth [0.8% Bacto-tryptone/0.5% yeast extract/0.5% NaCl (pH 7.6)].

Construction of yeast expression plasmid for protyrosinase from *A. oryzae*

The expression vector for protyrosinase, pGTy, was constructed by inserting the protyrosinase cDNA fragment [7] into the *NotI* site of pG3 [18], as described [7]. For the mutants, the absence of undesired mutations on the *melO* was confirmed by DNA sequencing before construction of the expression vector.

Site-directed mutagenesis

Site-directed mutagenesis of the *melO* gene was performed by the method of Carter et al. [19]; the following mutagenic primers were used: His63Asn, 5'-TACCTAGCCGcCTGaATGGT-GAGCC-3' (*melO* numbering, 172–197); His76Asn, 5'-TACA-ACAATTCgAatTGGTGGGG-3' (214–237); Cys82Ala, 5'-GGGGATACgctcAtCATGGAAAC-3' (236–258); His83Asn, 5'-GATACTGCaAcCATGGAAAC-3' (239–258); His84Asn, 5'-GATACTGCcAtaATGGAAACA-3' (239–259); His93Asn, 5'-CTTGTTTCCACgTGGaATCGTGCG-3' (261–285); His193Asn, GCTCAACAGaAtAACAAATTTtCTaGATGCCAATT-3' (568–601); His266Asn, 5'-GAACAGTTCaATCCACTcGAGT-CGGGT-3' (787–813); His290Asn, AAAGCCCCaATAATGA-CATGcATGTTCGcTATTGGTGG-3' (860–896); His294, 5'-ATAATGACATGaATCTCGcGAAATGGTGGAGTC-3' (869–900); His332Asn, 5'-TTTTACTTTaATCATCATTGCTTTAT-cGAtTATCTATTC-3' (985–1020); His333Asn, 5'-TTTTACT-TTCATaATTGCTTTATcGAtTATCTATTCT-3' (985–1021); Cys334Ala, 5'-TACTTTTCATCAcgcgTTTATTGAC-3' (988–1011); His347Asn, 5'-CAGACAATGaAtAAGAAGACAGATGCTAGcCAAATAACA (1031–1068); His462Asn, TTTG-CCTTGGCgATaTCGCAGACAGACaATACCGGCAAA-3' (1357–1394); His479Asn, GAATCTGTGCTctcgAGATGGaA-TGTACAGGGC-3' (1415–1446); His489Asn, TGCCAGAC-TaACCTAAGtActACAGCATTT-3' (1456–1485); His510Asn, 5'-AAAAGAAAAaATGCTAATAATGAATTcGCAGTGCA-C-3' (1519–1554); His518Asn, 5'-AATAATGAATTcGCAGT-GaACCTGCATACT-3' (1534–1563); His520Asn, AATAATG-AATTcGCAGTGCA-3' (1534–1569). Substituted nucleotides are indicated in lower case. The mutation was verified by DNA sequencing before construction of the expression plasmid pG3TY. *S. cerevisiae* YPH 250 strain (*MATa*, *ura3*, *lys2*, *trp1*, *his3* and *leu2*) was transformed with the plasmid pG3TY containing wild-type or mutated *melO* by the method of Ito et al. [20] and each transformant was selected on supplemented minimal medium [SMM; 0.67% yeast nitrogen base without amino acids/0.002% histidine/0.003% leucine/2% (w/v) glucose/0.002% adenine sulphate/0.002% uracil/2% (w/v) agar] at 30 °C.

Preparation of the recombinant protyrosinase expressed in *S. cerevisiae*

S. cerevisiae YPH 250 cells harbouring pG3TY were precultured in SMM tryptophan drop-out medium at 30 °C for 48 h. The culture broth was then inoculated in YPD medium [1% (v/v) yeast extract/2% (w/v) peptone/2% (w/v) dextrose] [21] at 30 °C. After 72 h, yeast cells were harvested by centrifugation at 2700 g for 15 min to give a precipitate. The collected cells

Figure 1 Structure of expression plasmid pETY for production of the thioredoxin–protyrosinase fusion protein in *E. coli* (A) and the expressed protein (B)

Protyrosinase cDNA (*melO*) was ligated downstream of the thioredoxin gene (*trxA*) at the *NcoI*–*Bam*HI site. Expression of the cloned gene was induced by providing a source of T7 RNA polymerase in the host cell under the control of the bacteriophage T7 transcription signal.

were vortex-mixed with glass beads and homogenized with 0.1 % (v/v) Tween 20 and the inhibitor mixture [22]. The homogenate was centrifuged for 15 min at 11 000 *g* and the supernatant was taken. Finally, the recombinant protyrosinase fraction was collected.

Construction of expression plasmid in *E. coli* cells

The cDNA was modified by PCR to remove the signal sequence and to introduce a start codon at the N-terminus of the protyrosinase by using a sense primer 5'-TACTTTGGTTTCA-CCATGGCCCTCAGTCG-3' that introduced an *NcoI* site. A Met start codon was created by the *NcoI* site at the N-terminus of protyrosinase. The amplified DNA was confirmed to lack the undesired mutation by sequencing. The expression plasmid for protyrosinase, pETY, was constructed by inserting the modified *melO* fragment into pET-32a(+) (Novagen, Madison, WI, U.S.A.) containing the T7 promoter/lac operator and T7 terminator and the thioredoxin gene [23] (Figure 1).

Expression of the recombinant protyrosinase as a thioredoxin fusion protein in *E. coli*

E. coli BL21(DE3) transformed with pETY was cultured at 37 °C in a 5 litre flask holding 1 litre of Luria–Bertani medium containing 50 mg/ml ampicillin until D_{600} reached 0.8, then isopropyl β -D-galactopyranoside was added to a final concentration of 0.1 mM and the incubation was continued for 23 h

at 18 °C (the agitation rate was 150 rev./min). The cells were harvested by centrifugation at 2700 *g* for 10 min and then used for purification of the protyrosinases.

Purification of the protyrosinase expressed as a thioredoxin fusion protein in *E. coli*

The cells were harvested by centrifugation at 2700 *g* for 10 min and washed with 40 ml of 50 mM Tris/HCl buffer, pH 7.5, then resuspended in 40 ml of the same buffer. They were disrupted by sonication twice at power setting 4 for 1 min with an Ultra S homogenizer (VP 30S; TAITEC, Koshigaya-shi, Saitama-prefecture, Japan). The cell homogenate was centrifuged at 6000 *g* for 10 min. CuSO₄ was added to the supernatant to a final concentration of 10 μ M and the mixture was incubated at 4 °C for 14 h. The free Cu²⁺ remaining after binding to the active site of protyrosinase was removed by adding EDTA (final concentration 2 mM). Protein purification was performed at 4 °C. The EDTA-treated solution was dialysed against 2 litres of 10 mM sodium phosphate buffer, pH 7.0, at 4 °C for 4 h. The dialysate was loaded on a Chelating Sepharose FF column (1.6 cm $\times$ 25 cm) loaded with Ni²⁺, then eluted with a linear gradient of imidazole (0–500 mM) in 10 mM sodium phosphate buffer, pH 7.0, containing 500 mM NaCl. The purified potentially active fraction was pooled and dialysed against 10 mM sodium phosphate buffer, pH 7.0.

Activation of the recombinant protyrosinase

Activation of the protyrosinase expressed in *S. cerevisiae* or *E. coli* was accomplished by acid shock in 200 mM sodium acetate buffer, pH 3.0, for 20 min as described [6, 7].

SDS/PAGE and Western blotting

SDS/PAGE was performed by the method of Laemmli [24]. Separated proteins on the polyacrylamide gel were transferred to PVDF membrane (Bio-Rad) and protyrosinase was detected with anti-(*Aspergillus* protyrosinase) antibody as described [7]. Bands were detected with horseradish-peroxidase-labelled goat anti-(rabbit IgG) (Bio-Rad), with 4-chloronaphth-1-ol (Bio-Rad) and H₂O₂ as substrates.

N-terminal amino acid sequence analysis

The tyrosinase, activated by acid shock, was precipitated with 5% (w/v) trichloroacetic acid; the precipitated protein sample was subjected to SDS/PAGE. The enzyme sample was transferred to PVDF membrane; the protein fraction was then subjected to sequence determination on an Applied Biosystems 473 A protein sequencer with a 610 data analysis system.

Molecular mass determinations

Molecular mass determinations of the tyrosinase activated by acid shock were performed by gel filtration on Superose 6 and by SDS/PAGE [24].

HPLC assay of the activated recombinant tyrosinase for mono-oxygenase activity

An HPLC assay of mono-oxygenase activity of the tyrosinase for L-tyrosine was performed at pH 5.5 by measuring the reaction product, L-dopa, with TSK gel ODS-80 Ts (Tosoh) by the method described previously [25]. One katal of L-tyrosine mono-

oxygenase activity was defined as the amount of enzyme required to form 1 mol of L-dopa from L-tyrosine at 40 °C and pH 5.5.

Determination of kinetic parameters for the activated recombinant tyrosinase

Kinetic parameters for the activated tyrosinase were assayed with the HPLC method by Hanes–Woelf plots [26].

Spectrophotometric determination of mono-oxygenase activity

The mono-oxygenase activity of the activated tyrosinase was determined spectrophotometrically with 10 mM L-Tyr at pH 5.5 by measuring A_{475} with a U-3000 (Hitachi) spectrophotometer by the previous method of Fujimoto et al. [27].

The oxidase activity of the activated recombinant tyrosinase with 10 mM L-dopa was also determined at pH 5.5 and 40 °C by measuring A_{475} with the same spectrophotometric method [27].

Atomic absorption spectrophotometry

Copper concentration was measured with a Shimadzu AA-660 (P/N 206-10000-02) atomic absorption spectrophotometer. The copper standard solutions from 0.1 to 10 p.p.m. were calibrated by atomic absorption spectrophotometry.

EPR spectra determination

EPR spectra were measured on a Varian E112 X-band EPR spectrometer; the temperature was controlled with an Oxford ESR-9 helium-flow cryostat [28]. The magnetic fields and microwave frequencies were measured by an Echo Electronic FEM2000 NMR gauss meter and a Takedariken TR5204 frequency counter respectively.

CD measurements

The CD spectra were measured with a Jasco J-700 spectrophotometer at 25 °C in a cuvette with a 1 mm path length. Concentrations of the enzyme were adjusted to 0.2 mg/ml protein in 5 mM Tris/HCl buffer, pH. 7.5. The contents of α -helix, β -structure and random coil of the enzyme were calculated with the SSE-338 program [29].

Determination of protein concentration

Protein concentrations were determined by the method of Bradford [30] with BSA (Sigma) as a standard.

RESULTS AND DISCUSSION

From a crystallographic analysis of *Palinurus interruptus* haemocyanin [31], one of the pair of copper ions, Cu(A), is surrounded by residues 196, 200 and 226; the other, Cu(B), is surrounded by residues 346, 350 and 386. Mutation positions were selected on the basis of a sequence comparison of the tyrosinase from *A. oryzae* [7] with other tyrosinases from *N. crassa* [8–11], *S. antibioticus* [13] and *H. sapiens* [12] (Figure 2). For the cysteine residue it is also assumed that a covalent Cys⁸²-Xaa-His⁸⁴ might be important for enzymic activity, as described for the thioether bridge between Cys-94 and His-96 in the Cys⁹⁴-Xaa-His⁹⁶ peptide of *N. crassa* tyrosinase [9]. Mutations were focused at positions of 18 histidine residues (His-63, His-76, His-83, His-84, His-93, His-193, His-266, His-290, His-294, His-332, His-333, His-347, His-462, His-479, His-489, His-510, His-518 and His-520) and two cysteine residues (Cys-82 and Cys-334).

Figure 2 Comparison of amino acid sequences of tyrosinases from *A. oryzae*, *N. crassa*, *S. antibioticus*, mouse and *H. sapiens*

Filled triangles show the His-63 (*A. oryzae* protyrosinase numbering), His-84, His-93, His-290, His-294, His-332 and His-333 residues deduced as new copper ligands in *A. oryzae* tyrosinase. The open triangle shows the Cys-82 residue deduced as a crucial site and a copper ligand of *A. oryzae* tyrosinase. Asterisks indicate identical amino acids in conservative regions. 1, *A. oryzae* tyrosinase [7]; 2, *N. crassa* tyrosinase [9]; 3, *S. antibioticus* tyrosinase [13]; 4, mouse tyrosinase [14,15]; 5, *H. sapiens* tyrosinase [12].

A Western blot analysis of the mutated products of the *melO* gene expressed in *S. cerevisiae* showed proteins with molecular masses of approx. 67 kDa that reacted with rabbit anti-(*A. oryzae* protyrosinase) serum, which is consistent with the apparent molecular mass of the native proenzyme, as shown in Figure 3. The activities of activated tyrosinases of the mutant enzymes after acid shock are summarized in Table 1. A number of the mutant enzymes (His63Asn, His84Asn, His93Asn, His290Asn, His294Asn, His332Asn, His333Asn and Cys82Ala) had no detectable activity, indicating that the mutated residues are essential for activity. A second group of mutant enzymes (His76Asn, His83Asn, His193Asn, His266Asn, His347Asn, His462Asn, His479Asn, His489Asn, His510Asn, His518Asn, His520Asn and Cys334Ala) retained full or partial activity. However, one single-residue mutant, His193Asn, exhibited complete destruction of the catalytic activity of mono-oxygenase for L-tyrosine and a partial destruction of oxidase for L-dopa. It is assumed that the His-193 residue might be a binding site of the enzyme for the aromatic substrate L-tyrosine.

We succeeded in overproducing the recombinant protyrosinase from *A. oryzae* as a thioredoxin fusion protein in *E. coli*. The protyrosinases were accumulated as a soluble fraction in these cells. The expressed recombinant fusion protyrosinases have no copper ions. As described for the purification of the recombinant protyrosinase in the Experimental section, the recombinant protyrosinases were added to make a final concentration of 10 μ M CuSO₄ and the mixture was incubated at 4 °C for 14 h; the binding of copper ions to the active sites of protyrosinase was then performed. In our simplified purification method, the protyrosinases were purified with a single step of Chelating Sepharose FF, which trapped the histidine tag in the anchor region to the C-terminal end of thioredoxin. The wild-type protyrosinase was purified 102-fold over the crude extract. Approximately 20 mg of wild-type protyrosinase was obtained from 1 litre of the culture of *E. coli*; the yield of protyrosinase was 53 % for a single-step purification.

Activation of the fusion protyrosinases expressed in *E. coli* was accomplished by acid shock at pH 3.0 for 20 min. The engineered thioredoxin-fusion tyrosinases expressed in *E. coli* were revealed after SDS/PAGE (Figure 4). The molecular masses of the subunits of activated tyrosinases after acid shock were determined as 83 kDa on SDS/PAGE. Gel filtration of the activated tyrosinase on a Superose 6 column gave an estimated molecular mass of 330 kDa (results not shown). The results suggested that

Figure 3 Western blot analyses of the wild-type and mutant activated tyrosinases from *A. oryzae* expressed in *S. cerevisiae* YPH250

Proteins were detected with rabbit anti-*A. oryzae* protyrosinase antibody as described in the Experimental section. Positions of the mutated amino acid residues are indicated above the lanes. Abbreviation: TY, tyrosinase.

Table 1 Relative activities of wild-type and mutant tyrosinases from *A. oryzae* expressed in *S. cerevisiae* for the mono-oxygenase with L-tyrosine, and oxidase activity with L-dopa at pH 5.5

Spectrophotometric determinations were performed at 475 nm [27].

Enzyme	Relative activity (%)	
	Monoxygenase with L-tyrosine	Oxidase with L-dopa
Wild type	100	100
His63Asn	0	0
His76Asn	120	147
His83Asn	122	109
His84Asn	0	0
His93Asn	0	0
His193Asn	0	38
His266Asn	132	105
His290Asn	0	0
His294Asn	0	0
His332Asn	0	0
His333Asn	0	0
His347Asn	82	89
His462Asn	8	16
His479Asn	3	21
His489Asn	7	25
His510Asn	141	104
His518Asn	10	63
His520Asn	29	53
Cys82Ala	0	0
Cys334Ala	79	72

the activated tyrosinase was composed of four identical subunits, each with a molecular mass of 83 kDa.

The N-terminal amino acid sequence of the activated wild-type tyrosinase precipitated with 5% (w/v) trichloroacetic acid was SDKIIHLTDD (single-letter amino acid codes), which is known to be the N-terminal sequence of thioredoxin including the 10 residues from the Ser-2 to Asp-11. Although the initiating methionine residue was encoded in the construct, it seems to have disappeared after cleavage of the protein.

The specific activity of the activated wild-type tyrosinase towards L-tyrosine mono-oxygenation, as determined by the HPLC method, was 1.69 kat/kg of protein. A K_m of 0.82 mM and a k_{cat} of 23.6 s⁻¹ for L-tyrosine mono-oxygenation were calculated from Hanes–Woolf plots.

An atomic absorption spectrophotometric determination of copper contents of the activated site-directed mutant tyrosinases His63Asn, His84Asn, His93Asn, His290Asn, His294Asn, His332Asn and His333Asn, expressed in *E. coli*, demonstrated the loss of binding of 1 g-atom of copper/mol of subunit as a consequence of the mutagenesis of histidine residues (Table 2). The copper content of the mutant tyrosinase Cys82Ala was also determined as 1 g-atom/mol of subunit. The copper content of the activated tyrosinases was somewhat lower than that of the protyrosinases (Table 2). Some of the single copper ions might have been removed from the mutant tyrosinases by the acid shock at pH 3.0. As expected from melanin formation, the eight mutant tyrosinases were devoid of any enzymic activity after acid shock at pH 3.0. An almost complete loss of the copper content was determined by atomic absorption spectrophotometry for seven double mutants, His63Asn/His290Asn, His63Asn/His294Asn, His63Asn/His332Asn, His63Asn/His333Asn, Cys82Ala/His290Asn, His84Asn/His290Asn and His93Asn/His290Asn.

The contents of secondary structures (α -helix, β -structure and random coil of mutant enzymes) by CD spectroscopy are shown in Table 3. The results for major global changes indicate that the increases in the content of α -helix were 4.4% for His63Asn, 3.3% for His93Asn, 3.3% for His290Asn and 4.2% for His333Asn compared with that of the control of the wild-type enzyme, and the decreases in the content were 4% for Cys82Ala, 2.4% for His84Asn and 2.3% for His294Asn. The results also indicated that the increases in the amount of β -structure were 3.4% for His84Asn and 7.3% for His93Asn; decreases in β -structure of 5.1% for His63Asn, 4.2% for His290Asn and 8.2% for His294Asn were also identified. Despite this, the results for α -helices and β -structures of the mutant enzymes His84Asn and His332Asn indicated that no substantial changes in the secondary structure occurred.

Copper ions in the (I) and (II) oxidation states are biologically important [32]. Basically, three different types of copper centre

Figure 4 SDS/PAGE of purified wild-type and mutant thioredoxin-fused tyrosinases from *A. oryzae* expressed in *E. coli*

Proteins were detected with Coomassie Brilliant Blue R-250 dissolved in 50% (v/v) methanol/9.5% (v/v) acetic acid and destained in 50% (v/v) methanol/7% (v/v) acetic acid. Abbreviation: Trx-TY, thioredoxin-fused tyrosinase.

Table 2 Atomic absorption spectrophotometric determination of copper content for wild-type and mutant tyrosinases from *A. oryzae* expressed in *E. coli*

The activation of protyrosinase expressed in *E. coli* was accomplished by incubation with an acidic buffer at pH 3.0 for 20 min. The copper contents of wild-type and mutant protyrosinases are shown in parentheses; the values without parentheses show the copper contents of wild-type and mutant tyrosinases after acid activation. Abbreviation: PCMB, *p*-chloromercuribenzoate.

Enzyme	Copper content (g-atom/mol subunit)	
Wild type	1.7	(1.9)
Wild type after treatment with PCMB	0.02*	(1.6)†
His63Asn	0.2	(0.9)
Cys82Ala	0.4	(0.6)
His84Asn	0.2	(0.7)
His93Asn	0.9	(1.0)
His290Asn	0.2	(0.6)
His294Asn	0.3	(0.8)
His332Asn	0.4	(0.4)
His333Asn	0.7	(0.8)
His63Asn/His290Asn	0.01	(0.01)
His63Asn/His294Asn	0	(0.05)
His63Asn/His332Asn	0.03	(0.03)
His63Asn/His333Asn	0.03	(0.08)
Cys82Ala/His290Asn	0.05	(0.07)
His84Asn/His290Asn	0.01	(0.03)
His93Asn/His290Asn	0.01	(0.05)
Apoenzyme	0.01	(0.02)

* After treatment with 0.1 mM PCMB at pH 3.5, activated tyrosinase was dialysed against 5 mM sodium acetate buffer, pH 3.5.

† After treatment with 0.1 mM PCMB at pH 7.0, protyrosinase was dialysed against 5 mM phosphate buffer, pH 7.0.

Table 3 Estimated secondary structure for CD determination of the activated tyrosinases from *A. oryzae* after acid shock at pH 3.0

Enzyme	Variant	Secondary structure (%)		
		α -Helix	β -Structure	Random
Activated tyrosinase	Wild type	25.7	41.7	32.6
	His63Asn	30.1	36.6	33.3
	Cys82Ala	21.7	43.0	35.3
	His84Asn	23.3	45.1	31.6
	His93Asn	29.0	49.0	31.1
	His290Asn	29.0	37.5	33.5
	His294Asn	23.4	33.5	33.0
	His332Asn	24.7	43.0	32.3
	His333Asn	29.9	39.1	30.9
	Wild type	26.7	41.1	32.2
Protyrosinase				

centre that is 'non-detectable' by EPR, apparently containing a pair of contiguous copper atoms.

The value of EPR spectroscopy in establishing electronic structures and their dependence on metal co-ordination-sphere composition and geometry can be illustrated by its application to several aspects of copper bio-inorganic chemistry [33]. In the wild-type tyrosinase from *A. oryzae*, the third type of copper (Type III) is silent towards EPR because of antiferromagnetic exchange coupling and/or rapid electron-spin relaxation between two neighbouring Cu(II) centres (Figure 5 and Table 4). The EPR spectra of the five mutant tyrosinases His63Asn, His93Asn, His290Asn, His294Asn and Cys82Ala are shown in Figure 5. Some of their EPR properties of copper, the anisotropic perpendicular and parallel g -values ($g_{\perp}$ and $g_{\parallel}$) of copper, and the hyperfine coupling constant parallel, $^{\text{Cu}}A_{\parallel}$, of the six mutants are shown in Table 4 and compared with that of the wild-type

are known. 'Blue' or type I copper occurs in the blue electron-carrying proteins such as stellacyanin, plastocyanin and azyrin. There is also 'non-blue' or type II copper and a type III copper

Figure 5 EPR spectra of the wild-type and mutant tyrosinases from *A. oryzae* expressed in *E. coli*

Asterisks indicate the positions of possible free-radical peaks.

Table 4 Parameters of EPR spectra for wild-type and mutant tyrosinases from *A. oryzae* expressed in *E. coli*

We deduced the copper ligand geometry environment from the correlation between $g_{\perp}$ and $A_{\parallel}$ values reported previously [34]. Abbreviation: n.d., not determined, because the preparations were partly insoluble at high concentration.

Enzyme	EPR spectra obtained			Copper ligand geometry
	$g_{\perp}$	$g_{\parallel}$	$A_{\parallel}$ (10^{-4} cm $^{-1}$)	
Wild type	EPR silent	EPR silent	EPR silent	
His63Asn	2.06	2.256	185	Square tetrahedral
Cys82Ala	2.07	2.253	190	Square tetrahedral
His84Asn	n.d.	n.d.	n.d.	
His93Asn	2.06	2.301	152	Distorted tetrahedral
His290Asn	2.08	2.301	150	Distorted tetrahedral
His294Asn	2.06	2.251	190	Square tetrahedral

tyrosinase. These results clearly indicate that the five mutants (His63Asn, Cys82Ala, His93Asn, His290Asn and His 294Asn) in which the single copper site is empty contain only 1 g-atom of

copper/mol of subunit of tyrosinase. This information might be valuable in deducing the locations of sites of reactivity on bound substrate molecules. They have a more or less axially symmetrical appearance with the parameters given. The values are very similar for the five mutant tyrosinases, suggesting that the remaining copper is in a similar ligand environment.

In a detailed analysis of copper ligand geometry [34], the correlations between $g_{\parallel}$ of copper(II) complexes and $^{\text{Cu}}A_{\parallel}$ arising from various factors have been given for copper(II) complexes. The authors noted that a reported complex with a thioether donor at the apex of a square pyramid whose base was the donor set N_3O_2 had $g_{\parallel} = 2.240$ and $A_{\parallel} = 0.0178$ cm $^{-1}$, which placed this complex in the normal square-planar CuN_2O_2 region. Tetrahedral distortion of a square-planar chromophore with any of the biomimetic (N, O, S) donors decreased $A_{\parallel}$ and increased $g_{\parallel}$, lowering the ($g_{\parallel}$, $A_{\parallel}$) point for that CuX_4 core. From the geometrical analysis of EPR by Sakaguchi and Addison [34], we deduced that the nitrogen or sulphur donors in the Cu(B) or Cu(A) ligand geometry of the three mutants His63Asn, Cys82Ala and His294Asn should be on a square-tetrahedral geometry and also that the nitrogen or sulphur donors in the Cu(B) or Cu(A) of the two mutants His93Asn and His290Asn should be on a distorted tetrahedral geometry, as shown in Table 4.

The EPR spectra of two mutants, His62Asn and His189Asn, of *S. glaucescens* were measured by Huber and Lerch [5]. The mutants had a more or less axially symmetrical appearance by these parameters. The values were similar for both materials, suggesting that the remaining copper atom was in a very similar ligand environment. They noted that the amide nitrogen group was not a good ligand for either Cu(I) or Cu(II). Both mutants of *S. glaucescens* showed drastically changed physicochemical properties: (1) no enzymic activity, (2) no reaction with H_2O_2 and (3) only one copper atom bound per molecule. The fact that most of this copper was detectable by EPR confirmed that one copper atom from either the Cu(A) site (Asn-62) or the Cu(B) site (Asn-189) had been selectively removed. From the $g_{\parallel}$ and $A_{\parallel}$ values and the energies of the d-d transition, the copper in both mutants could be defined as being in a slightly distorted tetragonal ligand environment. Applying the correlation between $A_{\parallel}/g_{\parallel}$, ligand type and geometry in copper complexes, possible copper ligands in *S. glaucescens* tyrosinase were either three nitrogen atoms and one oxygen atom or two nitrogen atoms and two oxygen atoms.

Spritz et al. [35] described the Cu(B) motifs of all known tyrosinases and polyphenol oxidases as differing from those of haemocyanin in that they contain four rather than three absolutely conserved histidine residues; in human tyrosinase these occur at His-363, His-367, His-389 and His-390. His-363 and His-367 are clearly similar to the copper-binding histidine residues of the haemocyanin Cu(B) motif. The His390Ala mutant abolished catalytic activity did not decrease copper binding, whereas the His-389 substitution abolished it. These results indicate that it is His-389, rather than His-390, that has a critical role in copper binding by human tyrosinase, even though it is His-390 that has been conserved in all of the putative copper-binding positions.

On the basis of the study of *Neurospora* tyrosinase with a molecular mass of 46 kDa including 2 g-atoms of copper/mol [8–11], it is conceivable that the activation of protyrosinase takes place by the active conformation of the thioether linkage between Cys-94 and His-96 [9]. However, the absence of a similar structure in the tyrosinase from *S. antibioticus* [13] makes such a function rather unlikely. After dye-sensitized photo-oxidation of the apoenzyme of *Neurospora* tyrosinase, three histidine residues (His-188, His-193 and His-289) were destroyed [10]. Copper

measurements of photo-oxidized, reconstituted apoenzyme demonstrated the loss of binding of one copper/mol of enzyme as a consequence of the photo-oxidation of histidine residues [10]. Lerch suggested that the three histidine residues destroyed might be ligands of one of the two active-site copper ions; His-306 of *Neurospora* tyrosinase was indicated as an active site by active-site-directed modification with catechol [10]. His-306 of *Neurospora* tyrosinase is an essential residue and corresponds to the active His-333 of the tyrosinase from *A. oryzae*. His-193 of *A. oryzae* tyrosinase, corresponding to the photo-inactivated His-188 of *N. crassa* tyrosinase, might not be an essential residue but a binding one for catalysis in the *A. oryzae* enzyme.

For Cu(A), we confirmed that three histidine residues, His-63, His-84 and His-93, are essential residues of the tyrosinase from *A. oryzae* as determined by site-directed mutagenesis. In the present paper we also found that Cys-82 is an essential residue for the catalytic activity of the tyrosinase activated at pH 3.0. *A. oryzae* tyrosinase was inhibited completely by *p*-chloromercuribenzoate. The most likely binding sites of the tyrosinase from *A. oryzae* for Cu(A) are His-63, His-84 and His-93, with the remaining conserved Cys-82 providing the fourth ligand. We confirmed by atomic absorption spectrophotometry for copper incorporation that Cu(A) is not always liganded by three histidine residues (His-63, His-84 and His-93) and one cysteine residue (Cys-82). Confirmation was obtained that the two mutants His290Asn and His294Asn contain one copper ion that is fully detectable by EPR (Figure 5 and Table 4). We deduced that the nitrogen or sulphur donors in the copper-ligand geometry of His290Asn and His294Asn should be in a square tetrahedral geometry and a distorted tetrahedral geometry respectively. The results of atomic absorption spectrophotometric determination on the double mutant Cys82Ala/His290Asn confirmed that there was no copper atom. We propose a new binding motif of Cu(A) of tyrosinase from *A. oryzae* in which the Cu(A) ion is co-ordinated by three histidine side chains, His-63, His-84 and His-93, and a single thiol group of Cys-82.

In *Aspergillus* tyrosinase, His-290, His-294 and His-333 are clearly similar to the copper-binding histidine residues of the haemocyanin Cu(B) motif. The His332Ala mutant abolished catalytic activity and decreased copper binding. These results indicate that His-332 has a critical role in copper binding by *Aspergillus* tyrosinase. We confirmed that three histidine residues, His-290, His-284 and His-333 for Cu(B), are essential residues for catalytic activity and copper ligands of the activated tyrosinase from *A. oryzae*. Furthermore, it was found in the present study that the His-332 residue is also an essential site of the tyrosinase from *A. oryzae*. We also confirmed that the His63Asn, Cys82Ala and His93Asn mutants contain one copper ion that is fully detectable by EPR (Figure 5 and Table 4). We confirmed that the double mutant His63Asn/His332Asn contains almost no copper ions (Table 2). We deduced that the nitrogen donors in the His63Asn and Cys82Ala mutants should have a square-tetrahedral geometry, and those in the mutant His93Asn should have a distorted tetrahedral geometry. Atomic absorption spectrophotometric results on the double mutant His63Asn/His332Asn showed no copper atom.

The imidazolate-bridged dimetallic centre in copper-zinc superoxide dismutase (EC 1.15.1.1) was a novel structural feature that had not previously been encountered in co-ordination chemistry [33]. The Cu(II) ion is co-ordinated by four histidine side chains, His-44, His-46, His-118 and His-61, and there is evidence for a fifth axial water ligand.

This is the first paper to determine the essential seven histidine residues, His-63, His-84, His-93, His-290, His-294, His-332 and

His-333, and the single essential cysteine residue, Cys-82, for tyrosinase activity of *A. oryzae*. It is concluded that one pair of copper ions, Cu(A), is liganded by three histidine residues, His-63, His-84 and His-93, and a single cysteine residue, Cys-82, whereas the other, Cu(B), is liganded by four histidine residues, His-290, His-294, His-332 and His-333.

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